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Leptin modulation of locomotor and emotional behaviors: The role of STAT3 signaling in dopamine neurons

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Leptin modulation of locomotor and emotional behaviors: The role of STAT3 signaling in dopamine neurons

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Résumé

La leptine circule en proportion de la masse graisseuse du corps et la transduction de son signal à travers la forme longue de son récepteur via un certain nombre de voies neurales , y compris MAPK, PI3-K ,AMPK et JAK2 - STAT3 . Il faut noter que STAT3 constitue une voie clée au récepteur de la leptine par laquelle la leptine module l'expression des gènes impliqués dans la régulation du bilan énergétique.

La plupart des recherches ont porté sur la fonction du récepteur de la leptine au sein de l' hypothalamus, en particulier la fonction du récepteur de la leptine dans le noyau arqué. Toutefois, les récepteurs de la leptine sont également exprimés sur les neurones dopaminergiques de l'aire tégmentale ventrale et la leptine agit sur cette région du cerveau pour influencer la prise alimentaire, la motivation, la locomotion, l'anxiété et la transmission de la dopamine. De plus, la leptine active la STAT3 dans les dopaminergiques et GABAergiques populations neuronales. Bien que ces résultats contribuent à notre compréhension des multiples actions de la leptine dans le système nerveux central, il reste à résoudre les cellules et la signalisation du récepteur de la leptine qui sont responsables des effets neurocomportementaux de la leptine dans le mésencéphale.

Visant à déterminer la contribution de la voie de signalisation STAT3 dans les neurones dopaminergiques du mésencéphale, nous avons généré une lignée de souris *knockout* conditionnel dans lequel l'activation du gène de STAT3 sur son résidu tyrosine 705 (Tyr 705) est absent spécifiquement dans les neurones dopaminergiques. Avec l'utilisation de ce modèle de souris génétique, nous avons évalué l'impact de l'ablation de la signalisation STAT3 dans les neurones dopaminergiques sur un certain nombre de fonctions liées à la dopamine, y compris l'alimentation, la locomotion, les comportements liés à la récompense, l'émotion et la

libération de dopamine dans le noyau accumbens. Fait intéressant, nous avons observé un dimorphisme sexuel dans le phénotype des souris $STAT3^{DAT\text{-}KO}$. L'activation de la voie de signalisation STAT3 dans les neurones dopaminergiques est responsable de l'action de la leptine dans la réduction de la locomotion, récompense liée à l'activité physique, et de l'augmentation de la libération et de la disponibilité de la dopamine chez les souris mâles. Cependant, il ne module pas le comportement émotionnel. D'autre part, les souris femelles $STAT3^{DAT\text{-}KO}$ augmentent les niveaux d'anxiété et les niveaux plasmatiques de corticostérone, sans provoquer de changements de la dépression. Cependant, la perte d'activation de STAT3 dans les neurones dopaminergiques ne module pas le comportement locomoteur chez les souris femelles. Notamment, les actions de la leptine dans le mésencéphale pour influencer le comportement alimentaire ne sont pas médiées par l'activation de STAT3 dans les neurones dopaminergiques, considérant que les souris mâles et femelles ont un comportement alimentaire normal.

Nos résultats démontrent que la voie de signalisation STAT3 dans les neurones dopaminergiques est responsable des effets anxiolytiques de la leptine, et soutient l'hypothèse que la leptine communique l'état d'énergie du corps (i.e. la relation entre la dépense et les apports énergétiques) pour les régions mésolimbiques pour atténuer les effets de motivation et de récompense de plusieurs comportements qui servent à réhabiliter ou à épuiser les réserves d'énergie. En outre, ce travail souligne l'importance d'étudier la modulation de la signalisation de la leptine dans différente types de cellules, afin d'identifier les voies de signalisation et les mécanismes cellulaires impliqués dans les différentes fonctions neuro-comportementales de la leptine.

Mots-clés: leptine, STAT3, dopamine, mésencéphale, récompense liée à l'activité physique, anxiété, comportement.

Abstract

The adipocyte-derived hormone leptin circulates in proportion to the body fat content and transduces its signal through the long form of its receptor via a number of neural pathways, including MAPK, PI3-K, AMPK and JAK2-STAT3. Of note, STAT3 constitutes a key pathway downstream to the leptin receptor by which leptin modulates the expression of genes involved in energy balance.

Most research has focused on leptin receptor function within the hypothalamus, in particular leptin receptor function within the arcuate nucleus. However, leptin receptors are also expressed on dopaminergic neurons of the ventral tegmental area, and leptin has been shown to target this brain region to influence feeding, motivation, locomotion, anxiety and dopamine tone. Moreover, leptin activates STAT3 in both dopaminergic and GABAergic neuronal populations. Although these findings contribute to our understanding of the multiple actions of leptin in the central nervous system, it remains to be resolved which cells and leptin receptor signaling pathway mediates the neurobehavioral effects of leptin in the midbrain.

Aiming at determining the contribution of STAT3 signaling in midbrain DA neurons, we generated a line of conditional knockout mice in which the main activation site of STAT3 gene (tyr 705) is absent specifically in dopaminergic neurons ($STAT3^{DAT-KO}$ mice). Using this genetic mouse model, we assessed the impact of ablation of STAT3 signaling in dopaminergic neurons on a number of dopamine-related functions, including feeding, locomotion, reward-related behaviors, emotion and nucleus accumbens dopamine release. Interestingly, we observed a sexual dimorphism in the phenotype of $STAT3^{DAT-KO}$ mice. STAT3 signaling in DA neurons mediates the actions of leptin in the midbrain to decrease locomotion and running reward, and to increase dopamine release and availability in male mice. However, it does not

modulate emotional behavior. On the other hand, STAT3^{DAT-KO} female mice exhibited increased anxiety-like behavior accompanied by increased plasma corticosterone levels, without changes in behavioral despair relative to littermate controls. However, loss of STAT3 activation in dopaminergic neurons does not modulate locomotor behavior in female mice. Notably, the actions of leptin in the midbrain to influence feeding behavior are not mediated by STAT3 signaling in dopaminergic neurons, as both male and female STAT3^{DAT-KO} mice have normal feeding behavior as compared to littermate controls.

Our results demonstrate that STAT3 signaling in dopaminergic neurons mediates the anxiolytic actions of leptin, and support the hypothesis that leptin communicates body energy status (defined as a relationship between energy intake and energy expenditure) to mesolimbic regions to adjust the motivational and rewarding effects of multiple behaviors that serve to either restore or deplete energy stores. In addition, this work highlight the importance of studying cell-type specific modulation of leptin signaling molecules to tease apart pathways and the mechanisms involved in the different neurobehavioral functions of this adipocyte-derived hormone.

Keywords: leptin, STAT3; dopamine, midbrain, running reward, anxiety, behavior.

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List of Abbreviations

5-HT: serotonin
6-OHDA: 6-hydroxydopamine
AAV: adeno-associated virus
ABA: activity-based anorexia
AgRP: agouti related protein
AMPH: amphetamine
AMPK: 5′adenosine monophosphate-activated protein kinase
ARC: hypothalamic arcuate nucleus
BBB: blood–brain barrier
BDNF: brain derived neurotrophic factor
BMR: basal metabolic rate
BNST: basal nucleus of the stria terminalis
BSR: brain-stimulation-reward
cAMP: cyclic adenosine monophosphate
CART: cocaine- and amphetamine-regulated transcript
CCK: cholecystokinin
CeA: central nucleus of the amygdala
CLi: caudal linear nucleus
CNS: central nervous system
CNTF: ciliary neurotrophic factor
CPP: conditioned place-preference
D1R: dopamine 1-like receptor
D2R: dopamine 2-like receptor
DA: dopamine
DARPP: dopamine- and cAMP-regulated phosphoprotein
DAT: dopamine transporter
db: diabetes gene
DIO: diet-induced obesity
DLS: dorsolateral striatum
DNA: deoxyribonucleic acid
DRN: dorsal raphe nucleus
ECBs: endocannabinoids
EGF: epidermal growth factor
EPM: elevated-plus maze
ER: estrogen receptor
ERK 1/2: extracellular signal-regulated kinase 1/2
FAA: food anticipatory activity
fMRI: functional magnetic resonance imaging
FR1: fixed ratio 1
FST: forced-swim test
GABA: γ-aminobutyric acid
G-CSF: granulocyte-colony stimulating factor
HF: high-fat
HPA: hypothalamic-pituitary axis

ICV: intracerebroventricular
IFN: interferon
IGF-1: insulin-like growth factor-1
IL: interleukin
IRS: insulin receptor substrate
JAK2: janus tyrosine kinase 2
KO: knockout
LepR: leptin receptor
LepRa: short-isoform of the leptin receptor
LepRb: long-isoform of the leptin receptor
LF: low fat
LH: lateral hypothalamus
LHA: lateral hypothalamic area
LPOA: lateral preoptic area
MAPK: mitogen-activated protein kinase
MHb: medial habenular nucleus
mTOR: mammalian target of rapamycin
NAc: nucleus accumbens
NE: norepinephine
NMDA: N-methyl-D-aspartate
NPY: neuropeptide Y
NT: neurotensin
NTS: nucleus of the solitary tract
ob: obese gene
OFT: open field test
OT: tuberculum olfactorium
PAG: periaqueductal gray
PFC: prefrontal cortex
PI3-K: phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA: cAMP-dependent protein kinase
PMT: pontomesencephalic tegmentum
POMC: proopiomelanocortin
PTP1B: protein-tyrosine phosphatase 1B
PVN: paraventricular hypothalamic nuclei
RLi: rostral linear nucleus
RRF: retrorubral field
RRF: retrorubral field
SH2: Src homology 2 domain containing proteins
SHP2: protein tyrosine phosphatase 2
SN: substantia nigra
SNc: substantia nigra pars compacta
SNr: substantia nigra pars reticulada
SOCS 3: suppressor of cytokine signaling 3
STAT: signal transducer and activator of transcription
$STAT3^{DAT\text{-}KO}$: dopamine-specific STAT3 knockout mice
$STAT3^{N\text{-}/\text{-}}$: neural STAT3 mutant mice

TH: tyrosine hydroxylase
TST: tail suspension test
Tyr: tyrosine
VMH: ventromedial hypothalamus
VTA: ventral tegmental area

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Chapter I

Introduction and Literature Review

1. General Introduction

1.1. Central regulation of energy balance

In order to maintain an ideal body weight, an organism must balance energy intake with energy expenditure. Under normal "steady-state" conditions, daily caloric requirements are estimated based on a balance between energy expenditure and energy needs, to maintain normal growth, thermogenesis, locomotion and reproduction (Bouchard and Perusse 1993, Wynne *et al.* 2005, Lenard and Berthoud 2008). Energy balance regulation depends on a complex interaction between central and peripheral signals to influence food intake and energy expenditure (Kalra *et al.* 1999, Wynne *et al.* 2005, Matsuda *et al.* 2011). Excessive caloric intake in relation to metabolic needs can lead to positive energy balance and, as a consequence, weight gain (Wynne *et al.* 2005).

In modern societies, it is very common to eat beyond need, given that food consumption is also a gratification for the palate (Cota *et al.* 2006). Along with overfeeding, sedentary life style greatly contribute to an imbalance in total daily energy expenditure, resulting in body fat gain (Levine *et al.* 1999), which will eventually cause obesity. On the other hand, body energy stores can be depleted when daily energy expenditure exceeds caloric consumption. The major components of total daily energy expenditure are: 1) caloric needs; 2) thermic effect of food; 3) locomotion-induced thermogenesis (D'Alessio *et al.* 1988, Levine 2004). Of note, in most cases, these homeostatic mechanisms matching energy expenditure to energy intake, regulates body weight tightly, aiming to maintain body weight over a long period of time. However, this is not always the case as in modern societies, it is very common to eat beyond physiological or nutritional needs.

To preserve energy homeostasis, peripheral signal informs the brain about the body energy stores through either the autonomic nervous system, or via central actions of nutrients, hormones and metabolites (Lenard and Berthoud 2008). Within the central nervous system (CNS), there are several structures involved in energy homeostasis, including the brainstem, parts of the cortex and limbic system, and the hypothalamus (Lenard and Berthoud 2008). In fact, the hypothalamus has been implicated in the control of food intake and body weight since a long ago, when experiments demonstrated that lesions of this brain structure lead to a dysregulation of food consumption (Brobeck 1946, Anand and Brobeck 1951). Within the hypothalamus, there are two primary populations of neurons located in the arcuate nucleus (ARC) which are responsible for the integration of peripheral signals of nutritional status (Cone *et al.* 2001): 1) the anorexigenic proopiomelanocortin (POMC)- and cocaine- and amphetamine-regulated transcript (CART)-containing neurons (Elias *et al.* 1998, Kristensen *et al.* 1998); 2) the orexigenic neurons coexpressing neuropeptide Y (NPY) and agouti related protein (AgRP) (Broberger *et al.* 1998, Hahn *et al.* 1998). These ARC neuronal populations form complex neural circuits with hypothalamic second-order neurons to modulate energy homeostasis (Lopaschuk *et al.* 2010). Thus, the above mentioned hypothalamic pathways allow transduction of peripheral signals into behavioral and metabolic responses aiming at maintaining body energy stores at a constant level.

1.2. Ingestive behavior: appetitive *versus* consummatory behavior

Given the ever-increasing rise in obesity in our society, mostly due to energy intake chronically exceeding energy output (Ogden *et al.* 2006), it remains very important to identify the mechanisms underlying ingestive and locomotor behavior. In fact, ingestive behavior encompasses all drinking and eating behaviors, and is thought to be comprised of two main

phases that contribute to the overall energy intake of animals: the appetitive and consummatory phases (Kearney 2010, Keen-Rhinehart *et al.* 2013). The appetitive phase is characterized by foraging behavior, when animal acquire and transport food, along with hoarding behavior, when the animal stores the food; the consummatory phase is the phase that follows, when the food is consumed, and involves basic motor movements that control chewing, swallowing, etc, as well as central mechanisms responsible for food consumption (Foltin 2001, Bartness *et al.* 2011). Both appetitive and consummatory behaviors can increase in conditions where energy reserves are further challenged, such as during pregnancy/lactation, exposure to cold temperatures, hunger and food cravings (the desire to immediately consume food and to replenish the energy stores) (Day and Bartness 2003). Although both consummatory and ingestive behaviors involve motivation, reward-related pleasure and locomotion, the important distinction between these two phases of ingestive behavior lies in the fact that consummatory behaviors also reflects the ability to perform motor actions; while appetitive behaviors reflects willingness to aquire fuels either for eating or for storage (Keen-Rhinehart *et al.* 2013). Despite the importance of appetitive behavior to the etiology of obesity and related diseases, more focus has been given to the neuroendocrine mechanisms underlying consummatory behavior (Kalra *et al.* 1999, Abizaid and Horvath 2012, Kageyama *et al.* 2012).

Appetitive ingestive behavior is a motivated behavior which aims to ensure that sufficient energy will be available to fulfill the energy needs of the body (Everitt *et al.* 1984). Therefore, this phase of ingestive behavior is greatly influenced by the dopaminergic reward circuit, which will be discussed in more detail later in this thesis. For now, it is sufficient to mention that food hoarding increases the activation of tyrosine hydroxylase (TH) (a rate limiting enzyme for the synthesis of dopamine) within dopaminergic neurons in the mesolimbic reward circuitry (Yang *et al.* 2011,

Zhang *et al.* 2011), suggesting that the act of hoard food can be naturally rewarding and reinforcing for animals which engage in such behavior.

1.3. Feeding behavior

Feeding is a physiological process required for survival (Blundell and Tremblay 1995). There are multiple and complex factors involved in feeding behavior, which are characterized by interactions between peripheral and central mechanisms that sense and respond to environmental changes, such as nutrient supply, temperature and plasma glucose, among others, to adjust energy intake (Stubbs 1999). In fact, the composition and amount of ingested food is variable between individuals, and as mentioned previously in this thesis, is not well correlated with daily energy expenditure (Edholm 1977, de Castro 1998). Thus, chronic consumption of excessive calories coupled with physical inactivity often observed nowadays, will eventually cause obesity. The "thrifty genotype" theory (Neel 1962) can serve as a possible explanation on how alterations in feeding behavior throughout time are contributing to the increased rates of overweight and obesity. According to this theory, for thousands of years our ancestors lived by hunting, farming and fishing to obtain food. However, these people experienced alternating periods of food abundance and starvation. Thus, to adapt to these extreme changes in caloric needs, our ancestors developed a thrifty genotype that allowed them to eat in abundance and store fat during times of plenty, so that they would not starve during times of famine (Neel 1962). With today's obesogenic environment, including less physical activity coupled to high availability of caloric foods, the "thrifty genotype" is no longer adaptive. In fact, the biological mechanisms stemming from this began to work against us by continuing to store calories in preparation for famine, by

promoting increased food seeking and ingestion, and thus contributing to increased body fat stores.

The decision whether or not to eat a palatable food depends on how the CNS processes and integrates information about different aspects of eating behavior with hormonal signals related to hunger and satiety to promote energy balance (Smith and Campfield 1993, Morton *et al.* 2006). Thus, nutrient sensing, absorption and repletion of the body energy stores regulates the expression and release of several metabolic hormones that play a fundamental role in the control of energy homeostasis by the CNS (Fernandes *et al.* 2013). So, when energy stores are depleted, the brain intercommunicates with peripheral signals to provide the energetic needs to the body, making sure that adequate amounts of calories are available to tissues via the circulation (Figlewicz *et al.* 1995, Riedy *et al.* 1995, Barrachina *et al.* 1997, Woods *et al.* 2000). Actually, under usual circumstances, blood energy supply does not necessarily decrease below the threshold to trigger a feeding episode - it is very common that animals initiate meals even though the body energy stores are full, suggesting that timing and frequency of meals are not always driven by energy needs (Woods *et al.* 2000). In the last decade, great progress has been made in the identification of these peripheral signals which affects food intake and energy balance by communicating metabolic information to the brain. Moreover, the realization that specific genes have a profound influence on food intake and body weight, played a pivotal role in generating new targets for drug development in the treatment of obesity and related disorders.

1.4. Locomotion: ambulatory activity and voluntary exercise

As previously mentioned, there are three main components of energy expenditure: 1) basal metabolic rate (BMR); 2) thermic effect of food; 3) activity-induced thermogenesis, including

both voluntary exercise and spontaneous locomotor activity, or "non-exercise activity thermogenesis" (Levine 2004). By definition, voluntary exercise can be seen as a purposeful activity and/or movement that expends a significant amount of energy (Knab and Lightfoot 2010); while ambulatory activity represents the energy expenditure associated with all activities we undertake, including those associated with mating, predator avoidance, food- and shelter-seeking (Garland *et al.* 2011). These processes can be very different among species and individuals, resulting in a large variation in daily energy expenditure and efficiency of energy stored. Efficient energy storage, calculated by dividing the excess calories stored by the excess calories consumed, is beneficial because it allows longer survival during periods of scarcity of food (Levine *et al.* 1999). However, as discussed in *Section 1.3*, in the obesogenic environment of modern societies where locomotion necessary for daily activities has diminished and high-density food supply has become abundant, efficient energy storage leads to energy imbalance and predisposes to obesity (Levine *et al.* 1999, Garland *et al.* 2011). This energetic efficiency with which non-exercise activities are performed is one of the major determinants of how much energy an individual will spend performing daily trivial activities. The link between non-exercise ambulatory activity and eating is highlighted in studies showing that severe underfeeding or food deprivation triggers substantial increases in locomotor activity, possibly due to an increase in motivation for foraging behavior in rodents (Overton and Williams 2004, Adan *et al.* 2011).

Similarly, voluntary exercise is important to human health for many reasons, including the prevention of obesity (Schrauwen and Westerterp 2000, Connelly *et al.* 2007). It is well known that insufficient physical activity disturbs energy balance, and thus contributes to increased overweight, obesity and associated comorbidities (Dishman *et al.* 2006). Conversely, exercise

reduces body weight and body fat mass in obese compared to lean rodents (Mayer *et al.* 1954), and is associated with increased BMR (Hill *et al.* 1983).

Peripheral and central metabolic mechanisms greatly influence physical activity behavior and in turn, physical activity affect the CNS. Exercise was shown to induce changes in central neuropeptide systems involved in the regulation of energy homeostasis (Levin and Dunn-Meynell 2004). Furthermore, regular physical activity protects the brain against the deleterious effects of poor lifestyle (including those elicited by consumption of a high-saturated fat diet) possibly by modulating molecular signaling pathways, known to be disrupted by the intake of junk food (Molteni *et al.* 2004, Wu *et al.* 2004).

2. Leptin overview

2.1. Leptin discovery and physiological actions

According to Kennedy's "lipostatic theory", the amount of food ingested is determined by a circulating "limiting factor" produced by the adipose tissue and sensed by the CNS, so that the brain monitors this blood metabolite to adjust the body fat stores accordingly (Kennedy 1953). In the late 50's, Hervey's parabiosis experiment (Hervey 1959), aiming at a better understanding of the mechanisms involved in the central regulation of food intake, strongly supported the lipostatic theory of body weight control. This parabiosis study of Hervey involved surgically connecting the circulatory system of two animals: lean and obese ventromedial hypothalamus (VMH)-lesioned rat, to produce a common blood supply. Hervey observed that the parabiosis significantly reduced both food intake and body fat mass of the parabiotic partners of obese VMH-lesioned rats, as compared to parabiotic partners of lean rats. This result suggested that a circulating humoral factor was produced in excess by the lesioned parabiont as body fat accumulated, and this factor was responsible for the reduced food intake and body weight observed in the non-lesioned parabiont (Hervey 1959). A couple of decades later, parabiotic experiments performed among wild-type and two different strains of mice with unknown genetic mutations, *ob/ob* and *db/db,* demonstrated that the obese gene (*ob*) mutation lacked the production of a circulating anorexic factor, whereas the diabetes gene (*db*) mutation impaired the response to this factor (Coleman 1973). This circulating factor was later identified by Jeffrey Friedman's laboratory and named leptin (from the Greek *leptos,* meaning thin) (Zhang *et al.* 1994). Therefore, leptin was the circulating satiety factor working as a negative feedback signal to control energy balance. However, it must be taken into consideration that both human and animals will eat beyond need should the opportunity arise. Thus, an important limitation to the

above-described studies is that a "circulating limiting factor" cannot be the only determinant of the amount of food consumed.

Leptin, a 16-kDa protein, is a hormone synthesized mainly by the adipose tissue that circulates in proportion to body fat mass and informs the CNS about the status of the body's energy stores, as well as acute changes in caloric intake (Zhang *et al.* 1994, Boden *et al.* 1996, Considine *et al.* 1996, Schwartz and Seeley 1997, Chan and Mantzoros 2005). It is secreted in a pulsatile fashion and has a significant diurnal variation with higher levels in late evening and early morning hours, possibly aiming to suppress appetite during the night while sleeping. (Sinha *et al.* 1996, Licinio *et al.* 1997). Leptin can be transported across the blood–brain barrier (BBB) to enter the brain, where it binds to specific leptin receptors (LepR), expressed throughout the body, to modulate a number of biological functions (Seeley and Woods 2003).

Early in the 90's it was observed that leptin injections reversed the obese phenotype of the leptin-deficient *ob/ob* mouse and also restored fertility to female *ob/ob* mice (Campfield *et al.* 1995, Halaas *et al.* 1995, Pelleymounter *et al.* 1995, Chehab *et al.* 1996). Importantly, additional studies determined that the *db* mutation resides in the gene encoding the leptin receptor (Chua *et al.* 1996, Lee *et al.* 1996) and that leptin administration to *db/db* mouse does not reduce food ingestion or body weight (Halaas *et al.* 1995). In summary, overfeeding will lead to increased body fat stores and consequently increased leptin levels. Increased plasma leptin will then activate LepR signaling within the brain, which will in turn, decrease food intake and body weight by stimulating energy expenditure.

Leptin has a number of other biological actions, including regulation of neuroendocrine function. It was previously reported that when starvation induces a fall in leptin, this acute energy deprivation will trigger a series of neuroendocrine responses, including decreases in reproductive

hormone levels in order to prevent energy-requiring processes, such as pregnancy; reductions in the metabolic rate by decreasing thyroid hormone levels; increases in growth hormone levels, that may mobilize energy stores; and reductions in growth-related processes by modulating insulin-like growth factor-1 (IGF-1) levels (Ahima *et al.* 1996, Cunningham *et al.* 1999, Amstalden *et al.* 2000, Chan *et al.* 2003, Chan *et al.* 2008). Other situations of extreme energy deprivation, such as eating disorders (i.e.: bulimia, anorexia nervosa), cachexia and exercise-induced amenorrhea are also associated with hypoleptinemia (Mantzoros *et al.* 1997, Audi *et al.* 1998, Licinio *et al.* 1998, Miller *et al.* 1998, Jimerson *et al.* 2000) and will indeed trigger a neuroendocrine dysfunction with subsequent anovulation and osteoporosis, suggesting that leptin is required for normal reproductive and neuroendocrine function (Welt *et al.* 2004). Moreover, leptin acts as a permissive signal that allows initiation of puberty, as its administration advances sexual maturation in mice (Barash *et al.* 1996, Ahima *et al.* 1997). Of note, leptin deficient *ob/ob* mice is sterile and its sterility can be reversed by leptin treatment (Chehab *et al.* 1996, Mounzih *et al.* 1997). Thus, leptin acts as a signal to the neuroendocrine reproductive system, so that under hypoleptinemia conditions, this adipocyte hormone acts as a metabolic gate to inhibit the neuroendocrine reproductive axis in males and females (Cunningham *et al.* 1999).

Leptin also plays a pivotal role in coupling the immune system and energy balance (Finck *et al.* 1998, Fantuzzi 2009, Procaccini *et al.* 2009), as inflammatory cytokines can influence leptinemia, and leptin can also induce the synthesis of inflammatory cytokines (Granowitz 1997, Sarraf *et al.* 1997, Loffreda *et al.* 1998, Yamaguchi *et al.* 1998). Other pleiotropic actions of leptin include the promotion of linear growth through its influence on energy balance, and the stimulation of secretion of pituitary growth hormone (Gat-Yablonski and Phillip 2008). Moreover, leptin signaling is critical for neural development, as leptin-deficient *ob/ob* and leptin

receptor-deficient *db/db* mice have reduced brain weight and DNA content - of note, this phenotype can be reversed in *ob/ob* mice by leptin administration (Steppan and Swick 1999). This adipocyte-derived hormone was also shown to promote mitosis in many different brain regions (Udagawa *et al.* 2006, Udagawa *et al.* 2007) and to stimulate the formation of neuronal projections among hypothalamic nuclei, thereby influencing the maturation of the hypothalamic feeding control circuitry (Bouret and Simerly 2007). Finally, leptin also plays a role in bone development, growth and homeostasis (Steppan *et al.* 2000, Driessler and Baldock 2010), thyroid function (Ghamari-Langroudi *et al.* 2010) and stress response (Ahima and Osei 2004).

2.2. Leptin receptor signaling

The LepR is the product of the *db* gene and belongs to cytokine class I receptor family (Myers 2004). The full-length receptor has similar signaling capacities as Interleukin 6- (IL6)-type receptor and its helical structure resembles the structure of this cytokine (Baumann *et al.* 1996, Tartaglia 1997). It has been shown to be expressed in the anterior pituitary gland, granulosa and theca cells of the ovarian follicle, several brain regions and other peripheral tissues (Zachow and Magoffin 1997, Finn *et al.* 1998).

Alternative splicing generates several isoforms of LepRs - to date, six splice variants of this receptor have been identified in several species, all of them capable of binding leptin (Chua *et al.* 1997, Tartaglia 1997). The short-isoform of the leptin receptor, LepRa, has a truncated intracellular domain and is detected in many organs and apparently lacks signaling capability (Campfield *et al.* 1996, Wang *et al.* 1997). However, it plays a pivotal role in transporting leptin across the BBB (Bjorbaek *et al.* 1998). Of note, the long isoform, LepRb, has the complete intracellular domain, mediates signal transduction and is highly expressed in the hypothalamus

(Lee *et al.* 1996, Elmquist *et al.* 1998, Chan *et al.* 2002, Zabeau *et al.* 2003). Other than the hypothalamus, leptin acts via the LepRb in the mesolimbic system, to modulate dopamine (DA)-related behaviors including food motivation, reward and emotion (Figlewicz 2003, Fulton *et al.* 2006, Hommel *et al.* 2006, Leinninger and Myers 2008, Scott *et al.* 2009, Leshan *et al.* 2010) and in the nucleus of the solitary tract (NST) of the brainstem to contribute to satiety (Robertson *et al.* 2008). Among other events, LepRb signal transduction is triggered by activation of signal transducer and activator of transcription (STAT), a protein key for leptin-induced gene transcription (Wang *et al.* 1997).

Except for LepRa, all other isoforms contain a conserved intracellular proline-rich box1 domain, required for recruiting janus tyrosine kinase 2 (JAK2), a tyrosine kinase associated to the receptor, which provides the receptor with the required kinase activity for signal transduction (Bjorbaek *et al.* 1997, Ghilardi and Skoda 1997). Thus, following leptin binding to LepRb, JAK2 is autophosphorylated and activated, promoting the subsequent phosphorylation of three LepRb tyrosine residues (Tyr985, Tyr1077, and Tyr1138). Each LepRb tyrosine phosphorylation site serve as docking sites for Src homology 2 (SH2) domain containing proteins, so that: Tyr985 leads to the recruitment of SH2-containing protein tyrosine phosphatase 2 (SHP2), which will trigger the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) (Li and Friedman 1999, Banks *et al.* 2000, Bjorbaek *et al.* 2001, Zhang *et al.* 2004). Alternatively, LepR ^{Tyr985} was shown to recruit suppressor of cytokine signaling 3 (SOCS3), thereby attenuating LepRb signaling (Banks *et al.* 2000, Bjorbak *et al.* 2000, Bjornholm *et al.* 2007); Tyr1077 recruits STAT5 (Gong *et al.* 2007); and Tyr1138 specifically binds and activates the latent transcription factor STAT3 (Vaisse *et al.* 1996, Eyckerman *et al.* 1999, Banks *et al.* 2000, Villanueva and Myers 2008). After binding to the LepRb, STAT3 is phosphorylated on tyrosine residue 705 by

JAK2. Once phosphorylated, STAT3 dimerizes and translocates to the nucleus to induce transcription of a number of genes, including SOCS3, which will in turn negatively feedback on LepRb signaling (Zabeau *et al.* 2003). Importantly, STAT3 is also activated by a variety of cytokines, members of the interferon (IFN) and interleukin (IL) families, including IL-6, IL-10, IL-2, by tyrosine kinase receptors, granulocyte-colony stimulating factor (G-CSF), epidermal growth factor (EGF), ciliary neurotrophic factor (CNTF), among others (Takeda *et al.* 1998, Akira 1999, Levy and Lee 2002).

LepRb signals by a number of neural pathways, including JAK2/STAT3 (Tartaglia 1997, Banks *et al.* 2000), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-K) (Kellerer *et al.* 1997, Niswender *et al.* 2001) and other signaling pathways, including mitogen-activated protein kinase (MAPK) (Rahmouni *et al.* 2009, Trinko *et al.* 2011), 5′adenosine monophosphate-activated protein kinase (AMPK), and the mammalian target of rapamycin (mTOR) (Robertson *et al.* 2008) ***(For leptin receptor signaling pathways, see Figure 1)***.

With respect to LepRb signaling in the modulation of energy balance, it was reported by Zhang and coworkers that conditional deletion of SHP2 in the forebrain, with subsequent impairment in ERK1/2 activation by LepR, induces obesity in mice, although the specific role of ERK1/2 in mediating the feeding effect of leptin needs further clarification (Zhang *et al.* 2004). Another important signaling pathway mediating leptin's effect on energy balance is PI3-K pathway (Niswender *et al.* 2001, Niswender *et al.* 2003). It was previously demonstrated that ICV administration of LY294002, a potent inhibitor of PI3-K signal transduction, prevented leptin-induced anorexia in rats (Niswender *et al.* 2001). In addition, it was suggested that JAK2 activation induces PI3-K activity through the insulin receptor substrate 2 (IRS-2) in the hypothalamus (Niswender *et al.* 2001). As shown in *Figure 1*, a key pathway downstream of the

LepRb by which leptin regulates gene expression and energy homeostasis, involves the activation of LepRb-STAT3 signaling pathway (Bjorbaek *et al.* 1997, Bates *et al.* 2003, Munzberg *et al.* 2003, Zabeau *et al.* 2003, Gao *et al.* 2004).

In humans, homozygous mutations of the *ob* gene leading to complete leptin deficiency and severe obesity have been described in extremely rare cases. Most obese humans are characterized by high circulating leptin levels (Considine *et al.* 1996) accompanied by leptin-resistance (El-Haschimi *et al.* 2000). Clinically, these patients with congenital leptin deficiency due to mutations in the *ob* gene or leptin resistance due to mutations of the *db* gene are obese due to severe hyperphagia (Strobel *et al.* 1998, Farooqi *et al.* 2007). Leptin replacement therapy to these patients will normalize body weight and food consumption by altering the rewarding value of the food and enhancing the response to satiety signals (Farooqi *et al.* 1999, Farooqi *et al.* 2007). Of note, for the vast majority of leptin-resistant obese humans leptin replacement is largely ineffective, inducing little if any weight loss (Heymsfield *et al.* 1999, Roth *et al.* 2008). Thus, leptin's anorectic effect is attenuated during conditions of high-fat (HF) feeding and diet-induced obesity (DIO) (Myers *et al.* 2012). Among the hypothalamic mechanisms involved in resistance to leptin's effect are: a) defects at/or downstream to the LepRb signaling, including impaired STAT3 activation; b) induction of inhibitors of leptin signaling by molecules such as SOCS 3 (Bjorbaek *et al.* 1999) and protein-tyrosine phosphatase 1B (PTP1B) (Zabolotny *et al.* 2002); c) alterations in leptin's transport across the BBB (Munzberg 2008, Myers *et al.* 2008). On the other hand, conditions such as dieting, which reduce body fat stores, lower plasma leptin levels and therefore disinhibit neural circuits driving feeding. Thus, overall leptin serves as a homeostatic regulator for long-term food intake and body weight maintenance (Ahima *et al.* 1996).

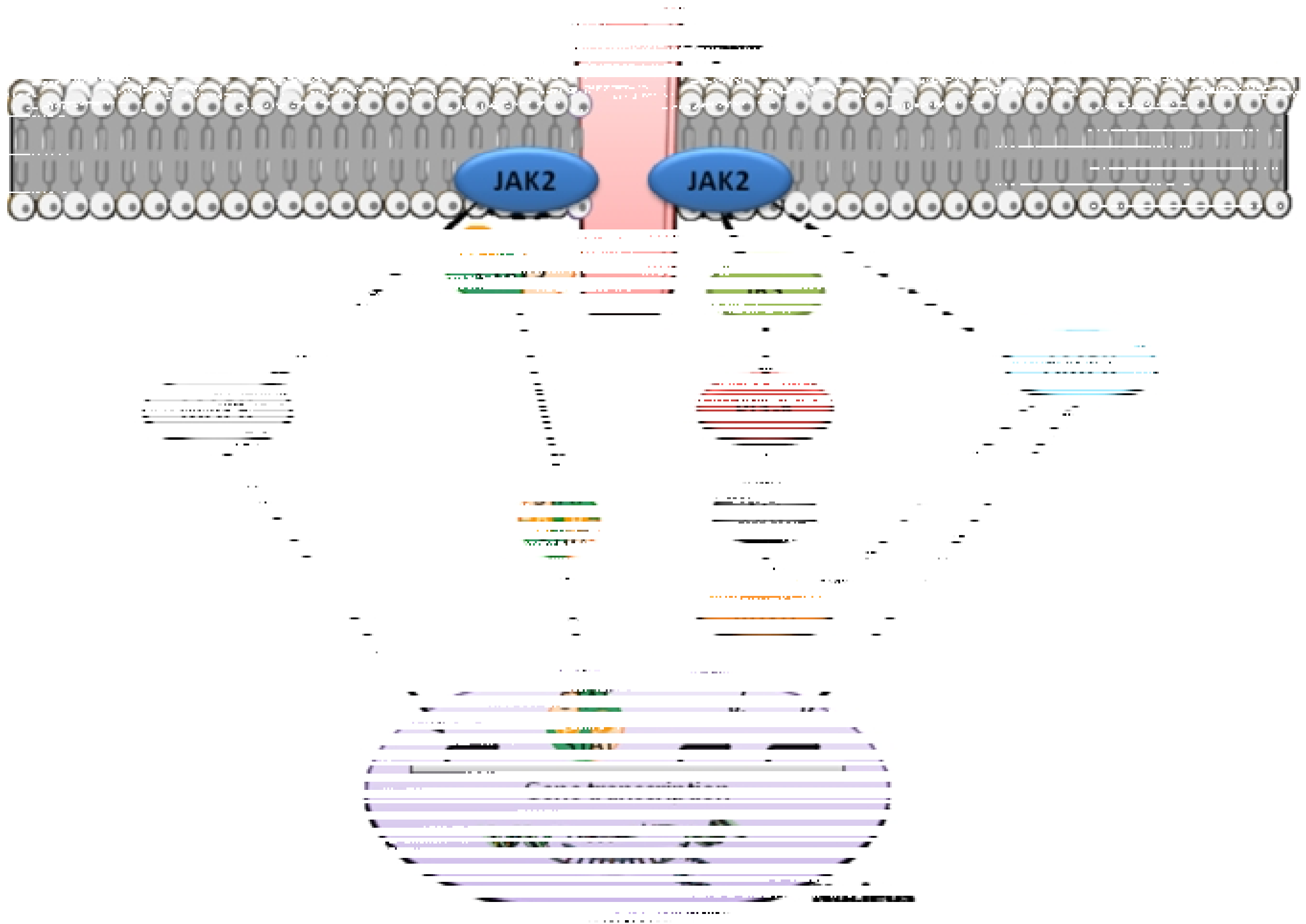

Figure 1: ***Leptin receptor signaling pathways***

2.3. Leptin activates STAT3 signaling in the hypothalamus

STATs are a family of cytokine-activated signaling proteins that can directly bind to DNA and modulate gene transcription (Gao *et al.* 2004). These proteins are characterized by a specific structure consisting of several highly conserved domains: an N-terminal domain, a helical coil domain, the DNA-binding domain, a linker domain, the dimerization domain and a transactivation domain (Horvath *et al.* 1995, Mao *et al.* 2005). The dimerization domain contains a SH2 domain, required for STATs to be recruited to the phosphorylated receptors and for the subsequent formation of dimers (Shuai *et al.* 1994). Thus, after its recruitment to the receptor, STATs becomes phosphorylated on a single tyrosine residue by the JAKs. The following events are: dimerization by reciprocal interaction between phophotyrosine-SH2 domain and nuclear translocation to induce target genes (Levy and Darnell 2002). Of note, all STATs are dimeric proteins in the absence of activating tyrosine phosphorylation (Braunstein *et al.* 2003).

Different STAT proteins were identified in mammals: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6. However, STAT3 has the most pleiotropic functions, playing an important role in embryogenesis, macrophage function, immune regulation, glial and neuron differentiation, among others (Bonni *et al.* 1997, Niwa *et al.* 1998, Takeda *et al.* 1999, Takizawa *et al.* 2001, Levy and Lee 2002, Moon *et al.* 2002, Yoo *et al.* 2002, Welte *et al.* 2003). Moreover, STAT3 is widely distributed throughout the CNS, and has been well implicated in glial and neuronal differentiation (Bonni *et al.* 1997, Takizawa *et al.* 2001, Moon *et al.* 2002) and, most importantly, in leptin-mediated energy homeostasis (Bates *et al.* 2003, Bates *et al.* 2004, Gao *et al.* 2004). STAT3 has a critical tyrosine residue (Tyr 705) in the SH2 region, which is essential for its activation (Calo *et al.* 2003). When phosphorylated/activated, STAT3 induces

the transcription of several genes, including SOCS3, which inhibits JAK2/STAT3 signaling and triggers central leptin resistance (Bjorbaek *et al.* 1999, Flier 2004, Howard *et al.* 2004).

Of note, STAT3 can be activated by a number of cytokines and stressors, via receptor-associated kinases (Akira *et al.* 1994, Wegenka *et al.* 1994, Zhong *et al.* 1994, Vaisse *et al.* 1996, Wishingrad *et al.* 1997, Peterson *et al.* 2000). Similarly, leptin can activate a number of STAT proteins (i.e.: STAT3, STAT5 and STAT6) (Ghilardi *et al.* 1996). However, only STAT3 is activated in the hypothalamus following leptin administration (Darnell 1996, Vaisse *et al.* 1996), giving strength to the notion that STAT3 plays a pivotal role in leptin's action in the hypothalamus to mediate energy balance.

Consistent with leptin's crucial role in the regulation of energy homeostasis and reproduction, neural STAT3 mutant mice (STAT3 $^{N-/-)}$ causes all of the major phenotypes of leptin signaling, recapitulating the obese and infertile phenotype of both LepRb deficient *db/db* mice and leptin deficient *ob/ob* mice (Gao *et al.* 2004). Of note, STAT3 $^{N-/-}$ are leptin-resistant, have reduced energy expenditure, decreased linear growth, increased corticosterone levels, infertility and became hypothermic after exposure to fasting or cold stress (Gao *et al.* 2004). Furthermore, mice with LepRb point mutations, that do not bind STAT3, are hyperphagic and obese with reduced energy expenditure. However, these mice are less hyperglycaemic as compared to *db/db* mice, and have normal reproductive and growth function (Bates *et al.* 2003, Bates *et al.* 2004). Importantly, the major role of STAT3 in energy homeostasis is further supported by evidence that other cytokines that activate STAT3, such as CNTF and IL-6, also decrease body weight (Lambert *et al.* 2001, Wallenius *et al.* 2002).

3. The mesolimbic and nigrostriatal dopamine systems

3.1. The ventral tegmental area (VTA) and substantia nigra (SN): receptors and neurotransmitters

In the ventral midbrain, areas containing dopaminergic neurons were classified into three nuclei: A8 cells in the retrorubral field (RRF), A9 cells in the SN, and A10 cells in the VTA and related nuclei (Oades and Halliday 1987, German and Manaye 1993). Neurons containing TH-positive cell bodies are mainly found in all VTA (Swanson 1982) and SN regions (mostly SN pars compacta) (Aumann *et al.* 2011). Of note, TH is the rate limiting enzyme in the synthesis of the neurotransmitters DA and norepinephine (NE), whose distribution in the midbrain parallels the distribution of DA (Bannon and Roth 1983). Eight major dopaminergic pathways were identified by dissecting DA circuits in the brain and the specific locations of the DA receptors in these circuits (Björklund *et al.* 1984). In the present section, I will focus on two of these pathways that are especially important: the nigrostriatal and the mesolimbic pathway, described below.

The nigrostriatal and mesolimbic systems are functionally interconnected (Haber *et al.* 2000, Everitt and Robbins 2005) and play complementary roles in the hedonic regulation of food intake, reward, motivation, emotion-related behavior, learning and locomotion (Barbeau 1974, McDonald and White 1993, D'Ardenne *et al.* 2008, Phillips *et al.* 2008, Smith and Villalba 2008). The SN lies in the midbrain immediately dorsal to the cerebral peduncles (Frankle *et al.* 2006) and consists of two parts with very different connections and functions: the dorsal part of the SN, known as SN pars compacta (SNc), contains most of the DA neurons of the A9 area (Anden *et al.* 1964, Dahlstroem and Fuxe 1964) and projects to the striatum; the ventral part, or SN pars reticulata (SNr), also contains some DA cells and comprises GABA neurons which projects to the thalamus (Deniau *et al.* 1978, Lynd-Balta and Haber 1994, Yung *et al.* 1998, Joel

and Weiner 2000). The main function of the SNc is motor control (Hodge and Butcher 1980), evidenced by the fact that degeneration of SNc DA neurons leads to the muscle tremors of Parkinson's disease (Kim *et al.* 2003, Pioli *et al.* 2008). However, besides locomotion, SN is also implicated in other behaviors such as addiction (See *et al.* 2007), reward-oriented behaviors (Hikosaka *et al.* 1993, Sato and Hikosaka 2002) and learning (Ljungberg *et al.* 1992, Da Cunha *et al.* 2003, Da Cunha *et al.* 2006). The VTA represents a significant portion of the extrapyramidal system of the basal ganglia, and is home to one of the major populations of DA cells in the brain (Kalivas 1993, Fields *et al.* 2007). It is located close to the midline on the floor of the midbrain, and surrounded by the SN (laterally), mammillary bodies and posterior hypothalamus (rostrally), and by the pons and hindbrain (caudally) (Oades and Halliday 1987). The VTA is composed by approximately of 55-60% dopaminergic, 5-33% GABAergic and 1-15% of glutamatergic neurons (Kalivas 1993, Margolis *et al.* 2006, Yamaguchi *et al.* 2007, Dobi *et al.* 2010). Of note, there's evidence indicating that some DA neurons may also use glutamate as a neurotransmitter (Descarries *et al.* 2008). Selective stimulation and intracellular recording of synaptic responses of VTA DA neurons suggested that both DA and glutamate were coreleased by the same cell (Sulzer *et al.* 1998, Joyce and Rayport 2000). Furthermore, the ability of DA neurons to release glutamate was explained by demonstrating that they selectively express VGLUT2, one of the three vesicular glutamate transporters (Dal Bo *et al.* 2004).

The VTA receives afferents from a numbers of brain regions, such as the amygdala (Wallace *et al.* 1992), the nucleus accumbens (NAc) (Heimer *et al.* 1991), the prefrontal cortex (PFC) (Sesack and Pickel 1992), the dorsal raphe nucleus (DRN) (Gervais and Rouillard 2000), the medial habenular nucleus (MHb) (Cuello *et al.* 1978), the lateral preoptic area (LPOA) and the medial hypothalamus (Swanson 1982). The major neuronal efferents of the VTA are divided into

mesolimbic and mesocortical projections. Mesolimbic projections include outputs to the NAc, tuberculum olfactorium (OT), amygdala, basal nucleus of the stria terminalis (BNST) and lateral septum, hippocampus, the medial part of the thalamus and supraoptic nucleus of the hypothalamus. Mesocortical projections include outputs to the prefrontal, entorhinal, cingulate and occipital cortices, in addition to the periaqueductal gray, parabrachial nucleus, locus coeruleus and dorsal and medial raphe nuclei (Swanson 1982, Oades and Halliday 1987, Pierce and Kumaresan 2006) ***(For mesocorticolimbic and nigrostriatal systems, see Figure 2)***.

Dopamine's action on neuronal circuits depends on two other neurotransmitters: glutamate and GABA (Beaulieu and Gainetdinov 2011). The main inhibitory inputs to the VTA are GABAergic and includes local interneurons and projections from the NAc, ventral pallidum and pontomesencephalic tegmentum (PMT) (Mao and McGehee 2010). As for the excitatory inputs to the VTA, the great majority are glutamatergic projections from the PFC, BNST, amygdala and also from the PMT (Mao and McGehee 2010). In fact, projections from the PMT are both GABAergic and glutamatergic and play an important role in reward-relevant behavior (Lanca *et al.* 2000, Corrigall *et al.* 2002).

Dopamine's physiological actions are mediated by G protein-coupled receptors, which according to their structural, pharmacological, and biochemical properties were divided into D1 and D2 classes of DA receptors (Andersen *et al.* 1990, Tiberi *et al.* 1991, Niznik and Van Tol 1992, Sokoloff *et al.* 1992, Civelli *et al.* 1993, Vallone *et al.* 2000). Dopamine signals through D1-like receptors (D1R) or D2-like receptors (D2R) to stimulate or inhibit, respectively, regional brain activity (Girault and Greengard 2004, Volkow *et al.* 2013). Among D1R are: DA receptors D1 and D5; among D2R are: DA receptors D2, D3 and D4 (Girault and Greengard 2004). According to autoradiography studies, D2R are expressed both post-synaptically on DA target cells and pre-

synaptically on DA neurons (Bouthenet *et al.* 1987, Sales *et al.* 1989, Chen and Pan 2000, Pickel *et al.* 2002, Sokoloff *et al.* 2006, Rondou *et al.* 2010). D2R are known to inhibit cyclic adenosine monophosphate (cAMP) production from adenylate cyclase. Conversely, the D1R family, activates G proteins to stimulate cAMP production from adenylate cyclase. These receptors are found post-synaptically on DA-receptive neurons (Beaulieu and Gainetdinov 2011). Moreover, D1R has been shown to be involved in the direct membrane depolarization of cholinergic cells (Aosaki *et al.* 1998, Pisani *et al.* 2000) and fast-spiking GABAergic interneurons (Bracci *et al.* 2002).

Dopamine receptors are highly expressed in the periphery and the CNS. In the brain, D1R is mostly expressed in striatal regions, NAc, SN, olfactory bulb, PFC and amygdala (Missale *et al.* 1998, Gerfen 2000, Rankin and Sibley 2010). As for the D2R expression, it is mostly expressed in the striatum, NAc, olfactory tubercle, SN, VTA, hypothalamus, cortex, hippocampus, septum and amygdala (Missale *et al.* 1998, Gerfen 2000, Vallone *et al.* 2000).

Previous neuroanatomical and functional studies have detected the presence of $GABA_A$ and $GABA_B$ receptors in the VTA, densely distributed in the paranigral nucleus (Bayer and Pickel 1991, Churchill *et al.* 1992, Ciccarelli *et al.* 2012). Importantly, a large interconnected network of GABAergic neurons in the VTA projects to the PFC and NAc (Van Bockstaele and Pickel 1995, Carr and Sesack 2000).

Among other neurotransmitters affecting DA neurotransmission in the midbrain, a subpopulation of DA neurons containing neurotensin (NT) and cholecystokinin (CCK) were already described in this region (Seroogy *et al.* 1987, Schalling *et al.* 1990, Kiyama *et al.* 1991, Binder *et al.* 2001). Moreover, the presence of nicotinic, endocannabinoid (ECB), serotonin (5-HT), N-methyl-D-aspartate (NMDA), glutamate and Mu opioid receptors were also detected in the VTA and NAc

(Doherty and Pickel 2000, Swanson and Kalivas 2000, Klink *et al.* 2001, Zheng and Johnson 2003, Pickel *et al.* 2004). Of note, 5-HT signaling was previously demonstrated to tightly regulate a number of DA-related functions in both inhibitory and excitatory ways (Fletcher *et al.* 1999, Fletcher *et al.* 2012).

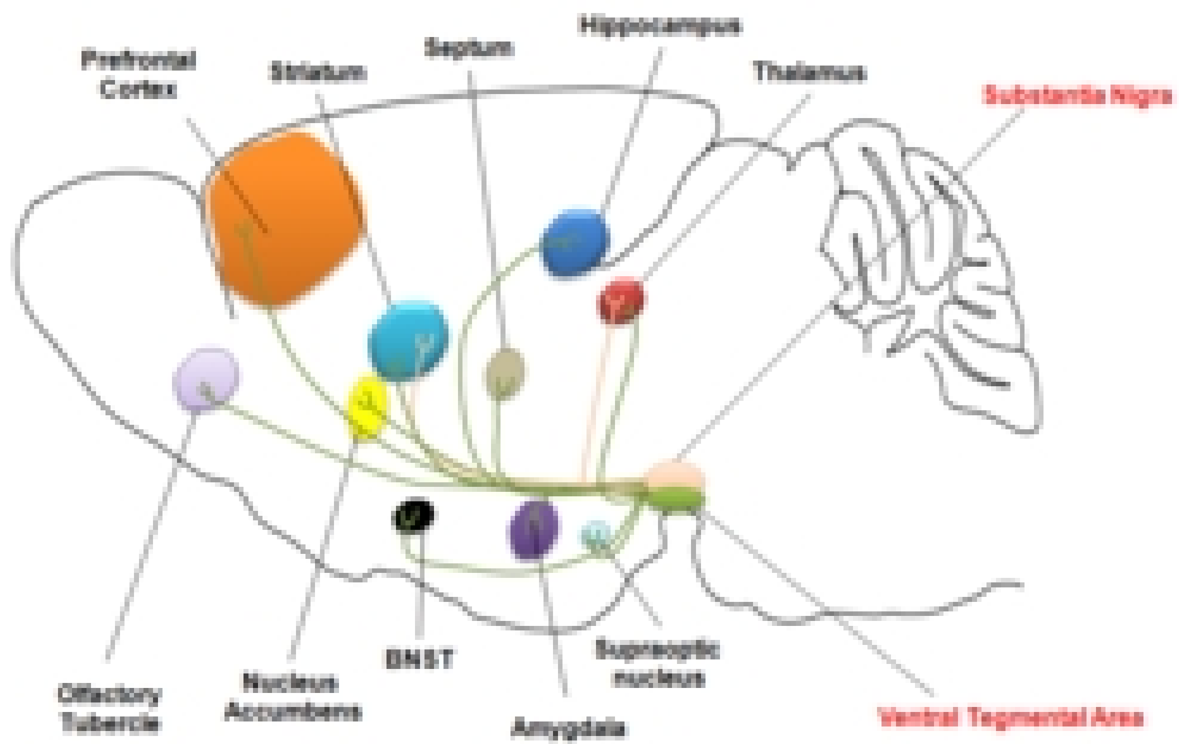

Figure 2: **Schematic representation of mesocorticolimbic and nigrostriatal systems.**

3.2. The dopaminergic reward circuit, feeding and running behavior

Rewards are defined as those objects or goals which we will work to acquire despite of time, energy or effort; simply because they are highly pleasurable (Schultz 2010). Rewards typically serve as reinforcers. By definition, a reinforcer is something that, when presented after a behavior, causes the probability of that behavior's occurrence to increase. Importantly however, not all rewards are reinforcers and not all reinforcers are rewards. A reward can be defined as a reinforcer only if it increases the probability of behaviors that lead to the procurement and/or consumption of the reward (Fulton 2010).

Given the importance of rewards to basic vital functions such as eating, drinking and reproduction, it has been proposed that there exists a neural system responsible for the reward processing (Shizgal 1997). This neural system was identified as the mesolimbic DA pathway, which responds to rewards through the neurotransmitter DA (Arias-Carrion and Poppel 2007, Phillips *et al.* 2008).

As described in a previous section, the mesolimbic pathway is one of the dopaminergic pathways in the brain whose neurons originate in the VTA and connect to the limbic system, including the NAc, the amygdala, the hippocampus and the PFC (Goodman 2008). Dopaminergic activity within the VTA depends on both inhibitory and excitatory inputs that are critically involved in brain mechanisms of reward, reinforcement and emotional arousal (Wise and Rompre 1989). The VTA also send dopaminergic projections to several brain areas that are part of the so-called "brain reward regions", which are interconnected in a highly complex circuitry allowing discerning and reacting to both reward and aversive stimuli in the environment (Russo and Nestler 2013). Of note, dopaminergic projections from VTA to the NAc represent the best characterized reward circuit in the brain (Koob 1992, Russo and Nestler 2013). The

identification of this circuit dates back to 1954, when the Canadian researchers James Olds and Peter Milner discovered that rodents will learn to work for direct electrical stimulation of certain brain regions, and such stimulation seemed to be highly rewarding (Olds and Milner 1954) - this experimental setup is referred to as brain-stimulation-reward (BSR). Similarly, there is data showing that brain self-stimulation also exert a powerful feeling of pleasure in humans (Bishop *et al.* 1963). Electrical brain stimulation is considered as one of the most powerful rewards since it directly activates the reward circuitry (Wise and Rompre 1989). BSR studies demonstrated that electrical stimulation of reward-related areas in the brain was rewarding and reinforcing, and thus, it has provided a useful tool for understanding how natural rewards are processed by the brain and the anatomical structures and neural mechanisms associated with reward circuitry (Rolls 1975, Wise 2002). In addition, BSR experiments provided first evidence that the reward circuitry is subdivided along functional and anatomical lines. Of note, besides playing a role in recognizing rewards in the environment and initiating their consumption, brain reward circuitry also modulates pleasure, motivation, value, and decision making (Koob and Le Moal 2008, Pujara and Koenigs 2014).

The "Dopamine hypothesis" postulates that DA, released in the mesolimbic pathway, promotes reward-related activities (Fibiger *et al.* 1987, Di Chiara and Imperato 1988). An extension of this hypothesis is the "Reward Deficiency Syndrome", stating that animals will engage in behaviors known to increase DA release solely to maintain high levels of this neurotransmitter (Palmiter 2007). In fact, there are two hypothesis on how DA and reward sensitivity influences the expression of motivated behaviors: 1) The above mentioned "Reward Deficiency Syndrome", which hypothesises that individuals with low dopaminergic function seek rewarding substances or behaviors to increase endogenous DA levels and improve mood (Blum *et al.* 2000); 2)

Conversely, when reward hypersensitivity is associated with increased dopaminergic transmission, individuals may feel more motivated to seek for additional reward stimuli solely due to the fact that a specific reward is such a powerful reinforcer (Davis *et al.* 2008).

As for the role of DA signaling in the pursuit of natural rewards, such as food, it was previously described that DA signaling in the NAc mediates the reinforcing effects of food (Wise 2008). On the other hand Baldo and coworkers demonstrated that bilateral injections of either D1R or D2R antagonist into the NAc show little effect on *ad libitum* food intake, although according to the authors, an important caveat to this interpretation is that the doses of D1R or D2R antagonist used may not have been high enough to completely block the effects of DA in food consumption (Baldo *et al.* 2002), suggesting that higher antagonist doses would have decreased feeding, Furthermore, when DA is depleted by intracranial injection of the DA-specific neurotoxin 6-hydroxydopamine (6-OHDA) rodents develop severe motivational and regulatory deficits in feeding and drinking and will die of starvation if DA is not replaced (Ungerstedt 1971, Zigmond and Stricker 1972, Marshall *et al.* 1974). Complementary to these studies are those on DA-specific deficient mice, suggesting that DA is pivotal for vital functions such as feeding, drinking and locomotion, but is not required for the development of neural circuits involved in these behaviors (Zhou and Palmiter 1995). In regards to feeding behavior, rodents lacking DA in dopaminergic neurons (but retaining noradrenergic neurons) can execute behaviors necessary to seek and ingest food, but they do not eat enough to survive (Szczypka *et al.* 1999). In light of the "DA hypothesis", this could be explained by a lack of motivation to respond to the presence of food - i.e.: the animal no longer shows goal-directed behavior because it does not anticipate a reward.

In fact, food has long been known as a powerful behavioral modulator. For example, behaviorist Edward Thorndike used food reward to motivate hungry animals learning to escape from early operant chamber (Thorndike 1898). Consistent with this, a study using an appetitive operant conditioning paradigm demonstrated that dopaminergic transmission within the NAc is required for food-seeking behavior through mechanisms related to hedonic value of food, suggesting DA as an important component of food motivation (Zhang *et al.* 2003). Similarly, dopamine transporter (DAT) KO mice, that show chronically elevated extracellular DA, display increased goal-directed behavior for food reward (Cagniard *et al.* 2006). Moreover, mesoaccumbens DA signaling has been implicated in behavioral responses to cues that predict reward and mechanisms involved in addiction (Robinson and Berridge 2003, Vezina 2004, Nicola *et al.* 2005, Hyman *et al.* 2006). Taken together, these studies suggest that DA is important to acquire information about cues and stimuli related to food rewards and to the behavioral responses to obtain these rewards (goal-directed behavior). However, apparently this neurotransmitter is not involved in the events related to food consumption *per se*.

Associations between the dopamine reward pathway and physical activity behavior have been found both in animal models and in humans. Consistent with that, voluntary wheel running exercise has two relevant components: a motor (movement) component and a motivational (rewarding) component (Knab and Lightfoot 2010), and has been suggested as a strong natural reinforcer and as such, a self-rewarding behavior. Moreover, voluntary exercise is related to changes in gene transcription in reward circuitry (Greenwood *et al.* 2011). Interestingly, a role for the DA system on the regulation of motivation for physical activity was previously described in studies of addiction and feeding showing that some animals will engage in physical exercise in order to obtain food (Pirke *et al.* 1993, Casper 2006, Palmiter 2007). Likewise, animals will

increase drinking and/or eating when these activities were conditioned with the opportunity to run on wheels (Premack *et al.* 1964), demonstrating that voluntary wheel running is a highly motivated behavior.

Notably, goal-oriented behaviors that impose high energy costs, such as running exercise are often motivated by psychological changes associated with prolonged physical activity, better known as "runner's high" (Panksepp *et al.* 2002, Dietrich and McDaniel 2004). Humans usually report this feeling during or right after long distance running, and it likely plays a major role in humans' motivation to keep running. In addition, rodents will readily begin to run when a wheel is introduced (Wagemaker and Goldstein 1980, Partin 1983, Eikelboom and Mills 1988, Looy and Eikelboom 1989), will indeed work by lever pressing to gain access to a running wheel (Belke 2006, Belke 2006, Belke and Christie-Fougere 2006) and spend more time in a place previously associated with the aftereffects of running (Lett *et al.* 2000, Lett *et al.* 2001), indicating that voluntary wheel running activity is naturally rewarding and reinforcing. Interestingly, rats trained to run long distances in a daily basis showed withdrawal signs, such as displaced aggression (i.e.: digging and biting in the test cage), when access to the running wheels is denied (Hoffmann *et al.* 1987). In addition, response to short-term wheel running deprivation is similar to response to short-term deprivation on other reinforcers (such as food): rodents will compensate with an elevation in the behavior when access is restored (Mueller *et al.* 1999). In agreement with this data, other reports demonstrated that a couple of days of running deprivation for rats with extensive wheel experience will induce a substantial increase in their subsequent daily running (Hill 1956, Hill 1958, Hill 1961, Mueller *et al.* 1997). Together, these data point to the conclusion that voluntary running exercise is a powerful natural reinforcer and shares neural reward pathways with other reinforced behaviors such as food (Chaouloff 1989). Consistent with

this idea, mice selectively bred for high wheel running exhibited increased running motivation mediated by reduced D1R signaling in the midbrain (Rhodes and Garland 2003) and as a result of this alteration, high-running mice are more motivated than the respective controls to seek for "running reward" (Rhodes *et al.* 2005). Other studies performed on this same mice suggest that selective breeding based on wheel running rates raise reward threshold in high-running mice by reducing the reinforcing value of shorter running durations (Belke and Garland 2007). The altered DA transmission in mice bred for high wheel running exercise can probably be explained by a decrease in DA concentration, decreased dopamine receptor densities, or reduced second-messenger signaling (Rhodes *et al.* 2005).

Besides being rewarding, when taken to extremes, voluntary wheel running activity can develop into an addictive-like behavior, as suggested by data obtained from surveys among highly committed human runners, reporting: 1) runner's high (a feeling or euphoria after a strenuous bout of exercise), 2) tolerance (the need to increase the distance run to achieve feelings of well-being), 3) addiction (difficulties in job performance and social interactions) 4) symptoms of withdrawal, including depression, irritability, and anxiety, when prohibited from running (Rudy and Estok 1989, Chapman and De Castro 1990, Furst and Germone 1993) and by the number of parallels between wheel running activity and drug-induced reward. For example, rats with a tendency toward addiction develop compulsive running, probably due to a downregulation of dopamine receptors (Werme *et al.* 1999). Moreover, similar to drugs of abuse, chronic running is related to increased delta FosB expression in the NAc of addiction-prone Lewis rats (Werme *et al.* 2002). Of note, the transcription factor delta FosB has been implicated in the development of drug addiction and control of the reward system in the brain (McClung and Nestler 2003, Nestler 2008). Importantly, there is growing data demonstrating that running and drugs of abuse activate

overlapping neural systems (Kanarek *et al.* 1998, Werme *et al.* 2000, Ferreira *et al.* 2006). For example, endurance exercise stimulates the release of endogenous opioid peptides in rats and humans (Christie and Chesher 1982, Janal *et al.* 1984, Werme *et al.* 2000, Angelopoulos 2001) and wheel running attenuates the rewarding effects of morphine in a conditioned place preference test (Lett *et al.* 2002).Providing further evidence of the parallel between addiction and voluntary exercise, experiments conducted in humans demonstrated that bouts of moderate exercise can help attenuate symptoms of daily cannabis use, nicotine and alcohol withdrawal (Ussher *et al.* 2001, Daniel *et al.* 2004, Ussher *et al.* 2004, Buchowski *et al.* 2011). Moreover, the beneficial effects of exercise as an adjunct in treatment of drug addiction was previously suggested to be related to its ability to facilitate DA transmission, normalize glutamatergic and dopaminergic signaling, and reverse drug-induced changes in chromatin via epigenetic interactions with BDNF in the reward pathway (Lynch *et al.* 2013). Thereby, as a natural reward, voluntary running exercise modulates the brain reward circuitry and changes the rewarding effect of addictive substances, thus contributing to reduce the incidence and severity of drug-addictive behavior.

4. Leptin signaling in the mesolimbic dopamine system

4.1. Leptin activates STAT3 signaling in extra-hypothalamic brain regions

Besides being influenced by satiety factors such as leptin, eating behavior is also modulated by dopaminergic neurons of the mesolimbic system (Cota *et al.* 2006, Fulton 2010, Fernandes *et al.* 2013). It is very well appreciated that LepRb activation in hypothalamic regions triggers STAT3 recruitment, phosphorylation and nuclear translocation to induce specific gene transcription and influence energy balance (Li and Friedman 1999). However, leptin also induces STAT3 phosphorylation at residue Tyr 705 in extra-hypothalamic regions.

More recently, a number of reports have focused on the role of LepRb signaling in brain sites other than the hypothalamus, including the hippocampus and VTA (Fulton *et al.* 2006, Hommel *et al.* 2006, Harvey 2007, Robertson *et al.* 2008), where leptin activates LepRb-STAT3 in both DA and GABAergic neuronal populations (Fulton *et al.* 2006). Consistent with this, LepRb mRNA was already detected in the VTA, SN, DRN, thalamus, cerebellum, NTS, among other areas (Elmquist *et al.* 1998, Figlewicz *et al.* 2003, Fulton *et al.* 2006, Hommel *et al.* 2006, Myers *et al.* 2009, Scott *et al.* 2009). Most importantly, colocalization of the LepRb and the dopaminergic marker TH was observed in different brain regions, including the hypothalamic preoptic nucleus, ARC, paraventricular hypothalamic nuclei (PVN) and caudal linear nucleus (Cli) of the midbrain. In addition, a small population of neurons coexpressing LepRb and TH was found in the DRN, periaqueductal gray (PAG), rostral linear nucleus (RLi) and retrorubral field (RRF) nucleus (Hakansson *et al.* 1998, Hay-Schmidt *et al.* 2001, Scott *et al.* 2009). However, not all these LepRb-expressing DA cells can sense peripheral leptin, as observed by lack of STAT3 phosphorylation following systemic leptin administration (Scott *et al.* 2009). In fact, besides the VTA/SN area (Figlewicz *et al.* 2003, Fulton *et al.* 2006, Hommel *et al.* 2006), LepRb- expressing DA neurons only induces pSTAT3 in the PAG and DRN, suggesting that

while many TH neurons express LepRb, very few cells are leptin-responsive (Scott *et al.* 2009) ***(For LepRb signaling in the midbrain, see Figure 3)***.

Interestingly, evidence for the significant role of DA in leptin action, comes from studies performed in mice lacking both leptin and DA. Contrary to leptin deficiency alone (*ob/ob* mice), mice lacking both leptin and DA are hypophagic and lean, developing obesity only when treated with the DA precursor L-DOPA. These findings are suggestive that dopaminergic neurotransmission is required for feeding in *ob/ob* mice (Szczypka *et al.* 2000). Moreover, *ob/ob* mice exhibit a substantial reduction in NAc DA release, do not sensitize to the locomotor-stimulating effects of AMPH (Fulton *et al.* 2006) and have their obese hyperphagic phenotype reversed by peripheral administration of D1R and D2R agonists (Scislowski *et al.* 1999).

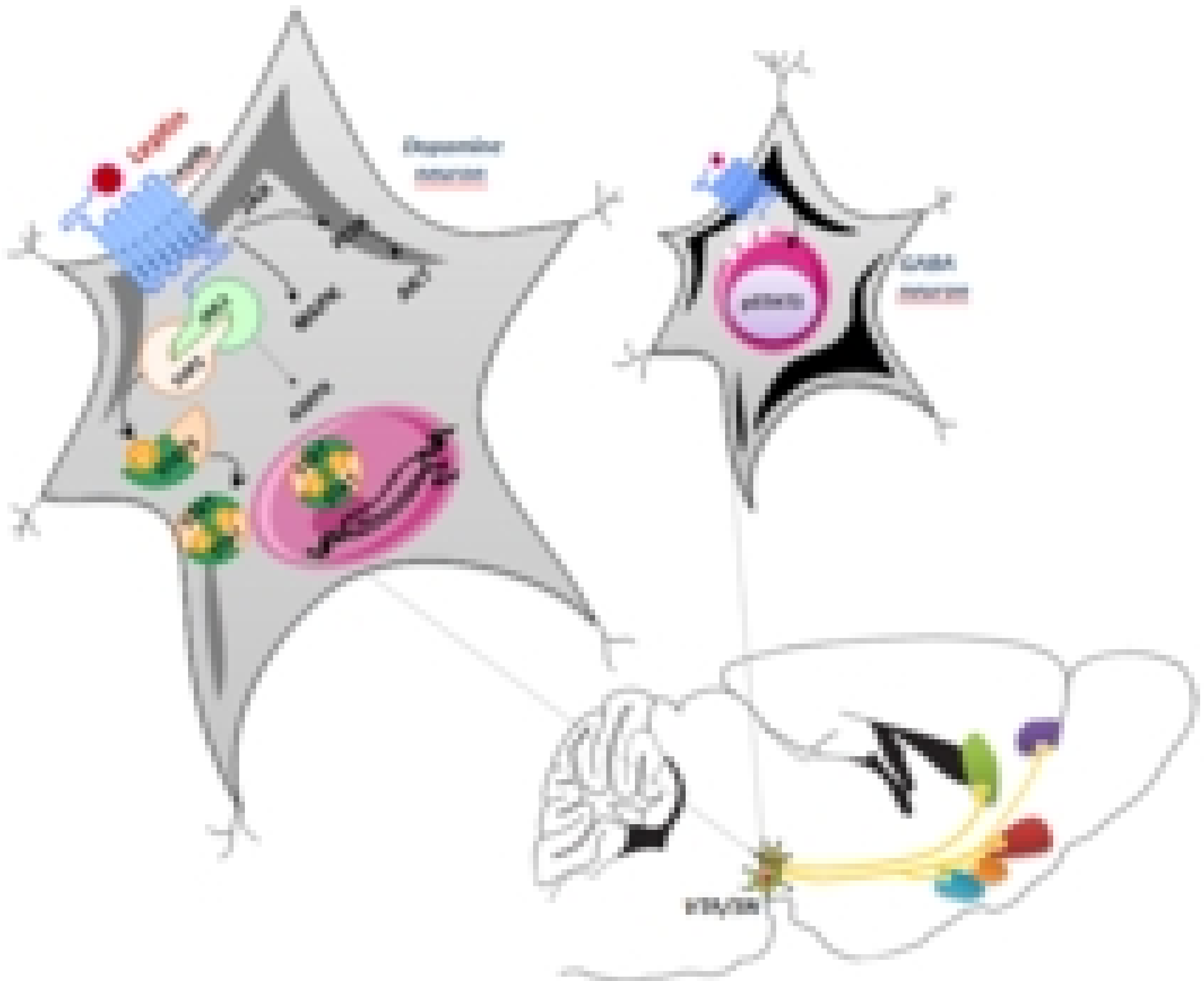

Figure 3: ***Schematic representation of leptin receptor signaling in dopaminergic neurons of the midbrain.***

4.2. Leptin and locomotion

Other than feeding, leptin influences a wide range of DA-related behaviors, including ambulatory activity (Fulton *et al.* 2006, Hommel *et al.* 2006) and voluntary wheel-running exercise (Bates *et al.* 2003, Coppari *et al.* 2005, Shapiro *et al.* 2011, Verhagen *et al.* 2011). However, there is inconsistency in the literature concerning leptin's effect on locomotion. Leptin-deficient *ob/ob* mice have reduced ambulatory activity (Clark and Gay 1972, Dauncey 1986) and reduced voluntary wheel running exercise (Jurgens *et al.* 2006), which can be reversed by leptin administration (Pelleymounter *et al.* 1995, Coppari *et al.* 2005, Fulton *et al.* 2006, Huo *et al.* 2009, Morton *et al.* 2011). Similarly, either an ICV leptin treatment (Ainslie *et al.* 2001, Choi *et al.* 2008) or leptin overexpression in the brain increases voluntary running exercise in rodents, while a partial blockade of LepRb diminishes this behavior (Matheny *et al.* 2009). On the other hand, other studies reported that an intraperitoneal leptin treatment in fed C57Bl/6 wild-type mice, did not affect either ambulatory activity or voluntary exercise (Pelleymounter *et al.* 1995, Morton *et al.* 2011). In contrast, leptin administration reduces hyperactivity in rodents (Exner *et al.* 2000, Morton *et al.* 2011, Verhagen *et al.* 2011) and lack of LepRb signaling in the VTA increases locomotion in male rats (Hommel *et al.* 2006).

Several factors may contribute to these inconsistent reports, including animal's physiological variation in leptin levels (Morton *et al.* 2011). Interestingly, fasting, a condition characterized by reduced leptinemia, is known to increase locomotor activity and voluntary exercise in rodents (Sakkou *et al.* 2007, Morton *et al.* 2011). Consistent with these findings, leptin was shown to abolish hyperactivity triggered by severe caloric restriction in both rodents and humans (Exner *et al.* 2000, Hebebrand *et al.* 2003).

The mechanisms involved in fasting-induced hyperactivity remain poorly elucidated and may represent a manifestation of increased food seeking behavior, driven by a reduced leptin signaling (Overton and Williams 2004, Williams *et al.* 2004). Alternatively, it may also involve increased hyperactivity-induced DA transmission in the reward circuitry (Mathes *et al.* 2010). However the specific role of leptin in the modulation of ambulatory activity and voluntary running exercise as well as the downstream signaling mechanisms needs to be further clarified.

4.3. The effect of leptin on reward

Observations that LepRb is expressed in the brain reward circuitry (Elmquist *et al.* 1998, Figlewicz *et al.* 2003) strongly suggest that there is a crosstalk between energy regulatory signals and the mesolimbic system.

Consistent with the notion that leptin influences the activity on neural circuits controlling food intake to impair the perception of food rewards, animal studies using the palatable food self-administration paradigm demonstrated that leptin inhibits motivated behavior for palatable food (Figlewicz 2003) and decreases operant responding for sucrose (Figlewicz *et al.* 2006, Davis *et al.* 2008). Furthermore, leptin reverses CPP for both sucrose and HF diet (Figlewicz *et al.* 2001, Figlewicz *et al.* 2004) and rodents fed a HF diet showed reduced CPP for AMPH (Davis *et al.* 2008). Notably, a human study using functional magnetic resonance imaging (fMRI) demonstrated that leptin administration to patients with congenital leptin deficiency increases the ability to discriminate between the rewarding properties of food (Farooqi *et al.* 2007), further highlighting the notion that leptin markedly affects food reward.

With respect to leptin's role in rewards other than food, it was previously reported that leptin decreases rewarding electrical brain stimulation (Fulton *et al.* 2000, Fulton *et al.* 2004), evoked

DA release in the NAc (Krugel *et al.* 2003) and inhibits DA neuronal firing (Hommel *et al.* 2006). In addition, leptin influences sexual motivation (Wade *et al.* 1997, Schneider 2006, Schneider *et al.* 2007) and drug self-administration. For example, this adipocyte hormone was shown to attenuate the effect of food restriction to cause heroin relapse (Shalev *et al.* 2001) and increased response to psychostimulant drugs, such as AMPH (Hao *et al.* 2004, Fulton *et al.* 2006), suggesting an excitatory role of leptin on reward pathways. Moreover, obese individuals self-administer drugs of abuse less often than lean individuals (Davis *et al.* 2010), suggesting a potential role for leptin in drug reward.

As previously mentioned in this thesis, voluntary wheel running exercise is a naturally rewarding and reinforcing behavior and shares similar neurobehavioral characteristics with either drugs or foods as reinforcers (Pierce *et al.* 1986, de Visser *et al.* 2007). Although a role for leptin in the modulation of running reward has yet to be described, Girard and coworkers reported that mice selectively bred for high wheel running activity have reduced plasma leptin levels which correlates with increased running speed and distance (Girard *et al.* 2007), suggesting enhanced motivation for foraging behavior (Exner *et al.* 2000). Alternatively, the low body fat and hypoleptinemia observed in high-runner rodents could be explained by "reward substitution", meaning that for these rodents, the rewarding value of running became much greater than the rewarding value of food (Meek *et al.* 2012). This "reward substitution" was also observed in studies conducted by Scarpace and co-workers, demonstrating that wheel running activity reduces the preference of rodents for a HF diet and modulates LepRb-STAT3 signaling in the brain reward circuitry (Scarpace *et al.* 2010). Thus, it is possible that, in the case of high-runner rodents, voluntary running is a highly valued reward, which can be even greater than food reward and is also modulated by leptin. Important to remember that mice bred for high rates of

running wheel activity have dysregulation of DA system in the brain, which is linked to a number of aberrant behaviors, such as addiction, hyperphagia and compulsive exercise (Mathes *et al.* 2010)

Leptin's ability to reduce reward-related behaviors occurs via an interaction with midbrain DA signaling pathways (Carr 2002). However, the specific cells and signaling pathways mediating the actions of leptin in the mesolimbic DA reward circuit remain to be better characterized.

5. Leptin's role in emotion and mood

The localization of the LepRb in limbic structures implicated in the control of mood and emotion, such as the hippocampus, cortex and amygdala, suggests a potential role for leptin in emotional behavior (Mercer *et al.* 1996, Elmquist *et al.* 1998, Leshan *et al.* 2006, Scott *et al.* 2009). Consistent with that, there is data showing that this adipocyte-derived hormone affects emotional behavior via its action in limbic regions (Harvey 2007). Moreover, analyses of leptin-induced c-fos mRNA expression revealed a link between behavioral actions of leptin and neuronal activity of specific limbic areas, particularly the hippocampus (Lu *et al.* 2006). Providing a connection between mood disorders and adiposity signals, many reports suggested that leptin has antidepressant-like properties (Charnay *et al.* 2000, Lu *et al.* 2006, Lu 2007, Garza *et al.* 2012, Guo *et al.* 2012, Milaneschi *et al.* 2012, Guo *et al.* 2013). Leptin administration reduces behavioral despair in rats via its action in the hippocampus (Sahay and Hen 2007) and stimulates hippocampal neurogenesis - a feature shared by antidepressants (Santarelli *et al.* 2003). In addition, *ob/ob* mice exhibited a depressive-like behavior in the forced-swim test (FST), as compared to their lean littermates (Collin *et al.* 2000), and peripheral leptin administration improved behavioral despair in rats (Lu *et al.* 2006). Briefly, FST or *Porsolt* test is a well characterized model used to screen depression-like behavior in rodents (Porsolt *et al.* 1978).

Chronic stress is a well known trigger for depression in humans (Lu 2007). In agreement, rodents exposed to chronic unpredictable stress or chronic social defeat stress showed decreased plasma leptin levels and increased despair, which was reversed by systemic leptin administration (Lu *et al.* 2006). In a more recent study, Yamada and coworkers demonstrated that peripheral leptin administration reduces depressive-like behavior in lean mice via an upregulation of brain derived

neurotrophic factor (BDNF) in the hippocampus (Yamada *et al.* 2011). Importantly, BDNF signaling in the hippocampus has been strongly implicated in the control of depression (Shirayama *et al.* 2002, Karege *et al.* 2005). Similarly, lack of LepRb signaling in the dorsal cerebral cortex and in the dentate gyrus in the hippocampus, also increases behavioral despair in mice (Guo *et al.* 2012). Furthermore, plasma leptin levels are reduced either in humans with major depression or in rodents with depression-like behavior, and both peripheral and hippocampal administration of this adipocyte hormone improved symptoms (Kraus *et al.* 2001, Atmaca *et al.* 2002, Westling *et al.* 2004, Jow *et al.* 2006, Lu *et al.* 2006). Consistent with these findings, *post-mortem* studies of depressed patients who committed suicide revealed a downregulation of leptin receptors in the frontal cortex (Lalovic *et al.* 2010).

Depressive disorder can often coexist with other mood disorders, such as anxiety (Sartorius *et al.* 1996). Providing another means of influencing the mesolimbic DA system, Liu and coworkers demonstrated that leptin has anxiolytic properties such that lack of LepRb signaling specifically in DA neurons induces an anxiogenic phenotype and increases burst firing of DA and D1R signaling in the central amygdala (CeA) (Liu *et al.* 2010, Liu *et al.* 2011). In agreement with that, both leptin receptor- (*db/db*) and leptin-deficient (*ob/ob)* mice displayed an anxiogenic phenotype (Finger *et al.* 2010, Dinel *et al.* 2011), which, in the case of the *ob/ob* mice, can be ameliorated by systemic leptin treatment (Asakawa *et al.* 2003). Providing further evidence of leptin as a neurotrophic hormone with anxiolytic and antidepressant properties, there is literature showing that depression and anxiety are common illnesses in patients with disorders characterized by hypoleptinemia, such as anorexia nervosa (Braun *et al.* 1994, Grinspoon *et al.* 1996, Pollice *et al.* 1997, Holtkamp *et al.* 2004, Hebebrand *et al.* 2007) and functional hypothalamic amenorrhoea (Fava *et al.* 1984, Warren *et al.* 1999). Furthermore, Lawson and

coworkers observed that low leptin levels are associated with increased symptoms of anxiety in women, independently of total body fat mass (Lawson *et al.* 2012).

In spite of all these findings, the neural pathways as well as the specific functional role of leptin receptor signaling in midbrain DA neurons in the regulation of emotional behavior needs further clarification.

Chapter II

Objectives & General Methods

6. Objectives

6.1. First Objective

Our first objective was to investigate whether STAT3 signaling in DA neurons could modulate the impact of leptin on feeding, food-motivated behavior, locomotion and NAc DA release. Moreover, we sought to determine if leptin influences running reward and if it depends on STAT3 signaling in midbrain DA neurons. This was accomplished by generation and phenotypic characterization of conditional KO mice lacking the main phosphorylation site (tyr705) of the STAT3 gene specifically in DA neurons, hereafter referred to as $STAT3^{DAT\text{-}KO}$ mice. This work constitutes the first article given in *Chapter III*: *"Leptin suppresses the rewarding effects of running via STAT3 signaling in dopamine neurons"* by Fernandes, M.F.; Sharma, S.; Mogra, S. and Fulton, S.

6.2. Second Objective

This study was undertaken to assess the contribution of STAT3 signaling in DA neurons on emotion-related behaviors. To address this question we generated DA-specific STAT3 KO mice ($STAT3^{DAT\text{-}KO}$) by crossing dopamine transporter DAT^{Cre} mice with $STAT3^{lox/lox}$ mice. Then, we studied the behavioral phenotype of $STAT3^{DAT\text{-}KO}$ female mice in anxiety and depression-like behaviors. Pharmacological and molecular approaches were used to shed light on the potential signaling mechanisms underlying the anxiogenic behavior observed in $STAT3^{DAT\text{-}KO}$ female mice. This study constitutes the second article given in *Chapter III*: *"Deletion of STAT3 in midbrain dopamine neurons increases anxiety-like behavior in female mice"* by Fernandes, M.F.; Sharma, S. and Fulton, S.

7. General methods

The *Cre-loxP* system overview and STAT3$^{DAT\text{-}KO}$ mice generation

The *Cre-loxP* system is a special type of site-specific recombination which allows for conditional mutagenesis of a gene of interest, thus representing a powerful tool in making alterations to the mouse genome (Nagy 2000, Novak *et al.* 2000). This technology allows the investigation of the role of specific genes in different regions or cell types, without confounding effects caused by systemic influences. It was first described in bacteriophage P1 and consists of a sequence-specific DNA recombinase (Cre) and a DNA sequence flanked by loxP sites, which is recognized by the Cre-recombinase (Zou *et al.* 1994). Because both Cre gene and loxP sites are not native to the mouse genome, they must be introduced by transgenic technology (Nagy 2000). Thus, when the enzyme Cre-recombinase is introduced to a system expressing loxP sites in the genome, it will catalyse a recombination event between the two loxP sites. Of note, the results of the recombination depend on the orientation of the loxP sites: 1) if both loxP-sites have the same orientation on a chromosome segment, the loxP-flanked DNA will be excised and eliminated; 2) if the loxP sites are oriented in opposite directions, Cre recombinase mediates the inversion of the floxed segment; 3) if the loxP sites are located on different chromosomes, Cre recombinase mediates a chromosomal translocation (Nagy 2000).

On the present work, Cre and loxP mice colonies were developed separately and crossed to produce a *Cre-lox* strain. More specifically, we generated STAT3$^{DAT\text{-}KO}$ mice, whereby dopamine transporter-Cre (DAT^{IresCre}) mice were crossed with STAT3$^{lox/lox}$ mice (obtained from Dr. Shizuo Akira, Osaka, Japan) in which loxP sites flank exon 22 of the STAT3 gene, encoding the tyrosine residue 705 ***(For breeding schematics, see Figure 4)***. Of note, DAT^{IresCre} (or DAT

knock-in) have Cre recombinase expression directed to DA neurons, without disrupting endogenous DAT expression (Backman *et al.* 2006, The Jackson Laboratory 2014).

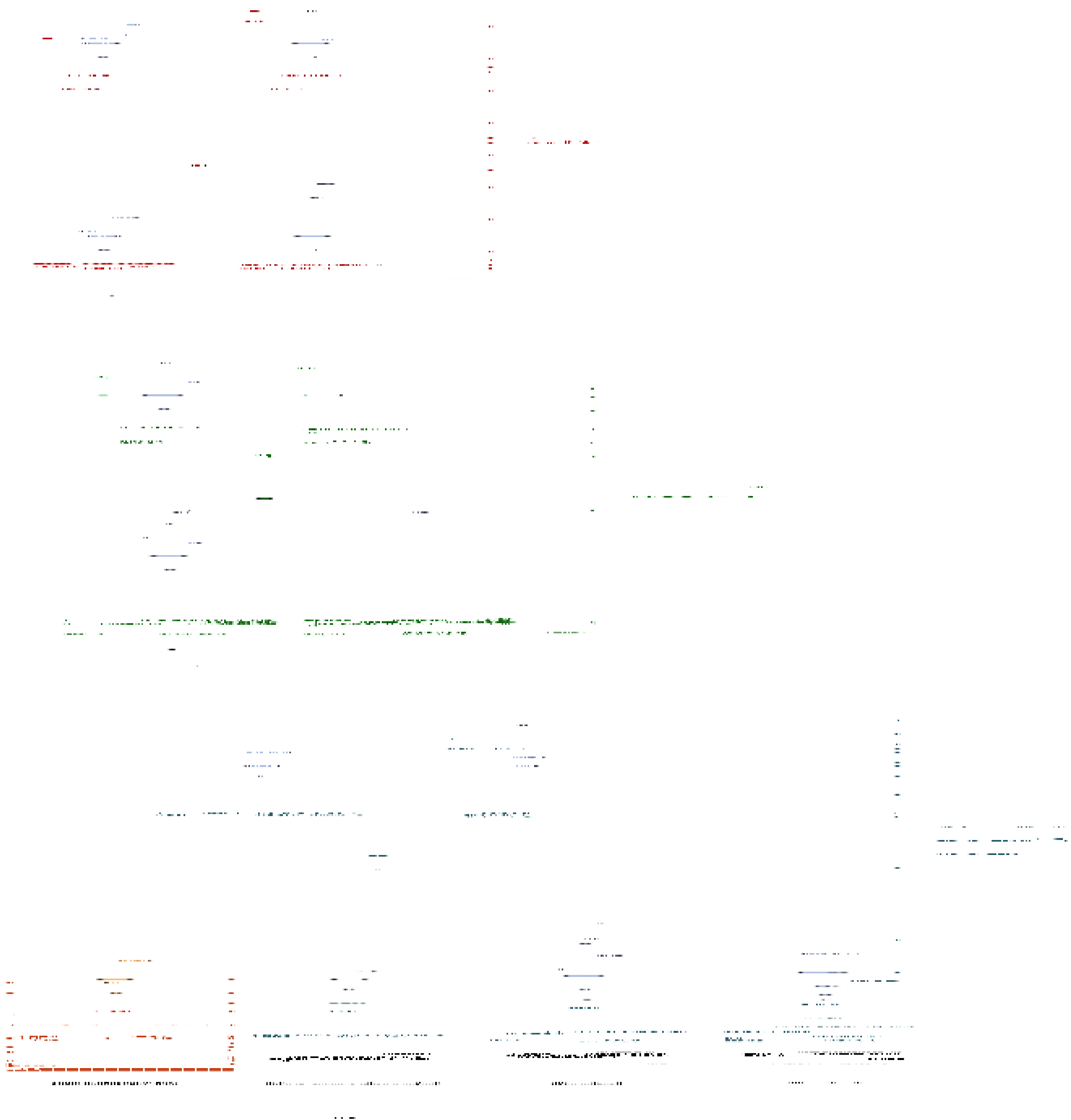

Figure 4. ***Breeding strategy to obtain STAT3^{DAT-KO} mice:*** 1) First step: to obtain male heterozygous *Cre* mice. Thus male heterozygous *Cre* were bred with female homozygous *Cre*

mice, to generate male heterozygous *Cre* mice; 2) Second step: to obtain male heterozygous *Cre* / heterozygous *Lox* mice. Thus, male heterozygous *Cre* / homozygous *Lox* were bred with female homozygous *Cre* / homozygous *Lox* ($STAT3^{lox/lox}$), to generate male heterozygous *Cre* / heterozygous *Lox* mice; 3) Third step: to obtain the heterozygous *Cre* / homozygous *Lox* mice ($STAT3^{DAT\text{-}KO}$ mice). Thus, male heterozygous *Cre* / heterozygous *Lox* were bred with female homozygous *Cre* / homozygous *Lox*, to generate $STAT3^{DAT\text{-}KO}$ and respective control mice.

Chapter III

Articles

8. Articles

8.1 Article #1

Contributions:

Maria Fernanda A. Fernandes, contributed by performing most of the experiments (95%), except for the qPCR to detect the disrupted STAT3 gene (performed by Shabana Mogra) and the fast-scan cyclic voltammetry (performed by Dr. Stephanie Fulton). Sandeep Sharma contributed for the setting up of the behavioral experiments. Maria Fernanda A. Fernandes also created the graphics and wrote the manuscript. Dr. Stephanie Fulton also contributed in the results discussion, redaction and correction of the manuscript.

Short article

Leptin suppresses the rewarding effects of running via Stat3 signaling in dopamine neurons

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Summary

Dopaminergic (DA) neurons of the midbrain are part of a neural circuit involved in reward and motivation. To determine the role of the Signal Transducer and Activator of Transcription 3 (STAT3) in this circuitry, we inactivated STAT3 signaling in DA neurons of mice ($STAT3^{DAT-KO}$ mice). Lack of STAT3 in DA neurons resulted in no changes in feeding, while it increased locomotion, running wheel exercise and running reward. Moreover, $STAT3^{DAT-KO}$ mice exhibited impairments in learning an appetitive operant conditioning task and reduced NAc DA release. Taken together, these results suggest that STAT3 mediates the action of leptin in the midbrain to decrease locomotor behavior and to increase DA tone, while it does not affect food intake, implying that different LepRb signaling molecules regulate distinct behavioral and biochemical actions of midbrain leptin. Additionally, STAT3 signaling in DA neurons enhances the rewarding value of running and facilitates instrumental learning. The findings described here shed light on the cells and neural pathways mediating the actions of leptin in the midbrain.

Highlights

► STAT3 signaling in midbrain dopamine neurons is a key modulator of spontaneous and voluntary physical activity ► Leptin in the ventral tegmental area abolishes the rewarding effects of running in a manner that depends on STAT3 in dopamine neurons ► Loss of STAT3 in dopamine neurons blunts DA overflow in the nucleus accumbens

Introduction

Behavior is a central component of energy balance and all behavior entails energy expenditure in the form of physical activity. Activity that increases the likelihood that a physiological need will be met is referred to as appetitive, and appetitive behaviors are crucial to the fulfillment of energy demands. Food-directed appetitive behaviors such as foraging, scavenging and hunting may benefit from increased physical activity and endurance (Bramble and Lieberman, 2004). The capacity for endurance running in cursorial mammals, particularly humans, is considered to have evolved as a means to increase the payoff of food-directed behaviors targeting distant or shifting food sources (Bramble and Lieberman, 2004; Carrier, 1984).

Physical activity is strongly influenced by the metabolic state of the organism. The adipocyte-derived hormone leptin is a critical signal controlling feeding and energy expenditure and a fall in leptin levels is a major component of the physiological response to fasting (Ahima et al., 1996). The influence of leptin on energy expenditure includes its ability to alter spontaneous locomotor activity and voluntary running in a manner that largely depends on food availability. Leptin increases locomotor activity and/or voluntary running during fed states in leptin-deficient humans (Farooqi et al., 1999; Licinio et al., 2004), *ob/ob* mice (Ahima et al., 1999; Morton et al., 2011; Pelleymounter et al., 1995), lean mice (Morton et al., 2011) and rats (Choi et al., 2008; Meek et al., 2012; Morton et al., 2011), or has no effect on running in fed wildtype mice (Morton et al., 2011; Pelleymounter et al., 1995). In contrast, leptin decreases locomotor activity and voluntary wheel running when food is restricted in *ob/ob* and lean mice (Morton et al., 2011) and rats (Exner et al., 2000; Verhagen et al., 2011). Physical activity escalates with food restriction, a phenomenon viewed as an expression of increased food acquisition behaviors (Bartness et al., 2011; Overton and Williams, 2004; Sakkou et al., 2007). That leptin could be involved in this

process is supported by evidence that circulating concentrations are inversely related to physical activity or voluntary running in humans (Franks et al., 2003; Hebebrand et al., 2003), rats (Exner et al., 2000; Hebebrand et al., 2003), wildtype mice (Morton et al., 2011; Thorburn et al., 2000) and in mice selectively bred for high running capacity (Girard et al., 2007). Furthermore, plasma leptin levels correlate negatively with marathon run-time independent of BMI (Bobbert et al., 2012) and with run speed and duration in high-running mice (Girard et al., 2007), suggesting that leptin could impact the motivational and rewarding effects of running. Indeed, endurance running is rewarding in humans ("runners high") (Morgan, 1985; Partin, 1983; Wagemaker and Goldstein, 1980) and rodents (Belke and Wagner, 2005; Collier and Hirsch, 1971; Kagan and Berkun, 1954; Novak et al., 2012), yet little is known about the neural pathways and signaling mechanisms involved.

Leptin modulates multiple components of brain reward circuitry (Fulton et al., 2000) and inhibits the rewarding effects of food (Figlewicz et al., 2004; Figlewicz et al., 2006). DA neurons of the midbrain ventral tegmental area (VTA) that project to limbic and cortical sites are essential for the control of appetitive and reward-relevant behaviors (Salamone and Correa, 2012; Schultz, 2013). Leptin receptors (LepR) are expressed on midbrain DA neurons (Figlewicz et al., 2003; Scott et al., 2009), and functional LepR signaling in DA neurons of the VTA is well-documented (Domingos et al., 2011; Fulton et al., 2006; Hommel et al., 2006; Liu et al., 2011). Leptin has anorectic actions in the VTA (Bruijnzeel et al., 2011; Hommel et al., 2006; Trinko et al., 2011) whereas LepR knockdown in the VTA increases food intake, spontaneous locomotor activity (Hommel et al., 2006) and food-motivated behavior (Davis et al., 2011). LepR signaling involves the activation of signal transducer and activator of transcription 3 (STAT3) (Banks et al., 2000; Tartaglia, 1997). Leptin phosphorylates STAT3 in DA and GABA neurons of the midbrain

(Fulton et al., 2006; Hommel et al., 2006), however leptin-induced pSTAT3 is observed only in a subset of LepR-positive DA neurons (Scott et al., 2009) suggesting that DA neurons responsive to leptin are heterogeneous and that LepR-STAT3 signaling may have unique neurobehavioral actions.

Few studies have investigated the mechanisms underlying the effects of leptin on locomotor activity. The hypoactivity of obese, leptin receptor deficient mice is corrected by restoration of LepR to the mediobasal hypothalamus (Coppari et al., 2005) or proopiomelanocortin (POMC) neurons (Huo et al., 2009) underscoring the role of the hypothalamus in leptin control of locomotor behavior. However, the divergent effects of leptin on physical activity in conditions where food is limited versus freely available suggest that separate neural processes are involved. We sought to investigate the absence of STAT3 in DA neurons in the control of physical activity, feeding and DA tone and to determine if leptin impacts the rewarding effects of running via STAT3 signaling in VTA DA neurons.

Experimental procedures

Animals

All experiments were carried out in accordance with the guidelines and approval of the Institutional Animal Care Committee of the CHUM Research Center. Floxed STAT3 mice (C57Bl6 background) in which loxP sites flank exon 22 of the STAT3 gene that encodes a tyrosine residue (tyr705) essential for STAT3 activation were graciously provided by Dr. Shizuo Akira (Osaka, Japan) (Takeda et al., 1998). Female mice homozygous for the floxed STAT3 allele were crossed with male mice heterozygous for the floxed STAT3 allele and heterozygous for the DAT::Cre transgene (B6.SJL-*Slc6a3*$^{tm1.1(cre)Bkmn}$/J) (Backman et al., 2006) to generate DAT^{Cre};$STAT3^{fl/fl}$ mice and littermate controls ($STAT3^{fl/fl}$ or $STAT3^{fl}$). In addition to littermate controls, DAT::Cre mice were used as secondary controls for wheel running experiments. Male mice were weaned at P28 and housed in a temperature and humidity controlled room that was maintained on a 12:12 hour reverse light/dark cycle. All experiments were conducted in the dark phase of the cycle.

Metabolic assessments

Mice were individually housed with free access to standard chow and water. Food intake and body weight was measured 3 times per week. Lean and fat mass were determined at 23 weeks of age using an Echo MRI quantitative nuclear magnetic resonance system (Echo Medical Systems, Houston, TX, USA). Metabolic efficiency (inverse of feed efficiency) was calculated for each subject by dividing energy intake (Kcal) over the 12-week period by body weight gain in grams. Following two days of habituation to cages, spontaneous locomotor activity was measured for 24 hours in mice (11-12 weeks of age) using automated metabolic cages (Accuscan Instruments

Inc., Columbus, OH, USA) consisting of 16 light beam arrays in x, y and z axes. Distance travelled (horizontal activity) was measured by computer-controlled software.

Voluntary wheel running

Voluntary wheel running was assessed in a separate cohort of individually-housed mice with continuous access to a low profile wireless running wheel in the cage (Med Associates, Inc., VT, USA). Wheel revolutions were constantly monitored via transmitters and recorded by data acquisition software (Med Associates Inc., St. Albans, VT, USA) on a nearby computer. Each full wheel revolution is equivalent 36.9 cm distance travelled. Mice had free access to food and water throughout and were not used for any other experiment.

Intra-cerebral cannulations and food intake

Anesthetised mice were placed in an ultraprecise mouse stereotaxic frame (Kopf; Tujunga, CA, USA) and maintained on Isoflurane (1.5%+oxygen). *VTA cannula implantation*: With bregma and lambda in the same horizontal plane, a bilateral guide cannula (26 gauge; 0.8mm distance between cannulae; Plastics One, Roanoke, VA, USA) was lowered in the VTA (AP, –3.4 mm; ML, ± 0.4 mm; DV, −4.7 mm). *Intracerebroventricular (ICV) cannula implantation*: A single guide cannula (26 gauge; Plastics One, Roanoke, VA, USA) was lowered into the right lateral ventricle (AP, –0.5 mm; ML, ± 1 mm; DV, −2 mm). Cannulae were anchored to the skull surface with dental cement and occluded with metal obturators of the same length. Following recovery and weight regain, ICV cannula placements were assessed by verifying the drinking response to injection of angiotensin II (10ng/1µl). Mice were habituated to a powder chow diet for 3 days for food intake measures. Powder food was placed in food cups that were fixed to the bottom of a larger container to catch spillage. Following an overnight fast, separate groups of mice received a bilateral VTA injection of leptin (200ng/500nl/side) or vehicle (PBS) or an ICV injection of

leptin (1ug/1ul; recombinant mouse leptin from Dr. A.F. Parlow, NIDDK) or vehicle right before dark cycle onset using internal cannulas (Plastics One) attached to 1µl Hamilton microsyringe.

Operant responding for food rewards

Mice were trained to press a lever for sucrose pellets according to published procedures (Sharma et al., 2012). Briefly, mice were trained in operant chambers, house in sound-attenuating boxes, equipped with two retractable levers (Med Associates Inc., St. Albans, VT, USA) and a pellet dispenser delivering 20mg sucrose pellets (Bio-Serv, Frenchtown, NJ, USA) to a receptacle positioned between the levers. One lever was designated active (triggers reward delivery) and other as inactive (no reward) in a counterbalanced manner. Operant training on a fixed ratio-1 (FR1) schedule of reinforcement (1 lever press = 1 sucrose pellet) was carried out for 8 days and the number of lever presses on each lever and rewards earned calculated by computer-controlled software (Med Associates Inc.)

Hedonic feeding – "Dessert test"

We utilized a paradigm in which sated rodents voluntarily over consume a palatable test diet (Choi et al., 2010). First, mice were entrained over 19 consecutive days to consume standard chow in a 4-hour window in the first half of dark cycle (Fig. 2E). Prior to test day (Day 20) mice were exposed to a small amount of high-fat/sugar food (D12231; Research Diets, Inc., New Brunswick, NJ) to prevent neophobia. On the day of testing, chow intake was measured each hour during 4 hours. Following the fourth hour of chow access, a separate set of food cups containing high-fat/sugar food were placed in each cage and weighed one hour later.

Running reward

A new cohort of singly-housed mice (11-14 weeks of age) had free access to homecage running wheels for 3 weeks. Mice were then subjected to a conditioned place preference (CPP) task using a three-compartment, automated mouse CPP apparatus (Fig. 3A; Med Associates Inc., St. Albans, VT, USA). Pre-test: Mice were confined to both the black and white compartments of the CPP apparatus for 5-min to permit habituation to each compartment. Mice were then allowed to move freely in the apparatus for 15-min during which the amount of time spent in each compartment was recorded. Conditioning trials: Mice had 2-hour access either to a running wheel (paired trial) or a locked running wheel (unpaired trail) and were then confined to either the black or white compartment for 30 min. Paired and unpaired conditioning trials took place in opposite compartments (counterbalanced across mice) on alternating days, thus each mouse ran every-other day (Fig. 3A). Post-test: Following the last day of conditioning (Day 14), mice were placed back in the open CPP apparatus for 15 min. The amount of time spent in each compartment was once again recorded and the proportion of time spent in the paired side was compared to that obtained during the pre-test.

Fast scan cyclic voltammetry

Carbon-fibre electrodes were constructed and prepared according to Kawagoe et al. (Kawagoe et al., 1993). Electrodes were calibrated *in vitro* using known concentrations of DA (1μM dopamine HCL; Sigma). Mice (6-7 weeks of age) were anesthetised with Isoflurane prior to decapitation. Of note, at high clinical concentrations (<0.7 mM), Isoflurane was shown to inhibit the release of glutamate without significantly affect dopamine release (Westphalen *et al.* 2013). Coronal slices (300μm) were cut using a vibratome at the level of the nucleus accumbens (NAc) (+0.90 to +1.30 mm from Bregma) in ice-cold, carbogenated ACSF containing 125 mM NaCl, 26 mM NaHCO3, 2.5 mM KCl, 2.4 mM CaCl2, 1.3 mM MgSO4, 0.3 mM KH2PO4 and 10 mM

D-Glucose. Fast-scan cyclic voltammetry was used to measure electrically-evoked DA release according to published procedures (Fulton et al., 2011). Briefly, DA release in the NAc core (Fig. 4A) was evoked at 5-min intervals by a single 400μA, 1.2 ms, rectangular electrical pulse delivered by an S-900 stimulator (Dagan Corporation, Minneapolis, MN) and a bipolar stimulating electrode (Plastics One, Roanoke, VI) placed ~200μm away from the carbon fibre electrode. The carbon fibre electrode potential was linearly scanned from -0.4 to +1.2 V and back to -0.4 V versus Ag/AgCl using a 400V/s scan rate controlled by an Axopatch 200B amplifier connected to a computer and pClamp 10 software through a DigiData 1200B analog to digital interface (Molecular Devices). One recording and stimulation site was tested per slice. DA overflow was evoked 5 to 8 times per slice, until recordings were stable. The mean values of the last three recordings from each slice were used for statistical comparisons. The sample size is indicated as n=(x;y), x referring to the number of slices and y the number of mice.

Western immunoblotting

Mice were decapitated under Isoflurane anaesthesia, brains rapidly dissected and immersed in -10°C isopentane and then stored at -80°C. Frozen brains were sliced into 0.5mm coronal sections using an aluminum brain matrix. Coronal sections were mounted onto slides and maintained on dry ice. Nuclei were micro-dissected using brain tissue punches (Stoelting Inc., Wood Dale, IL). Bilateral punches of 0.75mm diameter were obtained from the VTA and 1.0mm diameter punches from the NAc. Micro-dissected tissues were homogenized on ice in 100 ml of cell lysis buffer (100mM Tris, pH 7.5; 750mM NaCl; 5mM Na_2EDTA; 5mM EGTA, pH 7.5; 5% Triton x-100; 12.5mM sodium pyrophosphate; 5mM beta-glycerophosphate; 1mM, 5mM Na_3VO_4; 5μg/ml leupeptin) with added protease (PMSF 1mM) and phosphatase inhibitors (Sigma cocktails I and II) in 1.5 ml tubes using a motorized pestle. Tubes were centrifuged for

15 min at 14000 g. Protein concentrations were measured using BCA protein assay (Pierce Biotechnology, IL, USA). Protein samples were separated by electrophoresis on a 10-12% polyacrylamide gel and electro transferred to a nitrocellulose membrane (EMD Millipore Corporation, Billerica, MA, USA). Non-specific binding sites were blocked in TBS 5% low-fat milk and 0.1% Tween-20 or 5% BSA. Membranes were rinsed in buffer (0.1% Tween-20 in TBS) and then incubated with anti-DAT (1:1000; Millipore, EMD Millipore Corporation, Billerica, MA, USA), anti-DA D1A receptor (1:1000; Millipore, EMD Millipore Corporation, Billerica, MA, USA), anti-D2 receptor (1:1000; Millipore, EMD Millipore Corporation, Billerica, MA, USA), anti-TH (1:500; Millipore, EMD Millipore Corporation, Billerica, MA, USA) or anti-GAPDH (1:1000) followed by anti-rabbit, anti-rat or anti-mouse IgG horseradish peroxidase-conjugate (1:5000). After rinsing with buffer, immunocomplexes were visualized by chemiluminescence using the western lighting plus ECL kit (PerkinElmer, Waltham, MA, USA). Protein size was assessed using precision plus protein ladder (Bio-Rad, Bedford, MA, USA). The film signals were digitally scanned and then density quantified using ImageJ software. GAPDH (1:10000, Cell Signaling, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as an internal control, such that data were standardized according to GAPDH values.

Amphetamine locomotor sensitization

This experiment was performed as described previously (Fulton et al., 2006). A Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments) was used for automated measurement of locomotor activity following amphetamine treatment. Following a 2-day habituation period to CLAMS cages, mice were implanted with a 14-day osmotic mini-pump (Alzet model 1002, DURECT Corporation, Cupertino, CA, USA) into the intrascapular region under Isoflurane anaesthesia. Pumps delivered leptin (500 ng/hr; 12 μg/day) or vehicle (PBS) for

the duration of the experiment. Beginning the following day, each mouse received three IP injections, each separated by 2 days, in the following order: (1) 1 mg/kg amphetamine (low dose), (2) 4 mg/kg amphetamine (high dose), (3) 1 mg/kg amphetamine (repeat low dose). Locomotor activity was recorded for 2 hours following injections. D-amphetamine (Sigma-Aldrich, Dorset, UK) was dissolved in 0.9 % saline.

Statistical analyses

Data were analyzed using GraphPad 5 and are presented as mean±standard error of the mean (SEM). A two-way ANOVA with Bonferonni post-hoc tests was used to compare control and KO mice with regards to body weight, food intake, hedonic feeding, food-motivated operant responding, spontaneous locomotor activity and voluntary running. Unpaired t-tests were used for comparisons of body composition, metabolic efficiency, 24h-food intake following acute leptin injection, locomotor activity during the dark cycle, total running distance (12 weeks), running reward, electrochemistry and immunoblotting experiments. Area under the curve (AUC) was calculated for dark cycle spontaneous locomotor activity and amphetamine-induced locomotor sensitization and compared via unpaired t-tests. AMPH-induced locomotor sensitization to the second low-dose of amphetamine was assessed using a one-way ANOVA that compared activity counts between the first and second AMPH treatment for each treatment group. Criterion for significance was set to $p \leq 0.05$ for all comparisons.

Results and Discussion

Ablation of STAT3 in dopamine neurons

To remove STAT3 function in midbrain DA neurons we bred STAT3 mice in which loxP sites flank exon 22 of the STAT3 gene which encodes tyrosine residue 705 that is essential for LepR-STAT3 activation (Takeda et al., 1998) with heterozygous knock-in mice that express Cre recombinase under the control of the regulatory sequence of the dopamine transporter (DAT) (Backman et al., 2006). $STAT3^{fl/fl}$;DAT^{Cre} knockout mice (hereafter referred to as $STAT3^{DAT\ KO}$) are born at expected frequencies, are of normal size and appearance and have similar weaning weights (Fig. 1A, inset). A PCR product corresponding to the recombined STAT3 gene (ΔSTAT3) was observed in brain nuclei of $STAT3^{DAT\ KO}$ mice in which DAT is expressed, but was absent from control nuclei and tissues (Fig. S1A). Double immunohistochemistry for phospho-STAT3 (pSTAT3) and tyrosine hydroxylase (TH) in slices from leptin-treated mice confirmed that Cre recombination was specific to DA neurons (Fig. S1B). pSTAT3 nuclear staining was absent from DA (TH+) neurons of the VTA and substantia nigra (SN) of $STAT3^{DAT\ KO}$ mice, yet was apparent in non-dopaminergic (TH-) cells of the midbrain and throughout the mediobasal hypothalamus (MBH) (Fig. S1B). The number of pSTAT3-postive cells in the VTA and substantia nigra (SN) was reduced by 66.46±4.37% (n=4/group) relative to controls as determined by counts in a separate series of immunolabelled slices.

STAT3 in dopamine neurons controls body weight and composition

To determine the contribution of STAT3 signaling in DA neurons in the regulation of body composition and feeding, we measured body weight, fat and lean mass and food intake in chow fed mice (Fig. 1A-D). As of 19 weeks of age, $STAT3^{DAT\ KO}$ mice weighed significantly less than

controls (Fig. 1A), a difference associated with a reduction in total fat mass (Fig. 1B). Caloric intake normalized to body weight values was comparable between $STAT3^{DAT\ KO}$ mice and controls (Figure 1C). As shown in Figure 1D, metabolic efficiency (total caloric intake divided by body weight gain) was increased in $STAT3^{DAT\ KO}$ mice relative to controls indicating that $STAT3^{DAT\ KO}$ mice utilize more energy per input unit. These findings suggest that reduced body weight and fat composition of $STAT3^{DAT\ KO}$ mice is due to elevations in energy expenditure.

STAT3 in dopamine neurons controls spontaneous and voluntary physical activity

As midbrain DA neurons are strongly implicated in the control of locomotion, we next set out to determine if spontaneous locomotor activity is increased in $STAT3^{DAT\ KO}$ mice. Significant increases in horizontal movement (Fig. 1E) and total distance travelled (Fig. 1F) in the dark cycle demonstrate that nocturnal activity is elevated in $STAT3^{DAT\ KO}$ mice relative to controls. Previous work of Hommel et al. established that viral-mediated knockdown of LepR in the VTA increases both food intake and locomotor activity without affecting body weight (Hommel et al., 2006). Thus, findings here suggest that inhibition of locomotor activity by VTA leptin involves STAT3 signaling in DA neurons. Reduced leptin signaling is also linked with heightened voluntary running or exercise: Hypoleptinemia is associated with increased exercise (Franks et al., 2003) and faster marathon run-times (Bobbert et al., 2012) in men independent of BMI, with hyperactivity in individuals with anorexia (Hebebrand et al., 2003; Holtkamp et al., 2003) and increased wheel running in wildtype mice (Thorburn et al., 2000) and mice bred for high-running capacity (Girard et al., 2007; Vaanholt et al., 2008). In view of these findings, we next measured voluntary wheel running in a separate cohort of mice with free access to a home cage running wheel. $STAT3^{DAT\ KO}$ mice ran substantially more than controls as evidenced by an

increased number of wheel revolutions (Figure 1G). Remarkably, STAT3$^{DAT\ KO}$ mice ran ~11 km/day as compared to the ~6 km/day run by controls (Figure 1H). Similar wheel running activity between DAT::Cre mice and wildtype littermates confirmed that increased running activity of STAT3$^{DAT\ KO}$ mice is not attributable to the Cre transgene alone (Fig. S2).

We next sought to determine if hyperactivity of STAT3$^{DAT\ KO}$ mice is an expression of increased anxiety-like behavior in view of findings implicating LepR signaling in DA neurons in the anxiolytic actions of leptin (Liu et al., 2011). Using the elevated-plus maze (EPM), the most commonly used rodent test of anxiety, we found that proportion of time spent and entries made in the open arm was similar between STAT3$^{DAT\ KO}$ and controls (Fig. S3A-C). To confirm this result, we also used an open field (OF) test whereby reduced exploration in a novel, open arena reflects increased anxiety. Exploration in the OF was comparable between STAT3$^{DAT\ KO}$ and controls (Fig. S3D). Together, these findings demonstrate that loss of STAT3 in DA neurons increasing spontaneous locomotor activity and endurance running capacity in a manner unrelated to anxiety. These results are in accordance with observations that VTA leptin administration inhibits wheel running activity in a rat model of anorexia-induced hyperactivity (Verhagen et al., 2011). Hyperactivity is a detrimental clinical feature of 30-80% of patients with anorexia nervosa that can propagate negative energy balance (Davis et al., 1994). As anorexia-induced hyperactivity is tied to hypoleptinemia, findings here may also have implications for increased physical activity and motor restlessness in individuals with anorexia.

STAT3 in dopamine neurons does not mediate the anorectic effects of central leptin

Leptin has central actions to inhibit food intake when administered into the cerebral ventricles and into select brain nuclei such as the VTA. To ascertain if STAT3 signaling in DA

neurons mediates the anorectic effects of central leptin, we measured food intake following intra-VTA and ICV injection of leptin. Intra-VTA leptin attenuated chow intake relative to vehicle-injected controls to a comparable extent in STAT3$^{DAT\ KO}$ mice and control mice (Fig. 2A). Thus, similar to what has been reported in rats (Bruijnzeel et al., 2011; Hommel et al., 2006; Trinko et al., 2011) leptin has anorectic effects when injected into the VTA of mice. Similarly, reductions in food intake following ICV injection of leptin were not different between STAT3$^{DAT\ KO}$ mice and controls (Figure 2B). These data suggest that LepR-Stat3 signaling in DA neurons does not mediate the anorectic actions of leptin. LepR signal transduction also involves phosphatidylinositol 3-OH-kinase (PI3K) (Kellerer et al., 1997; Niswender et al., 2001) and extracellular signal-regulated kinase (ERK) signaling pathways (Rahmouni et al., 2009). Leptin not only activates Erk1/2 signaling in the VTA but pharmacological inhibition of Erk1/2 phosphorylation was shown to block the anorectic action of VTA leptin (Trinko et al., 2011). It remains to be determined if Erk1/2 signaling in DA and/or GABA neurons is involved in the effects of VTA leptin on feeding.

Absence of STAT3 dampens instrumental learning but does not affect hedonic feeding

Mesolimbic and nigrostriatal DA neurons are key modulators of ingestive behavior, and specifically the appetitive, conditioned and rewarding aspects of feeding (Fulton, 2010; Palmiter, 2007). Leptin decreases the rewarding effects of food (Figlewicz et al., 2004; Figlewicz et al., 2006) whereas viral-mediated knockdown of LepR in the midbrain increases food-motivated instrumental responding (Davis et al., 2011). To determine the role of STAT3 signaling in DA neurons in food reward, we used an effort-based, operant conditioning task whereby mice learn to press a lever in order to receive a sucrose pellet (Sharma et al., 2012). Mice were trained to respond on a fixed-ratio 1 (FR1) schedule of reinforcement such that each lever press was

reinforced with a 20mg sugar pellet. Training could not progress towards more complex schedules of reinforcement (e.g., progressive ratio) since $STAT3^{DAT\ KO}$ mice exhibited significant response impairments on the FR1 schedule as evidenced by a reduced number of lever presses (Fig. 2C) and decreased percentage of correct (active lever) responses (Fig. 2D) relative to controls. Numerous reports have tied diminished performance specifically in operant conditioning tasks to DA signaling deficits in the nucleus accumbens (NAc) (Nowend et al., 2001; Smith-Roe and Kelley, 2000; Steinberg et al., 2014), and thus $STAT3^{DAT\ KO}$ mice may have impaired NAc DA signaling. As an alternate method to investigate changes in food reward, we employed a hedonic feeding test whereby sated mice voluntarily overeat palatable food, also known as the dessert test (Fig. 2E) (Choi et al., 2010). As shown in Figure 2F, all mice consumed around 50% of their daily caloric intake within the 1st hour of exposure to the chow diet on the test day, eating progressively less over the remaining 3hours. As illustrated in Figure 2F, when exposed to sweetened high-fat food (dessert) during the last hour, both groups increased their caloric intake to a similar degree suggesting that STAT3 in DA neurons does not modulate hedonic feeding.

Leptin supresses the rewarding effects of running via Stat3 signaling in DA neurons

Endurance running or exercise can promote a lasting sense of well-being that includes feelings of euphoria and pleasantness (Janal et al., 1984) and reduced anxiety and stress (Morgan, 1985; Rosch, 1985), commonly referred to as the runner’s high (Boecker et al., 2008; Morgan, 1985; Partin, 1983; Wagemaker and Goldstein, 1980). Running also has natural rewarding properties in rodents: rats will work to have access to a running wheel (Belke, 1997; Pierce et al., 1986) and spend more time in a place previously associated with the aftereffects of wheel running (Belke

and Wagner, 2005; Lett et al., 2000). Addictive and compulsive-like behavior directed at reinstating or maintaining high levels of physical activity are reported in humans (Berczik et al., 2012; Davis et al., 1995) and rodents (Lattanzio and Eikelboom, 2003; Werme et al., 1999), and the rewarding effects of running can substitute for other rewards such as food (Routtenberg and Kuznesof, 1967; Satvat and Eikelboom, 2006) and drugs of abuse (Cosgrove et al., 2002; Kanarek et al., 1995). Running engages opioid (Lett et al., 2002; Vargas-Perez et al., 2004), DA (de Castro and Hill, 1988; Greenwood et al., 2011; Mathes et al., 2010; Vargas-Perez et al., 2004) and endocannabinoid systems (Chaouloff et al., 2011; Raichlen et al., 2012). The euphoric effects of running have been associated with increased opioid signaling in corticolimbic brain areas (Boecker et al., 2008), however, very little is known about the neural pathways and signaling molecules that give rise to the rewarding impact of running. We next set out to determine if running reward is increased in $STAT3^{DAT\ KO}$ mice and if leptin modulates the rewarding effects of running via the VTA in a manner that relies on STAT3 signaling in DA neurons. Using the conditioned place preference (CPP) test (Fig. 3A), a behavioral tool for examining changes in conditioned reward, our data demonstrate that the rewarding effect of running is increased in $STAT3^{DAT\ KO}$ mice as compared to controls (Fig. 3B,C). $STAT3^{DAT\ KO}$ mice spend more time in the paired side of the chamber previously associated with wheel running (Fig. 3C). Next, a separate cohort of mice with intra-VTA bilateral cannulae went through the conditioning procedure and then received an intra-VTA injection of leptin, at a dose shown to inhibit feeding (Fig. 2A) (Hommel et al., 2006), or vehicle prior to the CPP post-test. Once again, $STAT3^{DAT\ KO}$ mice treated with vehicle showed increased conditioned rewarding effects associated with wheel running as compared to controls (Fig. 3D). Confirming our hypothesis that the increased running reward observed in $STAT3^{DAT\ KO}$ mice is mediated by

LepR-STAT3 signaling in VTA DA neurons, VTA leptin completely suppressed the rewarding effects of running in control mice yet was unable to block running reward in STAT3$^{DAT\ KO}$ mice (Fig. 3E). In agreement with previous studies suggesting a role for mesolimbic DA signaling in wheel running performance (Dubreucq et al., 2013; Mathes et al., 2010), here we identify the influence of leptin to inhibit running reward via the VTA in a manner that is contingent on STAT3 in DA neurons.

Lack of Stat3 in dopamine neuron reduces dopamine overflow and expression of TH and D1R in the nucleus accumbens

The NAc is a critical efferent target of VTA DA neurons strongly implicated in motivation, appetitive responses and reward learning (Salamone and Correa, 2012; Schultz, 2013). As DA signaling in the NAc is important for sustaining reward-related operant responding (Salamone and Correa, 2012), our finding that operant performance for food is impaired in STAT3$^{DAT\ KO}$ mice (Fig. 2C,D) are suggestive of perturbations in NAc DA signaling. Correspondingly, complete leptin-deficiency was previously reported to supress DA overflow in the NAc and to diminish the expression of TH, the rate-limiting enzyme for DA biosynthesis (Fulton et al., 2006). To determine if lack of STAT3 modulates evoked DA overflow in the NAc we used fast scan cyclic voltammetry, an electrochemical method that permits real-time, quantal measurement of DA exocytosis. Axonal DA overflow in the NAc core of acute slice preparations was evoked with a stimulating electrode and recorded with a carbon fibre electrode (Fig. 4A). The NAc core was selected based on evidence linking this NAc sub-compartment to the regulation of wheel running (Greenwood et al., 2011; Vargas-Perez et al., 2003). As shown in Figure 4B and C, the amplitude of evoked DA overflow was significantly reduced in STAT3$^{DAT\ KO}$ mice relative to

controls. To determine if lower evoked DA overflow is coupled to changes in DA-relevant protein expression, we next carried out immunoblotting experiments. Protein levels of TH and D1 receptor were decreased in the NAc of $STAT3^{DAT\ KO}$ mice relative to controls (Fig. 4D). Similar reductions in D1R and TH expression in the NAc are reported in mice selectively bred for their high-running capacity (Knab et al., 2009). Collectively, these results identify a role for NAc DA signaling in running, physical endurance and the rewarding properties of running.

Lack of Stat3 in DA neurons impairs amphetamine-induced locomotor sensitization

To corroborate the electrochemical data showing attenuated DA overflow in the NAc we studied behavioral sensitization in response to the psychostimulant drug amphetamine (AMPH). The acute locomotor-activating effects of AMPH rely on its actions to increase extracellular DA in the mesoaccumbens pathway by blocking DA reuptake and eliciting reverse transport via DAT. Mice received systemic leptin or vehicle via subcutaneous osmotic mini-pumps for the 10-day sensitization protocol as reported previously (Fulton et al., 2006). As illustrated in Figure 4E, there was no difference in locomotor activity in response to low-dose AMPH between vehicle and leptin-treated mice of either genotype. As expected, locomotion was increased following repeat AMPH administration in control mice (sensitization effect - Figure 4F & G). However, vehicle-treated $STAT3^{DAT\ KO}$ mice failed to sensitize to AMPH as shown by similar locomotor responses to AMPH following the two injections (Fig. 4H). Despite the absence of AMPH sensitization in $STAT3^{DAT\ KO}$ mice, chronic leptin treatment restored AMPH locomotor sensitization in these mice, although not quite to levels observed in controls (Fig. 4H). These results suggest that STAT3 signaling in DA neurons is important but not necessary for leptin modulation of DA-dependent, AMPH-induced locomotor sensitization. In agreement with these

results, Leinninger et al. found that deletion of LepR in neurotensin neurons of the lateral hypothalamus (LH) that project to VTA DA neurons supresses the locomotor effects of AMPH and inhibit NAc DA overflow (Leinninger et al., 2011). Thus, leptin acts directly on DA neurons to modulate mesolimbic DA signaling and function, in part via STAT3 signaling, while also having important indirect actions via LH inputs to these neurons.

General Discussion

The present findings uncover a new function for leptin in the control of the rewarding effects of running and identify the key contribution of LepR-STAT3 signaling in DA neurons to this process. STAT3 loss-of-function elicited substantial increases in spontaneous locomotor activity and voluntary endurance running capacity without affecting the anorectic actions of leptin or hedonic feeding, results that highlight the involvement of DA-specific STAT3 in the central control of physical activity. By tying behavioral changes to decreased NAc DA overflow and a dampening of mesolimbic DA function our findings also reveal STAT3 as an important regulator of mesoaccumbens DA tone and highlight the contribution of midbrain DA signaling in the rewarding impact of running.

The present data suggest that reduced LepR-Stat3 signaling in midbrain DA neurons could be an important element mediating restriction-induced hyperactivity and the coinciding fall in leptin levels. Furthermore, as chronic (but not acute) exercise by itself has been shown to suppress leptin levels in men (Perusse et al., 1997), endurance training may be inherently reinforcing by inhibiting LepR-STAT3 signaling in DA neurons. Although energetically costly, the capacity for endurance running in cursorial mammals is considered to enable food acquisition when food is at a distance or requires pursuit (see examples of distance hunters in Carrier; (Bramble and Lieberman, 2004; Carrier, 1984). Correspondingly, the rewarding effects of running or the runner's high may have evolved to encourage stamina and therefore increase the probability of return on this energetic investment. The motivational effects of physical activity would accordingly rise during conditions when food is scarce (or costly) to promote activity-based behaviors such as hunting, scavenging or food hoarding (accumulation) then decrease as a function of food availability and increasing leptin. Interestingly, food hoarding increases with

food restriction and weight loss (Cabanac and Swiergiel, 1989), is inhibited by leptin (Buckley and Schneider, 2003; Keen-Rhinehart and Bartness, 2008) and was previously speculated as the underlying behavioral function of a brain reward circuit inhibited by leptin (Fulton et al., 2000). Otherwise, the rewarding effects of running could presumably encourage physical activity and thus help to counter overweight, obesity and associated health risks. Conversely, in some individuals the rewarding properties of physical activity can foster excessive and compulsive patterns of exercise that are characteristic of an addiction (Berczik et al., 2012). Consistent with findings here, an intriguing new report shows that exercise addiction in men is associated with low, fat-adjusted leptin levels (Lichtenstein et al., 2014). This finding may also have relevance to the issue of hyperactivity associated with hypoleptinemia in anorexia since individuals with anorexia exhibit heightened sensitivity to rewards (Jappe et al., 2011). It is not known if there are genetic or physiological factors that contribute to a larger drop in leptin levels in response to food restriction or exercise in normal or pathological states that could increase the propensity for physical activity and sensitivity to its rewarding effects

In view of its well-established role in reward-relevant behaviour, motivation and addiction, the contribution of DA in leptin control of running reward is not surprising. Previous studies implicated opioids in frontolimbic circuitry in the euphoric effects of running in humans (Boecker et al., 2008) and endocannabinoid signaling via CB1 receptors in the VTA that modulate DA neurotransmission in the running performance of mice (Dubreucq et al., 2013). Endocannabinoid, DA and opioid systems converge in basal ganglia circuitry and several studies highlight their interaction in the control and drug and food reward. CB1 signaling in the VTA fine-tunes phasic DA release and thereby contributes to appetitive behaviors and reward-relevant learning. Striatal DA can modulate the release of opioid peptides that have been connected to the

hedonic effects of rewarding stimuli. Parceling out the precise contribution of each these substrates, the manner in which they cooperate and the corticolimbic neuronal populations involved in the rewarding effects of running requires further investigation.

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Supplemental Information

Leptin modulates locomotor activity and running reward via STAT3 signaling in midbrain dopamine neurons

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Supplemental Experimental Procedures

Cre-mediated recombination

Genomic DNA (200ng) was isolated using from brain tissue punches and peripheral tissue dissections using Trizol and subjected to PCR to detect recombination ("ΔSTAT3") (Takeda et al., 1998) using the following primers: a- 5'-CAC ACA AGC CAT CAA ACT CTG GTC TCC-3' (specific for exon 23 of the STAT3 gene) b- 5'-GAT TTG AGT CAG GGA TCC ATA ACT TCG -3' (specific for loxP site upstream of the targeting construct).

Immunohistochemistry

$STAT3^{DAT\ KO}$ mice and controls were injected IP with either leptin (5 mg/kg body weight) or PBS following an overnight fast. Recombinant leptin was obtained from Dr. A.F. Parlow (National Hormone and Peptide Program, National Institute of Diabetes and Digestive Kidney Diseases, Torrance, California). Two hours after treatment, mice were anesthetised with sodium pentobarbital (50mg/kg, IP) and perfused transcardially with 0.9% ice-cold saline followed by 10% neutral buffered formalin. Tissue preparation for pSTAT3 staining was as described (Fulton et al., 2006). Following pSTAT3 primary antibody labeling, sections were rinsed and incubated with a secondary biotinylated anti-rabbit antibody (1:1000; Vector Laboratories), labeled with avidin-biotin complex, and then stained with nickel-enhanced diaminobenzidine (DAB). Floating sections were blocked in avidin/biotin solution followed by 3% goat serum, and then incubated in anti-TH antibody (1:1000, mouse, Millipore, EMD Millipore Corporation, Billerica, MA,

USA) overnight at 4°C. Sections were rinsed and incubated with a secondary biotinylated anti-mouse antibody (1:300; Vector Laboratories), labeled with avidin-biotin complex, and then stained with Vector-VIP substrate. In a separate series of slices (n=4/group) stained for pSTAT3 alone as above, the number of pSTAT3+ cells were counted using ImageJ software in slices at coronal levels corresponding to plates 59 to 61 of the mouse brain atlas (Paxinos and Franklin, 2004).

Elevated plus maze test (EPM)

The EPM apparatus consists of two closed arms that oppose two open arms in a plus design (Med Associates, Inc., St Albans, VT, USA). Decreased time spent in the open, exposed arm is an indicator of increased anxiety-like behavior. The apparatus is placed 60 cm above the floor and has a video camera fixed overhead. Each mouse was placed in the middle of the maze facing the open arm opposing the experimenter. Movement in the maze was recorded and tracked for 5 min by an overhead video camera connected to a PC with Ethovision XT software (Med Associates, Inc., St Albans, VT, USA).

Open field test

As an additional test of anxiety-like behavior, $STAT3^{DAT\ KO}$ mice and controls were tested in the open field test. The open field consists of a Plexiglas box (50 x 50 x 30 cm) in a lit room. Each mouse was placed in the middle of the arena and allowed to explore the field for 5 min. Movement in the field was recorded and tracked by an overhead video camera connected to a PC with Ethovision XT software (Med Associates, Inc., St Albans, VT, USA).

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Figure Legends

Figure 1. **STAT3 in DA neurons regulates body weight, locomotor activity and voluntary wheel running.** (A) Body weights of control (n=7) and $STAT3^{DAT\ KO}$ mice (n= 8). *Inset*: Weaning weight (n=20/group) (B) Total lean and fat mass of control (n=7) and $STAT3^{DAT\ KO}$ (n=8) mice at 23 weeks of age. (C) Caloric intake (chow) normalized by body weight in control (n=7) and $STAT3^{DAT\ KO}$ (n=8) mice (D) Metabolic efficiency (total intake divided by weight gain) of control (n=7) and $STAT3^{DAT\ KO}$ (n=8) mice. (E) 24h ambulatory activity in control and $STAT3^{DAT\ KO}$ mice (n=9/group). (F) Dark phase locomotor activity is elevated in $STAT3^{DAT\ KO}$ mice relative to controls (n=9/group) (G) *Ad libitum* voluntary wheel running is increased in $STAT3^{DAT\ KO}$ (n=12) as compared to controls (n=7) (H) $STAT3^{DAT\ KO}$ (n=12) mice run much longer distances each day relative to controls (n=7). Mean±SEM; *p<0.05; ** p<.001; ***p<0.001

Figure 2. **STAT3 in dopamine neurons does not mediate the anorectic effects of central leptin and hedonic feeding but impairs food-motivated operant learning** (A) Food intake of control and $STAT3^{DAT\ KO}$ mice receiving an intra-VTA injection of leptin (200ng/500µl/side) or vehicle (n=7-11/group) (B) Food intake of control and $STAT3^{DAT\ KO}$ mice receiving an ICV injection of leptin (1µg/1µl) or vehicle (n=4/group) (C) Lever press responses on a fixed-ratio (FR)-1 operant conditioning task in control and $STAT3^{DAT\ KO}$ mice (n= 9/group; main effect of genotype). (D) Percentage of correct and incorrect responses by Day 8 of testing (n= 9/group) (E) Layout of the hedonic feeding test where mice learn to consume their daily chow ration in a 4-h time window (F) Test day intake of chow and sweetened high-fat diet ("dessert") in control and $STAT3^{DAT\ KO}$ mice (n= 7/group). Mean±SEM; *p<.05; ** p<.01; ***p<0.001

Figure 3. **The rewarding effects of running are increased in STAT3$^{DAT\ KO}$ mice and are inhibited by leptin in the VTA**. (A) Schema demonstrating the conditioned-place preference (CPP) task used to measure running reward. Alternating conditioning trials (paired one day, unpaired next) lasted 14 days (B) Control (n=16) and STAT3$^{DAT\ KO}$ (n=12) mice show significant preference for the "paired" side of the chamber associated with wheel running. (C). Preference for the paired side of the chamber is enhanced in STAT3$^{DAT\ KO}$ mice (n=12) relative to controls (n=16). (D) In a separate cohort, running CPP was assessed 1 hour after intra-VTA injection of vehicle in control (n=9) and STAT3$^{DAT\ KO}$ (n=8) mice. As in B, STAT3$^{DAT\ KO}$ mice show increases running CPP relative to controls (E) Intra-VTA leptin blocked the rewarding effects of running in control mice (n=14) but not in STAT3$^{DAT\ KO}$ (n=16) mice. Running CPP was tested one hour after intra-VTA leptin (200ng, 500µl/side) injection. Mean±SEM; *$p<0.05$; ** $p<0.01$; ***$p<0.001$

Figure 4. **Lack of STAT3 in DA neurons decreases DA overflow in the NAc core and blocks amphetamine locomotor sensitization.** (A) Illustration of brain slice preparation with placement of carbon fibre recording and stimulating electrodes for measurement of DA overflow using fast-scan cyclic voltammetry. (B) Representative current-time plot showing subsecond stimulation-evoked DA release and reuptake in a control and STAT3$^{DAT\ KO}$ mouse. Background current waveforms obtained immediately before the stimulation were subtracted from current waveforms obtained after stimulation to generate DA cyclic voltammograms. Oxidation current peaks for DA were obtained at potentials of -300 to -500 mV (versus Ag/AgCl) corresponding to 3.5–4.5 ms in the voltage waveform. The peak oxidation currents derived from voltammograms

were converted to concentration by electrode calibration and used to generate current-time plots (C) DA overflow (mean of peak values from current-time plots) is reduced in $STAT3^{DAT\ KO}$ mice (n=6;3) relative to controls (n=6;3). (D) TH and D1 receptor protein expression is lower in in the NAc of $STAT3^{DAT\ KO}$ mice as compared to controls (n=5-10/group). Protein quantifications were normalized to GAPDH values. (E) AMPH locomotor sensitization: Locomotor activity over time following the first dose of AMPH. (F). Locomotor activity over time following the second dose of AMPH. (G). Area under the curve (AUC) locomotor activity data for control mice treated with leptin (n=9) or vehicle (n=9). Locomotor activity was increased following the second injection of AMPH in both groups of control mice, indicative of sensitization. (H) Area under the curve (AUC) locomotor activity data for $STAT3^{DAT\ KO}$ mice treated with leptin (n=6) or vehicle (n=7). $STAT3^{DAT\ KO}$ mice treated with vehicle failed to sensitize to repeat AMPH whereas leptin treatment to these mice restored AMPH sensitization. Mean±SEM; *$p<0.05$; ** $p<0.01$.

Supplementary Figures

Supplementary Figure S1. **Cre mediated STAT3 recombination.** (A) PCR detection of STAT3 gene recombination. The recombined STAT3 gene (ΔSTAT3) was observed only in tissues/nuclei of STAT3DAT KO mice in which DAT is expressed. (B) Double immunohistochemistry for TH (VIP, purple) and pSTAT3(tyr705) (ni-DAB, black) from leptin treated STAT3DAT KO mice (n=4/group) reveals the absence of pSTAT3 from TH+ (dopamine) neurons (open arrows) and the presence of pSTAT3 in TH- cells (closed arrows) of the VTA, SN and MBH of $STAT3^{DAT\ KO}$ mice. CX: cortex; HPC: hippocampus; Liv: liver; MBH: mediobasal hypothalamus; Pan: pancreas; OB: olfactory bulb; VTA: ventral tegmental area; SN: substantia nigra

Supplementary Figure S2**, related to Figure 1.** Similar wheel running activity between DAT::Cre and wildtype littermates. (n=4-5/group).

Supplementary Figure S3**, related to Figure 1. Comparable anxiety-like behavior in STAT3$^{\mathbf{DAT\ KO}}$ mice and controls.** (A) Heat maps showing locomotor activity in the EPM in a STAT3$^{DAT\ KO}$ mouse and control. (B,C) Anxiety-like behavior as measured in the EPM was similar between STAT3$^{DAT\ KO}$ mice and controls (n=11-12/group). Percentage of open arm and open arm time were not different between genotypes (C) No differences were observed in anxiety-like behavior in the open field test. Exploratory activity (distance travelled) was comparable between STAT3$^{DAT\ KO}$ mice and controls (n= 5-6/group).

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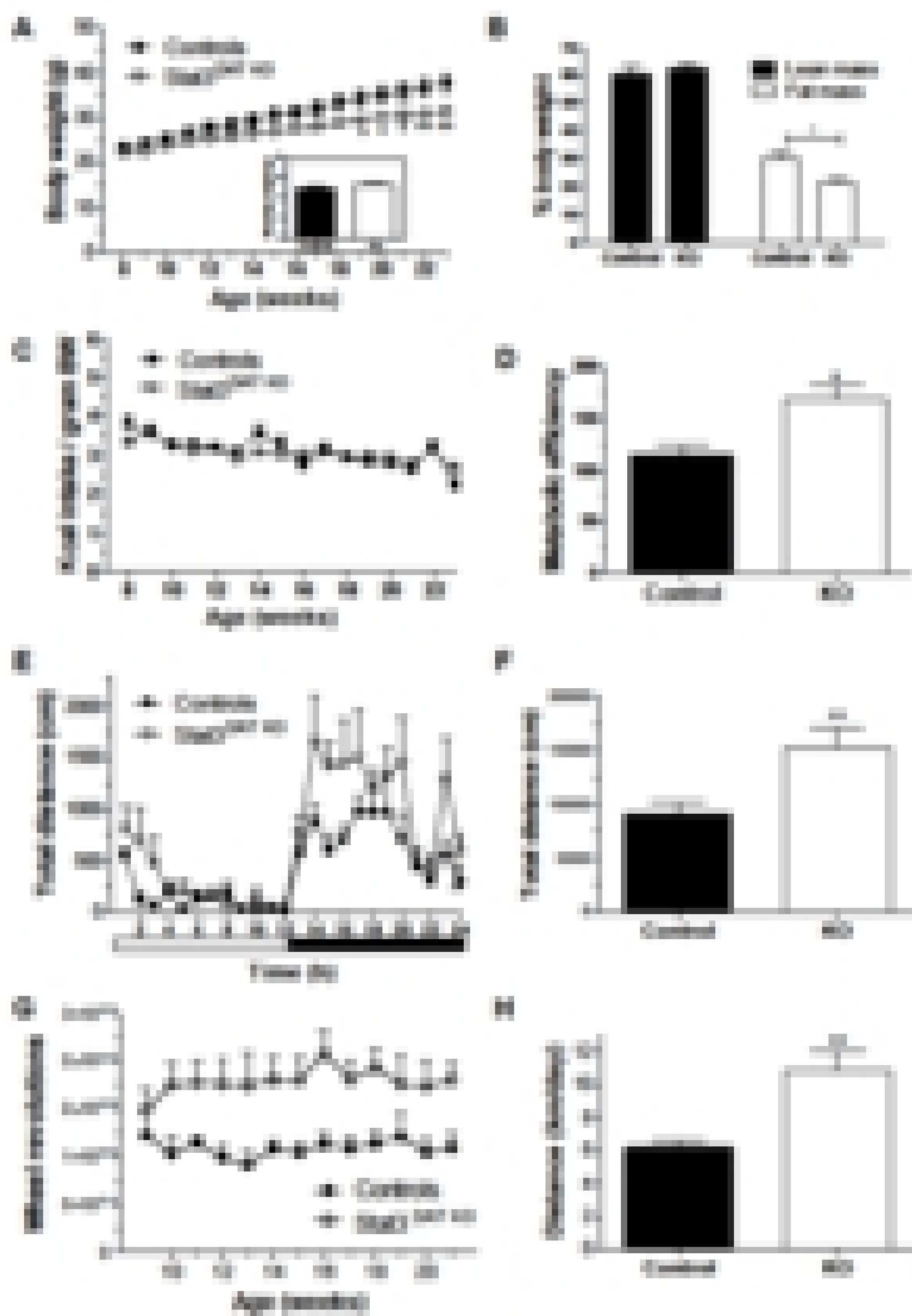

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Figure 1

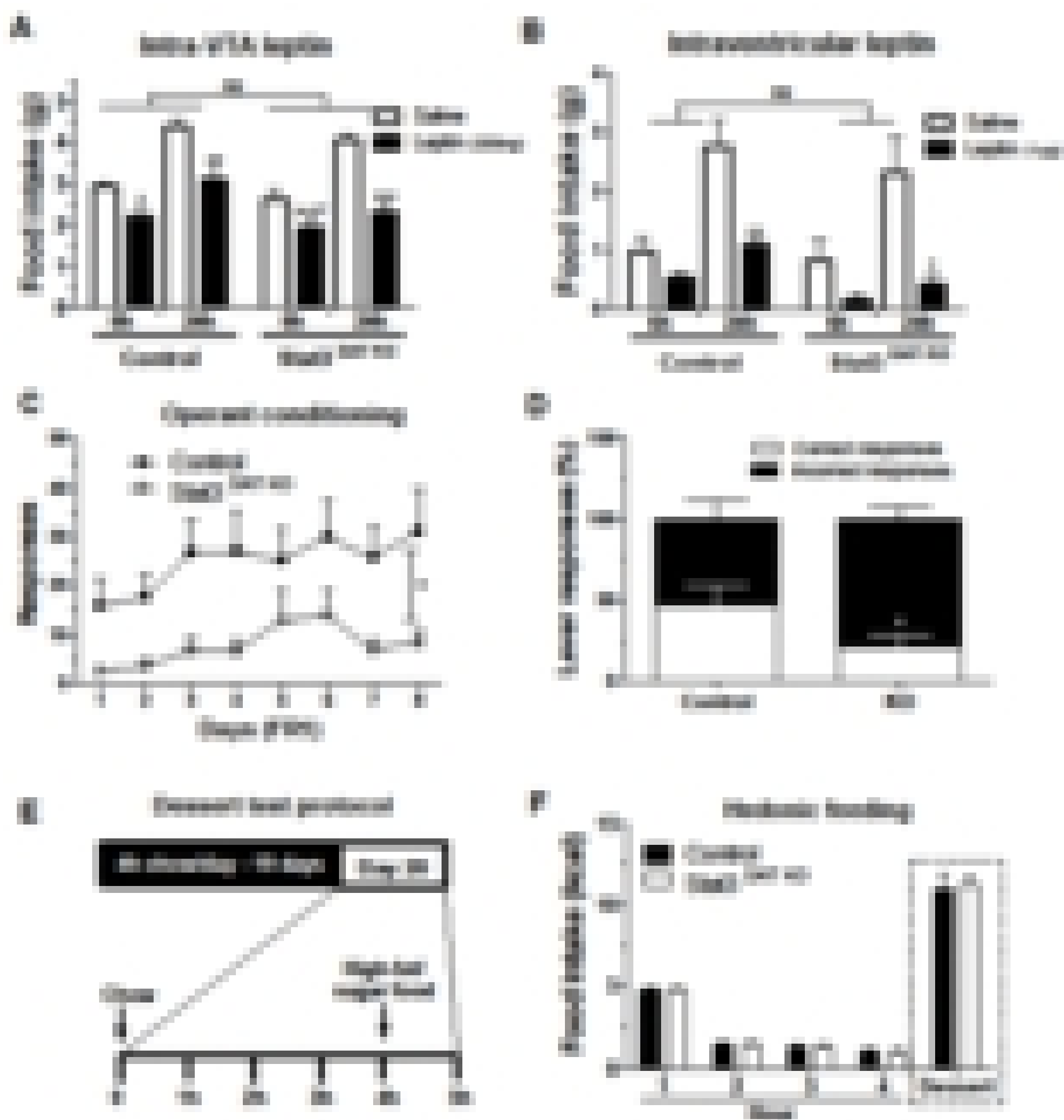

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Figure 2

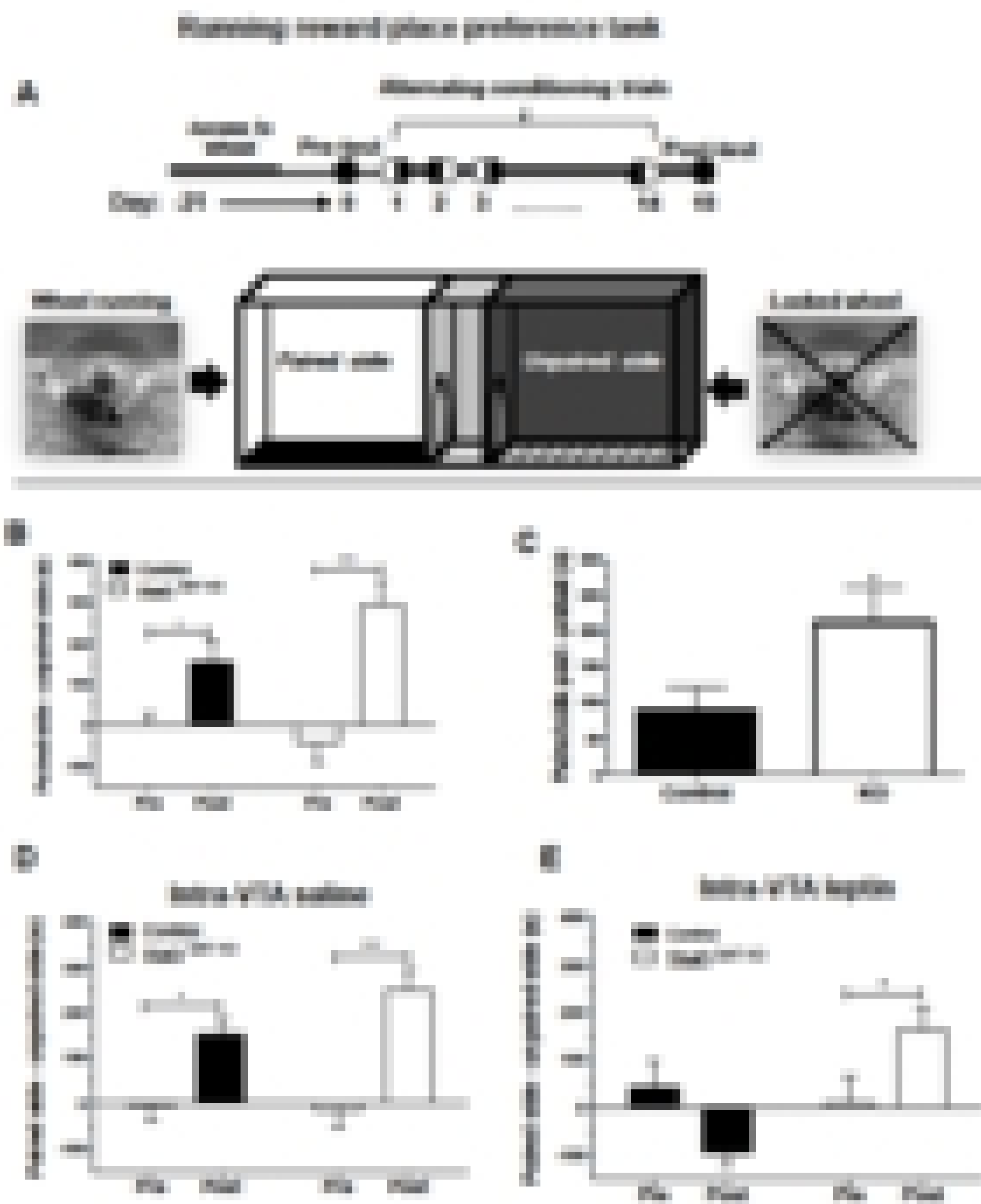

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Figure 3

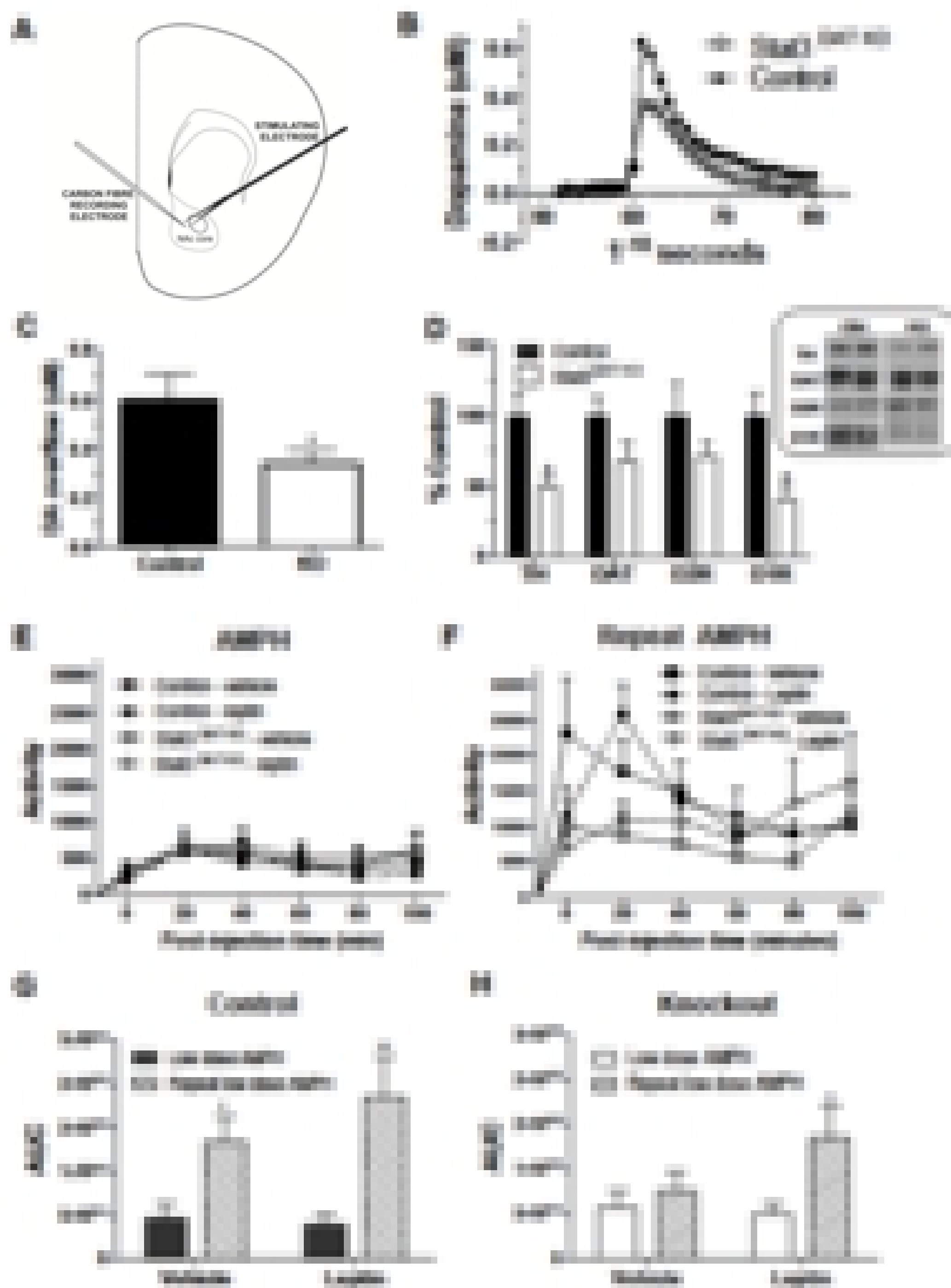

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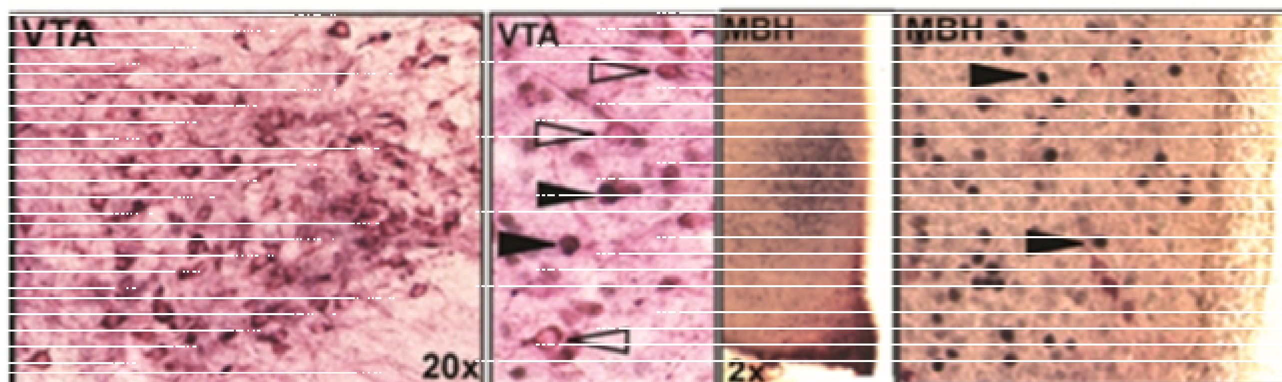
Figure 4



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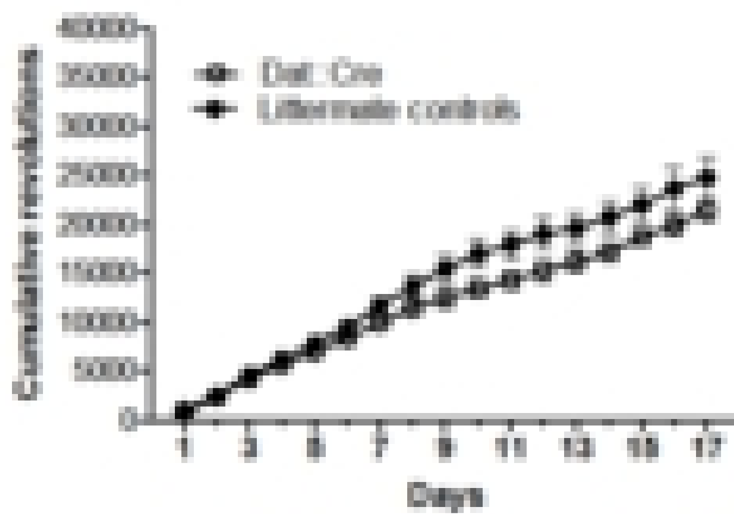
Supplemental Figure 1



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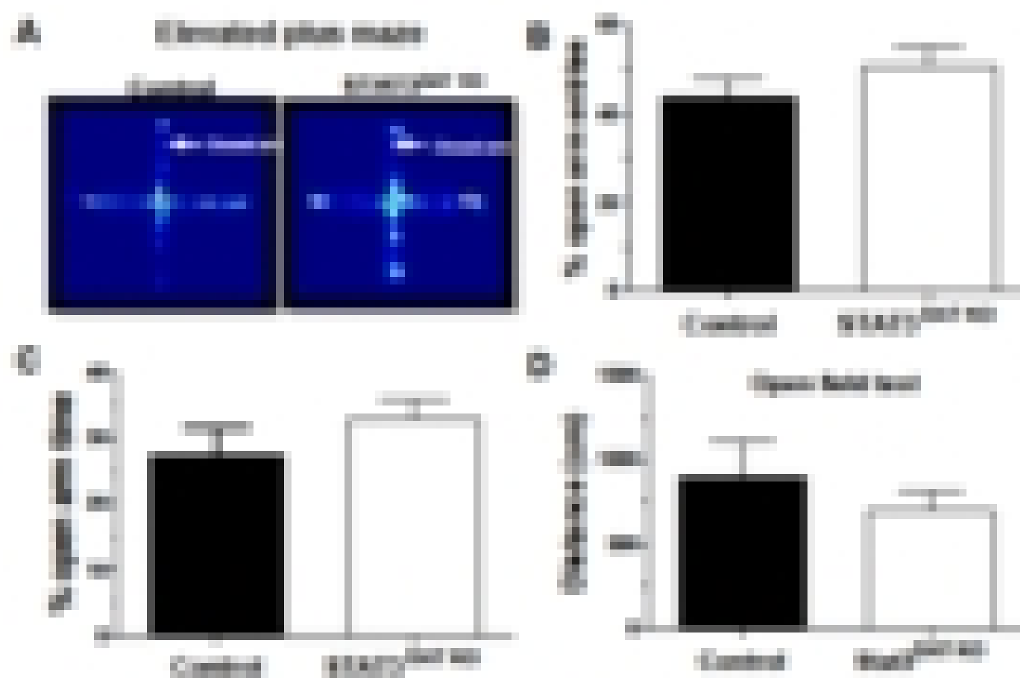
Supplemental Figure 2



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Supplemental Figure 3

8.2 Article #2

Contributions:

Maria Fernanda A. Fernandes, contributed by performing most of the experiments (97%), creating the graphics and writing the manuscript. Sandeep Sharma helped setting up the elevated plus maze (EPM) and open field (OFT) tests. Dr. Stephanie Fulton contributed in the results discussion, redaction and correction of the manuscript.

Deletion of STAT3 in midbrain dopamine neurons increases anxiety-like behavior in female mice

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Abstract

The adipocyte-derived hormone leptin targets the long form of its receptor in the central nervous system to produce several behavioral actions compatible with the maintenance of energy balance. Leptin activates a number of signaling pathways including JAK2-STAT3, MAPK, PI3-K and AMPK. JAK2-STAT3 constitutes a key pathway by which leptin modulates gene expression and energy balance. Leptin has anxiolytic effects which have been tied to LepRb signaling on mesolimbic dopamine neurons that project to the central nucleus of the amygdala, however, it remains to be resolved which leptin signaling pathways are involved. To assess the contribution of STAT3 signaling in dopaminergic neurons on anxiety-related behavior we generated a dopamine-specific STAT3 knockout ($STAT3^{DAT\text{-}KO}$) by crossing dopamine transporter DAT^{Cre} mice with $STAT3^{lox/lox}$ mice. $STAT3^{DAT\text{-}KO}$ female mice exhibited normal feeding, locomotion, body weight and behavioral despair relative to littermate controls. However, $STAT3^{DAT\text{-}KO}$ mice showed increased anxiety-like behavior in both the elevated plus maze and open field tests, as well as increased plasma corticosterone levels. Blocking dopamine D1-receptors in the central nucleus of the amygdala by local microinjection of the D1-receptor antagonist SCH23390 reversed the anxiogenic phenotype of $STAT3^{DAT\text{-}KO}$ mice. These results suggest that activation of STAT3 in midbrain dopamine neurons that project to the central amygdala mediates the anxiolytic effects of leptin.

Keywords: leptin, dopamine, central amygdala, anxiety

Introduction

The *ob* gene product leptin is a well described satiety factor which acts via the long form of its receptor (LepRb) to inform the hypothalamic centres about the status of body fat stores (Inui, 1999; Zhang *et al*, 1994). Leptin's actions in the hypothalamus as an "anti-obesity" hormone have been exhaustively studied. However, LepRb is distributed throughout the brain, including mesolimbic regions involved in emotional behavior (Elmquist *et al*, 1998; Leshan *et al*, 2006; Mercer *et al*, 1996; Scott *et al*, 2009). Via the LepRb, leptin targets the ventral tegmental area (VTA) to inhibit feeding-related behaviors (Davis *et al*, 2011; Hommel *et al*, 2006), locomotion (Hommel *et al*, 2006; Verhagen *et al*, 2011), and anxiety (Liu *et al*, 2011).

Of note, the therapeutic targets for obesity and mood disorders, such as anxiety, are the same (Zheng *et al*, 2006) and a strong relationship between feeding and anxiolytic behavior has already been described (Pallister and Waller, 2008; Swinbourne and Touyz, 2007). Consistent with this idea, previous studies pointed out to the anxiolytic effects of leptin in rodents and humans. There is data showing that plasma leptin levels in women is strictly related to symptoms of anxiety, independent of body fat composition (Lawson *et al*, 2012). Peripheral administration of leptin was previously demonstrated to decrease anxiety-like behaviors in mice (Finger *et al*, 2010; Liu *et al*, 2010; Lu *et al*, 2006). Moreover, leptin-deficient *ob/ob* mice display an anxiogenic phenotype, which can be reversed by systemic leptin administration (Asakawa *et al*, 2003; Finger *et al*, 2010). Although the specific signaling mechanisms by which leptin modulates anxiety-like behavior needs further clarification, Liu and coworkers demonstrated that inhibition of dopaminergic transmission via dopamine D1-receptor (D1R) may be involved (Liu *et al*, 2011).

Estrogen acts downstream of the LepRb to directly activate signal transducer and activator of transcription-3 (STAT3) pathway in a leptin-independent manner and thus, to influence gene transcription (Gao and Horvath, 2008). Similarly to the leptin receptor, estrogen receptors (ER) are also expressed in both dopaminergic and non-dopaminergic neurons in the midbrain. More specifically, ERs were detected in and around the A8, A9, and A10 dopaminergic cell groups in rodents (Creutz and Kritzer, 2002). Moreover, both leptin and estrogens significantly contribute to the incidence and intensity of mood disorders, including anxiety and depression, with females being at higher risk than males (Lund *et al*, 2005; Walf and Frye, 2006; Williams, 2010). Based on these findings, it can be speculated that estrogens and leptin may act synergistically to modulate STAT3 signaling in DA neurons. However, further experiments are required to test this hypothesis.

Given that STAT3 constitutes a predominant signal whereby leptin regulates gene expression (Bates *et al*, 2003); leptin induces STAT3 phosphorylation in dopamine (DA) and GABA neurons of the VTA (Fulton *et al*, 2006); and that both leptin and estrogen activates STAT3 signaling pathway, we hypothesized that this transcription factor might be a strong potential candidate molecule to mediate the anxiolytic effects of leptin in female mice. Using Cre-LoxP cell-specific gene targeting to abolish STAT3 activation in DA neurons, our goal was to determine whether LepRb-STAT3 could mediate the role of leptin in the midbrain to affect emotional processes.

Materials and methods

Animals

DA-specific STAT3 KO mice ("STAT3^{DAT-KO}") were generated as described (Fernandes *et al*, 2014, *submitted to Cell Metabolism*). Briefly, STAT3^{DAT-KO} mice have been generated by crossing the DAT^{Cre} mice with STAT3$^{lox/lox}$ mice, which have the main activation site of STAT3 gene (tyr705) flanked by loxP sites. STAT3^{DAT-KO} mice and littermate controls were housed in groups of five at 22-24°C on a 12:12 h light–dark cycle (lights on at 10:00 am) with ad libitum access to food and water. Mice were genotyped using PCR-based procedures. Estrous cycle was not monitored during these studies. Therefore, the effects of inactivation of STAT3 in DAT expressing cells on anxiety- and depression-like behaviors were determined in randomly cycling females. All animal procedures were approved by the CRCHUM Animal Care Committee.

Long term food intake and body weight

Mice were individually housed with free access to standard chow and water. Food intake and body weight was measured 3 times per week.

Ambulatory activity

Following two days of habituation to cages, spontaneous locomotor activity was measured for 24 hours in automated Accuscan metabolic cages (Accuscan Instruments Inc., Columbus, OH, USA) consisting of 16 light beam arrays in x, y and z axes. Distance travelled (horizontal activity) was measured by computer-controlled software.

Open field test (OFT)

To assess anxiety-like behavior, STAT3^{DAT-KO} mice and controls were tested in both the OFT and elevated plus maze (below). The open field consisted of a Plexiglas box (50 x 50 x 30 cm) in a brightly lit room. Each mouse was placed in the middle of the arena and allowed to explore the field for 5 min. Movement in the field was recorded and tracked by an overhead video camera connected to a PC with Ethovision XT software (Med Associates, Inc., St Albans, VT, USA).

Elevated plus maze test (EPM)

The EPM test served as an additional measure of anxiety-like behavior. The EPM apparatus consists of two closed arms that oppose two open arms in a plus design (Med Associates, Inc., St Albans, VT, USA). Decreased time spent in the open, exposed arm is an indicator of increased anxiety-like behavior. The apparatus is placed 60 cm above the floor and has a video camera fixed overhead. Each mouse was placed in the middle of the maze facing the open arm opposing the experimenter. Movement in the maze was recorded and tracked for 5 min by an overhead video camera connected to a PC with Ethovision XT software (Med Associates, Inc., St Albans, VT, USA).

Forced swim test (FST)

The FST was used to screen depressive-like behavior in STAT3^{DAT-KO} and littermate controls. In this test, animals display "behavioral despair" as indicated by increased immobility and less escape-oriented behaviors. When forced to swim in a glass cylinder filled with water in which they are confined mice eventually cease escape attempts and become immobile. The increasing immobility time reflects a state of despair. For this experiment, all mice were forced to swim in a glass cylinder (height, 15 cm; diameter, 12 cm) containing water (23°C) at a 10-cm depth. A

video camera located above the apparatus recorded each test. The duration of immobility during the last 4 min of the 6-min testing period (2 min habituation) was calculated.

Tail suspension test (TST)

The TST test served as an additional measure of depressive-like behavior. In this test, mice first struggle in an attempt to escape and over time animals become immobile, which is believed to reflect a state of "behavioral despair". Mice were suspended by the tail to a movement-sensitive hook for 10 min. Movement of the mouse during the procedure is automatically recorded via the transmission of electrical signals to a central unit. When movement was lower than a pre-established threshold, the mouse was considered immobile.

Surgical procedures

For intra-CeA cannula implantation, animals were anesthetized with Isoflurane (1.5%+oxygen) and placed in a stereotaxic frame (Kopf; Tujunga, CA, USA). Unilateral guide cannulae (26 gauge; Plastics One, Roanoke, VA, USA) were lowered into both sides of the CeA, using the following coordinates: AP, –1.25 mm; ML, ± 2.70 mm; DV, −4.2 mm (Crock *et al*, 2012). Cannulae were anchored to the skull surface with dental cement and occluded with metal obturators of the same length. Following cannula implantation mice were individually housed and body weights monitored daily to ensure appropriate post-surgical weight recovery. Mice recovered for at least 7 days before testing. At the end of the experiment, the cannulae placement was confirmed by the administration of methylene blue dye (0.3ul/side). Mice without staining restricted to the CeA (n=3) were excluded from subsequent analysis.

D1 dopamine receptor antagonist (SCH23390) injections in the CeA

For this experiment all mice were subjected to repeated handling to allow habituation and remained in their respective home cages until the infusion of the D1R antagonist SCH23390 (Sigma-Aldrich, St Louis, MO, USA) or vehicle (isotonic sterile saline) bilaterally into the central nucleus of the amygdala (CeA) (0.1ug/ul; 0.3ul/side delivered over two minutes). Following injection, all mice returned to their home cages and were tested in the EPM 30 minutes following the injections, as previously described (Liu *et al*, 2011).

Basal and stress-induced corticosterone measures

Both basal and stress-potentiated plasma corticosterone levels were measured. To measure basal corticosterone, blood samples were obtained via cardiac puncture at the time of sacrifice. For restraint stress experiments, mice were restrained in decapicones (Braintree Scientific Inc.,Braintree, MA, USA) for 30 min, and blood samples were obtained immediately after via cardiac puncture. All animals were sacrificed during the light cycle, at 6 am, when corticosterone levels are lower, according to the circadian pattern of corticosterone secretion. Plasma was separated by centrifugation and measured by an ELISA corticosterone kit according to manufacture instructions (Enzo Life Sciences, Farmingdale, NY, USA). Each sample was measured in triplicates. Inter assay variability was less than 10%.

Western immunoblotting

Mice were decapitated under Isoflurane anaesthesia and brains rapidly dissected and stored at -80°C. Frozen brains were sliced into 0.5mm coronal sections using a brain matrix. Coronal sections were mounted onto slides maintained on dry ice. Bilateral punches were obtained from

NAc (figure 18, Mouse Brain Atlas), DLS (figure 21, Mouse Brain Atlas) and CeA (figure 39, Mouse Brain Atlas) (Franklin and Paxinos 2008) using brain tissue punches (Stoelting Inc., Wood Dale, IL). Microdissected tissues were homogenized on ice in 100 ml of cell lysis buffer (100mM Tris, pH 7.5; 750mM NaCl; 5mM Na_2EDTA; 5mM EGTA, pH 7.5; 5% Triton x-100; 12.5mM sodium pyrophosphate; 5mM beta-glycerophosphate; 1mM, 5mM Na_3VO_4; 5µg/ml leupeptin) with added protease (PMSF 1mM) and phosphotase inhibitors (Sigma phosphatase inhibitor cocktails I and II) in 1.5 ml aliquots using a motorized pestle. Aliquots were centrifuged for 15 min at 14000 g. Protein concentrations were measured using BCA protein assay (Pierce Biotechnology, IL, USA). Protein samples were separated by electrophoresis on a 12-10% polyacrylamide gel and electro transferred to a nitrocellulose membrane (EMD Millipore Corporation, Billerica, MA, USA). Non-specific binding sites were blocked in TBS 5% low-fat milk and 0.1% Tween-20 or 5% BSA. Membranes were rinsed in buffer (0.1% Tween-20 in TBS) and then incubated with anti-DAT (1:1000; Millipore, EMD Millipore Corporation, Billerica, MA, USA), anti-D2 receptor (1:1000; Millipore, EMD Millipore Corporation, Billerica, MA, USA), anti-TH (1:500; Millipore, EMD Millipore Corporation, Billerica, MA, USA), anti- pDARPP-32 (Thr 34) (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by anti-rabbit, anti-rat or anti-mouse IgG horseradish peroxidase-conjugate (1:5000). After rinsing with buffer, immunocomplexes were visualized by chemiluminescence using the western lighting plus ECL kit (PerkinElmer, Waltham, MA, USA). Protein size was assessed with a precision plus protein ladder (Bio-Rad, Bedford, MA, USA). The film signals were digitally scanned; density quantified using ImageJ software. GAPDH (1:10000, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as an internal control, such that data were standardized according to GAPDH values.

Statistical analyses

Data were analyzed using GraphPad 5 and presented as means and respective standard errors of the mean (SEM). Unpaired t-tests were used to compare control and $STAT3^{DAT-KO}$ mice in all studies, except for 24h-ambulatory activity and food intake studies, which were analysed using 2-way ANOVA with Bonferoni post-hoc tests. Criterion for significance was set to $p \leq 0.05$ in all comparisons.

Results

Absence of STAT3 signaling in dopamine neurons enhances CORT levels and anxiety-like behavior. Basal plasma corticosterone (CORT) levels were elevated in female STAT3^{DAT-KO} mice as compared to respective controls at the middle of the light cycle (6am) (***p< 0.001, Figure 1A). Consistent with these findings, 30-minutes of restraint increased corticosterone levels in both groups, however, stress-induced elevations of CORT were more pronounced in STAT3^{DAT-KO} mice (***p< 0.001, Figure 1B). To determine the role of STAT3 signaling in DA neurons in anxiety-like behavior, STAT3^{DAT-KO} and control mice were tested in an OFT and EPM - two well-validated behavioral tests of anxiety (Archer, 1973; Carobrez and Bertoglio, 2005; Rodgers and Dalvi, 1997; Stanford, 2007). Our results demonstrated that STAT3^{DAT-KO} mice avoid the center of the open field arena and spend more time in the periphery as compared to controls (***p < 0.001, Figure 1C and 1D). In addition, as shown in, Figure 1E both percentage of time spent into the open arm and percentage of entries made into the open arm of an EPM is lower in STAT3^{DAT-KO} mice as compared to controls (**p<0.01. Figure 2E). Taken together, our results reveal an anxiogenic phenotype in STAT3^{DAT-KO} female mice.

STAT3^{DAT-KO} mice display normal locomotor activity, body weight and food intake

To determine if the increased anxiety-like behavior was a result of changes in locomotor activity we examined 24 hours locomotor activity in STAT3^{DAT-KO} mice and respective controls (Figure 2A and 2B). STAT3^{DAT-KO} mice and littermate controls exhibited similar locomotor activity during 24 hours (Figure 2A) and similar locomotor activity during the dark (active) phase (Figure 2B). Moreover, we assessed the effects of loss of STAT3 in DA neurons on long-term chow intake and body weight regulation. There is no difference in body weight and food consumption between genotypes (Figure 2C).

Lack of STAT3 in dopamine neurons does not affect depressive-like behavior

To determine if the absence of STAT3 signaling in DA neurons impacts depressive-like behavior, mice were tested in the FST and TST, two well-validated tests of behavioral despair in mice (Castagne *et al*, 2010, 2011). As shown in Figure 3A and 3B, there were no differences in immobility time, an index of "behavioral despair", in either the FST and TST between genotypes, demonstrating that loss of pSTAT3 in DA neurons does not influence depressive-like behaviors .

Lack of STAT3 in DA neurons alters the expression of DA-related proteins in the CeA

To determine if changes in the expression of DA-related proteins could be involved in the heightened anxiety of $STAT3^{DAT\text{-}KO}$ mice we studied the expression of TH, DAT and phospho-DARPP32(thr34) protein in the CeA. As shown in Figure 4, TH and DAT expression is significantly decreased in the CeA of $STAT3^{DAT\text{-}KO}$ female mice as compared to controls (***$p<0.001$, Figure 4) whereas phospho-DARPP32 (thr34), a marker of D1R activation, was increased in the CeA of $STAT3^{DAT\text{-}KO}$ mice relative to controls (*$p<0.05$). The increases in pDARPP32(thr34) observed are suggestive of increased CeA D1R signaling in $STAT3^{DAT\text{-}KO}$ female mice.

D1R antagonist (SCH23390) administration in the CeA reverses the anxiogenic phenotype of $STAT3^{DAT\ KO}$ mice

As we obtained biochemical evidence to suggest that D1R signaling is increased in the CeA of $STAT3^{DAT\text{-}KO}$ mice, we next set out to determine if blocking D1R in the CeA could reverse the increased anxiety-like behavior of $STAT3^{DAT\text{-}KO}$ mice. Mice received a bilateral injection of the D1 receptor antagonist SCH23390 (0.3 µg per mouse) or vehicle into the CeA and were then screened for anxiety-like behavior 30 minutes later. Supporting our earlier results, saline-treated

STAT3$^{DAT\ KO}$ mice spent less time (*$p<0.05$, Figure 5A) and made less entries (*$p<0.05$, Figure 5B) into the open arm as compared to controls. There were no differences in either the percentage of time spent in the open arm (Figure 5A) or percentage of entries made into the open arm (Figure 5B) of the EPM between SCH23390-treated STAT3^{DAT-KO} and control mice. In addition, the percentage of time spent in the open arm (Figure 5A) or percentage of entries made to this arm (Figure 5B) was similar between vehicle- and SCH23390-treated control mice. However, we observed that SCH23390-treated STAT3^{DAT-KO} mice increased the percentage of time spent in the open arms (**$p<0.01$, Figure 5A) as well as the percentage of entries made into this arm (*$p<0.05$, Figure 5B), as compared to vehicle-treated STAT3^{DAT-KO} mice. Thus, D1 blockade in the CeA reversed the increased anxiety-like behavior of STAT3$^{DAT\ KO}$ mice.

Discussion

Leptin has been shown to have anxiolytic actions mediated by LepRb signaling on mesolimbic dopamine (DA) neurons that project to the central nucleus of the amygdala (CeA) (Liu *et al*, 2011). Here we find that absence of STAT3 signaling, an important signaling molecule downstream of LepRb, specifically in DA neurons induces anxiety in female mice by increasing DA signaling in the CeA. However, depressive-like behavior is similar between $STAT3^{DAT\text{-}KO}$ mice and respective controls, suggesting that STAT3 signaling in dopaminergic neurons specifically modulates anxiety in female mice.

Besides its well-described role in controlling food intake and body weight, the adipocyte-derived hormone leptin also plays an important role in the modulation of mood (Leshan *et al*, 2006; Liu *et al*, 2010; Scott *et al*, 2009). Leptin's anxiolytic properties has already been described (Liu *et al*, 2010). Furthermore, systemic leptin ameliorates anxiety-like behavior in *ob/ob* mice (Asakawa *et al*, 2003) and reduced depressive-like behavior in rodents (Lu *et al*, 2006). However, the specific functional role of LepRb signaling in midbrain DA neurons in the regulation of emotional behavior as well as the signaling molecules involved remained elusive until now. Our findings that lack of STAT3 signaling in DA neurons of female mice results in increased anxiety-like behavior (OFT and EPM) and elevated basal and stress-induced corticosterone levels without changes in behavioral despair are consistent with those of Liu and coworkers, showing that LepRb KO in DA neurons increase anxiety but not despair in male mice (Liu *et al*, 2011). Alternatively, the anti-depressant effects of leptin appear to be more associated with hippocampal leptin signaling (Guo *et al*, 2013; Harvey, 2007). In contrast with Liu and coworkers, we failed to observe an anxiogenic phenotype in $STAT3^{DAT\text{-}KO}$ male mice. The difference between our results and those from Liu and collaborators as well as the sexual

dimorphism observed in anxiety-like behavior in STAT3^{DAT-KO} mice can be explained from a few speculative perspectives. First, the authors do not mention the reason why they chose to include only male mice in their study, given that there is literature showing that females are much more prone to be affected by mood disorders than males (Bekker and van Mens-Verhulst, 2007; Kessler *et al*, 2005). Furthermore, a dense population of estrogen-expressing neurons was observed in the CeA of female rats (Pfaff and Keiner, 1973) and estrogen increases extracellular DA levels by inhibiting the dopamine transporter (DAT) (Watson *et al*, 2006). Of note, estrogen modulation of DAT seems to occur either via an interaction between estrogen receptors (ERs) and DAT, or between ERs and a variety of signaling mediators responsible for second messengers and response cascades affecting DAT (Alyea *et al*, 2008). Thus, with regards to the gender differences observed between male and female STAT3^{DAT-KO} mice, a possible explanation could be related to the previously suggested gender differences in estrogen's effect on dopaminergic function (Alyea *et al*, 2008). One hypothesis is that estrogen may be contributing to increase extracellular DA concentrations in the CeA of STAT3^{DAT-KO} female mice by inhibiting DA reuptake. Moreover, given the growing literature suggesting marked sexual dimorphisms in brain wiring, morphology and chemistry (Cosgrove *et al*, 2007; De Vries and Boyle, 1998), one can speculate that the gender differences observed in our studies may be related to a difference in the number of ERs in the CeA of males *versus* females; an altered sensitivity to the anxiolytic actions of estrogen or even due to a different effect of estrogen in male and female brain. However, more research is necessary to test these hypotheses.

In agreement with the anxiogenic phenotype observed in STAT3^{DAT-KO} female mice, our results showed an increased basal and restraint stress-induced plasma corticosterone levels in mutant mice as compared to littermate controls. These findings are consistent with previous *in vivo* and

in vitro studies suggesting that leptin inhibits corticosterone secretion by the adrenal gland (Ahima *et al*, 1996; Nowak *et al*, 2002; Pralong *et al*, 1998; Szucs *et al*, 2001). On the other hand, other studies reported that central leptin increases the activity in the hypothalamic-pituitary-adrenal axis (HPA) axis which in turn activates corticotropin-releasing hormone (CRH) neurons to drive corticosterone secretion in male rats (Morimoto *et al*, 2000; van Dijk *et al*, 1997). The apparent discrepancy between these findings can be due to the metabolic status of the animal, gender and/or species differences.

To try to identify potential signaling molecules underlying the anxiogenic behavior of $STAT3^{DAT\text{-}KO}$ mice, our immunoblotting studies found that absence of STAT3 signaling in DA neurons reduces DAT and increases phospho-DARPP32 (Thr34) protein levels in the CeA of $STAT3^{DAT\text{-}KO}$ female mice. Of note, DARPP-32 is a critical downstream target of D1R: D1R signaling increases cAMP levels and PKA activity via the stimulation of adenylyl cyclase by stimulatory G-proteins. PKA-catalyzed phosphorylation will then activate DARPP-32 (Beaulieu and Gainetdinov 2011) Thus, increased pDARPP32(thr34) is suggestive of enhanced D1R activation, which our findings suggest could be due to increased extracellular DA concentrations elicited by reduced DAT function. A very speculative hypothesis on the link between STAT3 and DA reuptake would be that STAT3 binds to the DAT promoter to regulate its function. This hypothesis needs to be tested in future studies.

Taken into consideration previous data demonstrating that absence of LepRb in DA neurons potentiates anxiogenic behavior and D1R signaling in the CeA (Liu *et al.* 2011), we next investigated whether increased D1R in the CeA was contributing to anxiety-like behavior in $STAT3^{DAT\text{-}KO}$ mice. By blocking DA transmission specifically in the CeA via an intra-CeA administration of a D1R antagonist (SCH23390) we observed that the anxiogenic phenotype of

STAT3^{DAT-KO} female mice normalized. This finding is consistent with observations in DA-specific LepRb KO mice (Liu *et al*, 2011). Of note, the effect of D1R antagonist could include a mixture of postsynaptic and presynaptic mechanisms, blocking the activity of D1R localized at postsynaptic elements of CeA neurons as well as at presynaptic nerve terminals.

Importantly, the results of this study unravel the specific signaling pathway by which leptin acts on dopaminergic neurons to modulate anxiogenic behavior. Taken together, our findings suggest that the anxiolytic actions of leptin in female mice are mediated by increased LepRb-STAT3 signaling in midbrain DA neurons that target the CeA to decrease D1R signaling, perhaps via increased DA reuptake.

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Figure Legends

Figure 1: **Absence of STAT3 signaling in dopamine neurons enhances CORT levels and anxiety-like behavior.** (A) Basal plasma corticosterone levels (ng/ml, ± SEM) is increased in $STAT3^{DAT\text{-}KO}$ mice (n=4) as compared to controls (n=4). ***$p < 0.001$. (B) 30’ restraint stress-induced plasma corticosterone levels (ng/ml, ± SEM) is significantly increased in $STAT3^{DAT\text{-}KO}$ mice (n=6) as compared to littermate controls (n=6). ***$p < 0.001$ (C) Significant decrease in percentage of time spent in the centre of an open field arena in $STAT3^{DAT\text{-}KO}$ mice (n=10) as compared with the control group (n=8). ***$p < 0.001$. (D) Percentage time spent in the periphery of an open field arena is increased in $STAT3^{DAT\text{-}KO}$ mice (n=10) as compared to littermate controls (n=8). ***$p < 0.001$. (E) Percentage of open arm time and open arm entries is decreased in $STAT3^{DAT\text{-}KO}$ mice (n=7) as compared to littermate controls (n=8). **$p<0.01$, percentage open arm time; **$p<0.01$.

Figure 2. **$STAT3^{DAT\text{-}KO}$ mice display normal locomotor activity, body weight and food intake.** (A) 24h-locomotor activity, expressed as total distance travelled during the test in $STAT3^{DAT\text{-}KO}$ mice and respective controls (n=7-9). (B) Dark (active) phase locomotor activity in $STAT3^{DAT\text{-}KO}$ mice and respective controls (n=7-9). (C) Average caloric intake normalized by body weight of $STAT3^{DAT\text{-}KO}$ female mice and littermate controls on a regular chow diet (n=8-9).

Figure 3: **Lack of STAT3 in dopamine neurons does not affect depressive-like behavior.** (A) Total immobility time in the forced swim test is similar between $STAT3^{DAT\ KO}$ (n=6) and control

mice (n=4). (B) Immobility time of STAT3$^{DAT\ KO}$ (n=4) mice was comparable to controls (n=4) in the tail suspension test.

Figure 4: **Lack of STAT3 in DA neurons alters the expression of DA-related proteins in the CeA.** TH and DAT protein levels in CeA of STAT3$^{DAT\ KO}$ mice (n=5-7) were reduced as compared to controls (n=4). ***$p < 0.001$. Phosphorylation of DARPP32 at Thr 34 was increased in STAT3$^{DAT\ KO}$ mice (n=4) relative to controls (n=4). *$p<0.05$.

Figure 5: **D1R antagonist (SCH23390) administration in the CeA reverses the anxiogenic phenotype of STAT3$^{DAT\ KO}$ mice.** (A) Schema illustrating the placement of cannulae in the CeA (B) Percentage of time spent in the open arm of an EPM is comparable between STAT3$^{DAT\ KO}$ (n=5) and control mice (n=5) treated with SCH23390 (0.3 µg per mouse). Percentage of time spent in the open arm of an EPM is decreased in saline-treated STAT3$^{DAT\ KO}$ mice (n=5) as compared to saline-treated controls (n=5). *$p<0.05$. Similar proportion of time spent in the open arm time in saline- (n=5) versus SCH23390-treated (n=5) control mice. Increased percentage of time spent in open arm in SCH23390-treated STAT3$^{DAT\ KO}$ mice (n=5) as compared to saline-treated STAT3$^{DAT\ KO}$ mice (n=5). **$p<0.01$. (C) Percentage of entries made to the open arm of an EPM is similar between STAT3$^{DAT\ KO}$ (n=5) and control mice (n=5) treated with SCH23390. Percentage of entries to the open arm of an EPM is decreased in saline-treated STAT3$^{DAT\ KO}$ mice (n=5) as compared to saline-treated controls (n=5). *$p<0.05$. Comparable percentage of open arm entries in saline- (n=5) and SCH23390-treated (n=5) control mice. Increased

percentage of open arm entries in SCH23390-treated $STAT3^{DAT\ KO}$ mice (n=5) as compared to saline-treated $STAT3^{DAT\ KO}$ mice (n=5). *$p<0.05$.

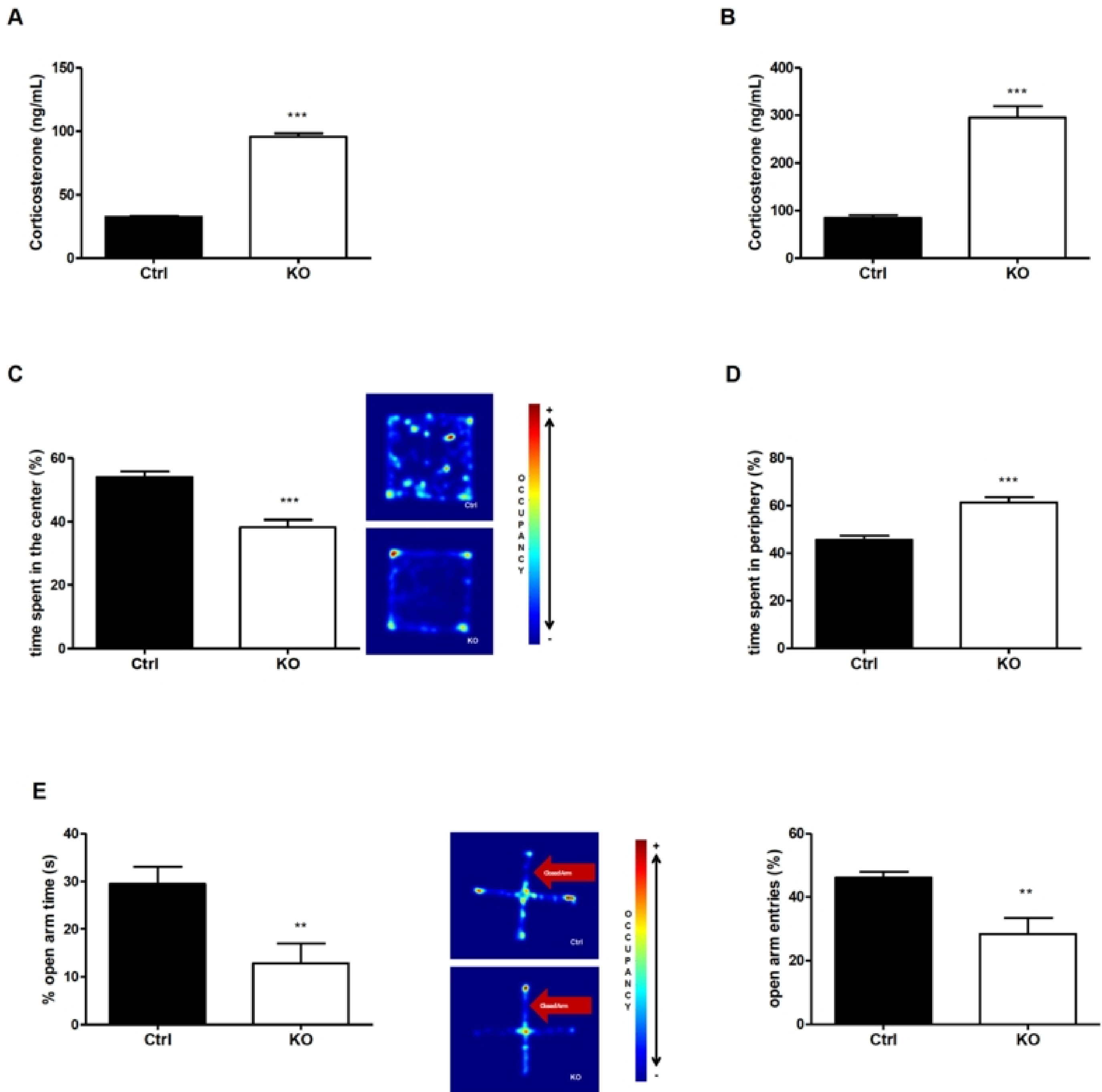

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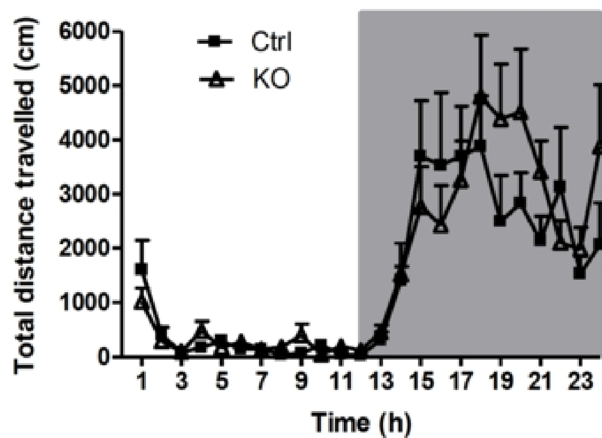
Figure 1

A



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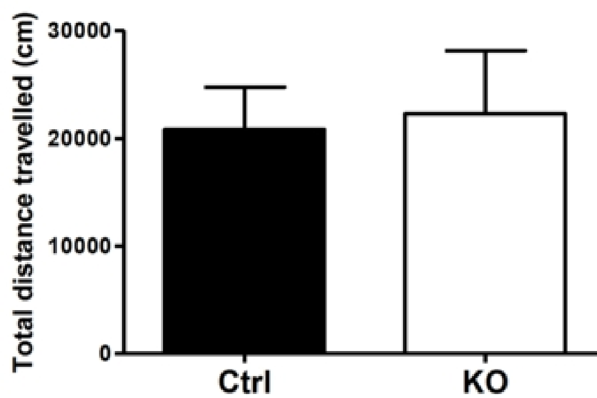

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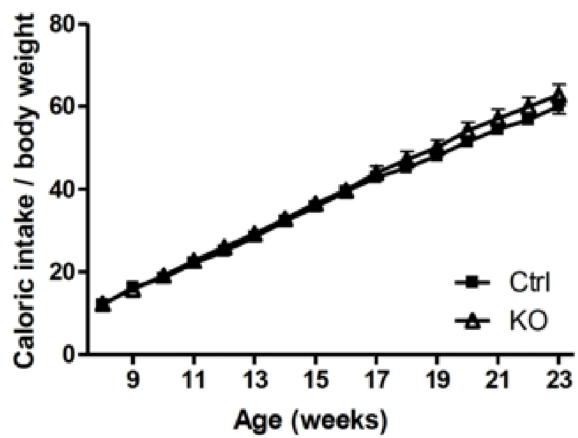

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Figure 2

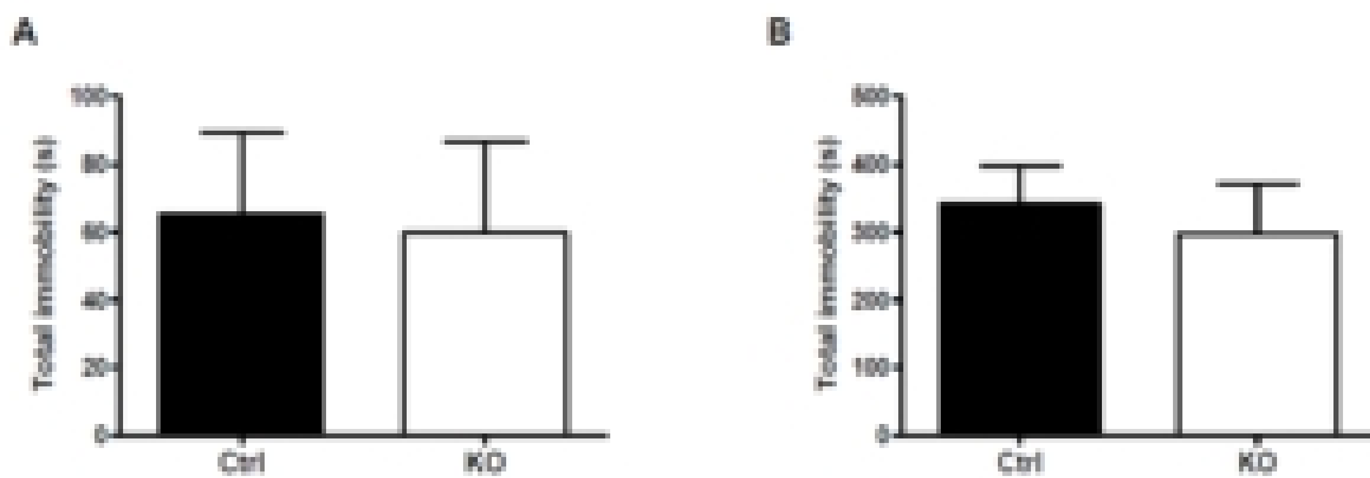

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Figure 3

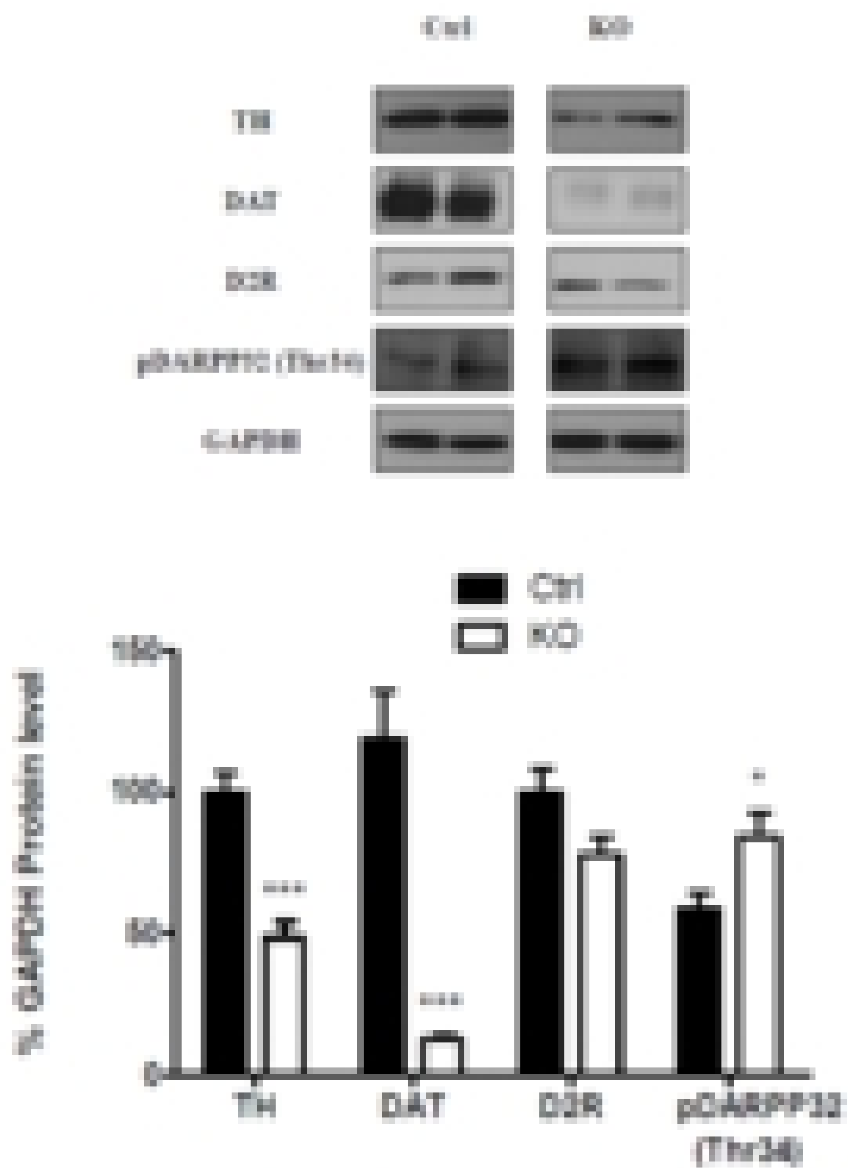

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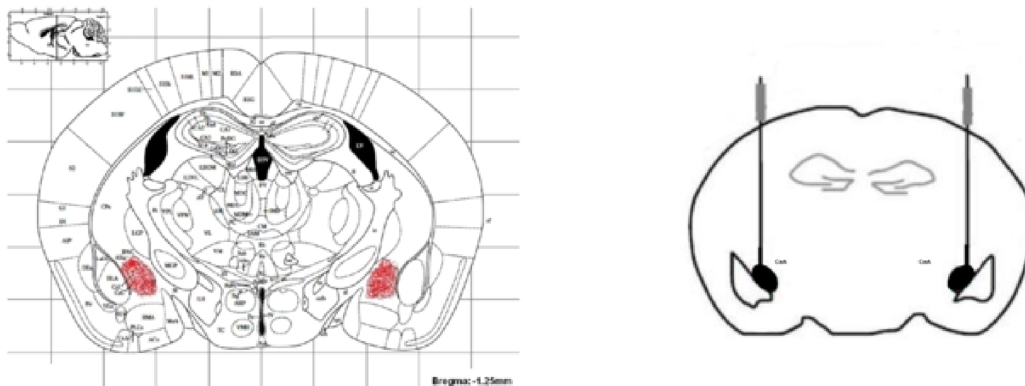
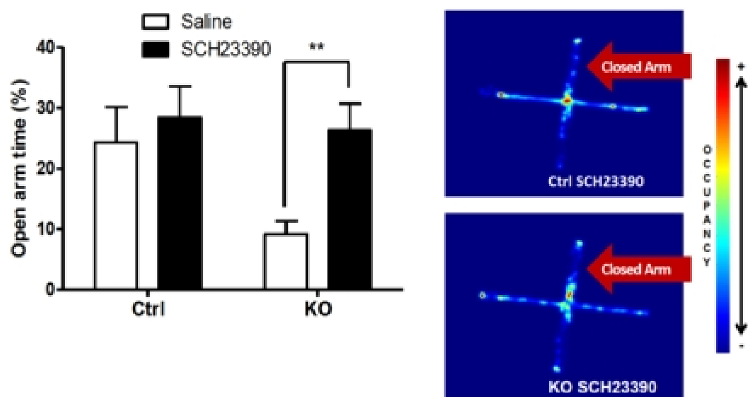
Figure 4

A



B



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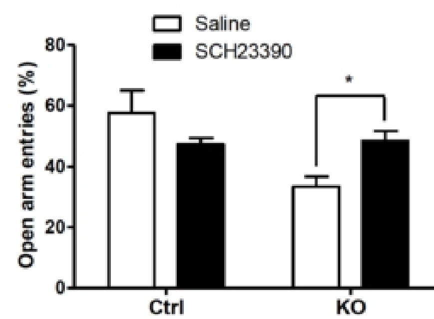

Fernandes *et al.*

Figure 5

Chapter IV

General Discussion & Conclusion

9. General Discussion

The studies described in this thesis hone in on the LepRb signaling mechanisms by which leptin affects mesolimbic DA signaling previously described by Fulton *et al*, Hommel *et al* and Domingos *et al*. (Fulton *et al.* 2000, Fulton *et al.* 2006, Hommel *et al.* 2006, Domingos *et al.* 2011). The objective of the present work was to further define the specific cells and signaling mechanisms by which leptin impacts the mesolimbic DA system. More specifically, this thesis tested the hypothesis that leptin acts in the midbrain to modulate multiple behaviors via STAT3 signaling in DA neurons. Thus, to isolate the STAT3 gene and study its role in the brain DA system, we generated a line of conditional KO mice lacking the main activation site of STAT3 gene (tyr705) selectively on DA neurons, as described in *General Methods*. It is important to highlight that STAT3 ablation was observed in DA neurons within the VTA/SN. No significant Cre-mediated STAT3 recombination was observed in other regions ***(Article#1, supplemental fig. 1)***. Of note, we are the first to identify a role for DA-specific STAT3 signaling in the modulation of locomotion, running reward, anxiety-like behavior and DA tone.

9.1. Role for STAT3 signaling in DA neurons in locomotion and running reward

The first study described in this thesis shows for the first time that STAT3 signaling in DA neurons mediates the action of leptin in the VTA to decrease spontaneous and voluntary locomotor activity and running reward, and to decrease DA overflow in the NAc of male mice. Moreover, our findings provide firm evidence that the influence of leptin in the midbrain to decrease food intake does not require STAT3 signaling in DA neurons.

Previous reports demonstrated that a direct intra-VTA leptin injection induces short-term decreases in food consumption in rats (Hommel *et al.* 2006, Bruijnzeel *et al.* 2011), while viral-mediated LepRb knockdown in the VTA has an opposite effect by increasing food intake without affecting body weight (Hommel *et al.* 2006). Thus, we sought to determine the contribution of STAT3 signaling in DA neurons on the previously described anorectic effects of leptin in the midbrain. We observed that either a single ICV or intra-VTA leptin injection reduced food intake to a similar extent in both control and $STAT3^{DAT\text{-}KO}$ mice. Our results are the first to show that leptin acts in the VTA to decrease food intake in mice, and imply that pSTAT3 in DA neurons does not mediate the acute anorectic effect of leptin. Alternatively, Hommel and coworkers found that adeno-associated virus (AAV)-mediated LepRb knockdown in the VTA, targeting both dopaminergic and non-dopaminergic neurons in this region, increases food intake (Hommel *et al.* 2006). This raises the possibility that either non-dopaminergic neurons (i.e.: GABA neurons) or other LepRb signaling pathway modulates the effect of leptin in the midbrain to decrease food consumption. Consistent with this hypothesis, Trinko and coworkers demonstrated that the anorexigenic effects of an intra-VTA leptin injection are probably mediated by ERK1/2 pathway, and STAT3 (tyr705) activation is not sufficient for the effects of leptin in the VTA to reduce food intake (Trinko *et al.* 2011). Alternatively, JAK2 signaling pathway has also been suggested as a possible mediator of the anorectic effects of leptin in the VTA (Morton *et al.* 2009).

We next checked long-term food intake and body weight of control and $STAT3^{DAT\text{-}KO}$ mice fed a chow diet and we observed that $STAT3^{DAT\text{-}KO}$ male mice exhibited reduced body weight and reduced adiposity in spite of no changes in caloric intake, when normalized by the body weight, suggesting that STAT3 signaling in DA neurons are not involved in long-term food intake. Our

findings are in agreement with previous studies by Davis and coworkers showing that knockdown of LepRb in the midbrain does not affect feeding (Davis *et al.* 2011), and suggest that signaling pathways other than STAT3 mediates the effects of leptin in the VTA to reduce feeding. To determine whether the observed reduced body weight/body fat mass in $STAT3^{DAT-KO}$ mice is due to increased energy expenditure, we assessed metabolic efficiency in these mice. Metabolic efficiency, defined as the ratio of calories consumed divided by body weight gain over a specific time interval, constitutes a initial method from which we can infer change in energy expenditure (Butler and Kozak 2010, Ellacott *et al.* 2010). Our data show that metabolic efficiency, was enhanced in $STAT3^{DAT-KO}$ mice fed a standard chow diet, as compared to littermate controls. Given that normalized chow intake is not different between genotypes, we hypothesized that the decreased body fat mass and increased metabolic efficiency observed in $STAT3^{DAT-KO}$ mice might be due to increased locomotion. To address this question, we examined ambulatory activity in $STAT3^{DAT-KO}$ and littermate controls. We found a significant increase in dark-phase (active cycle) locomotor activity in $STAT3^{DAT-KO}$ male mice relative to controls. This is in agreement with a previous report that viral-mediated VTA LepRb knockdown stimulates locomotor activity in male rats (Hommel *et al.* 2006), and thereby suggests that DA-specific STAT3 signaling mediates this effect. Furthermore, increased ambulatory activity in $STAT3^{DAT-KO}$ male mice may contribute to the observed enhanced metabolic efficiency. Of note, there's growing evidence of a link between metabolic signals and DA function, indicating a potential role for this neurotransmitter in energy balance. It has been suggested that the DA system acts to optimize the utilization of body energy resources, as DA favors energy expenditure (Beeler *et al.* 2012). In fact, dopamine can shift obese animals from a state of behavioral energy conservation to a state of behavioral energy expenditure (Beeler *et al.* 2012).

Consistent with this idea, dopamine was shown to increase resting energy expenditure in humans(Ruttimann *et al.* 1991, Nakagawa *et al.* 1994) As previously mentioned in this thesis, LepRb are also expressed on midbrain DA neurons and activation of these receptors on dopaminergic cells decreases dopamine function (Hommel *et al.* 2006, Figlewicz and Benoit 2009) , enhances locomotor activity and energy expenditure (Ribeiro *et al.* 2011).

VTA DA neurons also projects to the caudal brainstem, and VTA-SNc DA neurons modulate locomotor behaviors via projections to the basal ganglia which projects down to the brainstem mesencephalic locomotor region (Kravitz *et al.* 2010, Stephenson-Jones *et al.* 2011). Therefore, it could be possible that inactivation of STAT3 in midbrain DA neurons that project to the brainstem mediates the increases in locomotion observed in $STAT3^{DAT\text{-}KO}$ mice and thus, contributes to increased energy expenditure.

Food intake is regulated by an interaction between homeostatic and hedonic pathways. Moreover, leptin is involved in the signaling of food reward (Fulton *et al.* 2000, Fulton *et al.* 2004), and reduces the hedonic response to palatable foods (Rangel 2013). Thus, we assessed hedonic-based feeding behavior in control and $STAT3^{DAT\text{-}KO}$ mice using the "Dessert-Effect" test. In this test paradigm, the intake of a palatable food (“dessert”) is assessed in calorically-sated mice (Davis *et al.* 2012). Our results demonstrated that there are no differences in the hedonic responses to palatable food between genotypes, suggesting that STAT3 signaling in DA neurons does not modulate either the incentive motivational properties of food or food hedonics. Likewise, we did not observe differences in other aspects of feeding behavior, including food anticipatory activity (FAA), restriction-induced feeding and food preference between $STAT3^{DAT\text{-}KO}$ and control mice ***(Figures 7, 8, 9, respectively Appendix I)***. Thus, taken together, these

results strongly suggest that STAT3 signaling in DA neurons does not modulate any aspect of feeding behavior in mice.

As discussed in previous sections, leptin also affects energy balance by influencing the rewarding effects of food as well as goal-oriented behaviors that contribute to obtaining food (Rangel 2013). Accordingly, LepRb knockdown in the midbrain increases the motivation to obtain food (Davis *et al.* 2011). To test our hypothesis that STAT3 signaling in DA neurons mediates leptin's effect in food reward, we assessed food-motivated learning in $STAT3^{DAT\text{-}KO}$ and control mice. Interestingly, $STAT3^{DAT\text{-}KO}$ male mice show impaired food-motivated learning in a Fixed Ratio 1 (FR1) schedule of reinforcement. During appetitive instrumental learning tasks, animals are expected to learn to emit a behavior (lever press) to obtain a reinforcer (i.e.: sucrose pellet) (Robinson *et al.* 2007). A number of reports suggested that DA signaling (mainly in the NAc) is greatly involved in instrumental (i.e.: operant conditioning) and *Pavlovian* or classical conditioning (i.e.: CPP) learning, invoked during acquisition of behaviors associated with food reward (Wise *et al.* 1978, Smith and Schneider 1988, Beninger and Miller 1998, Di Chiara 2002, Wise 2004). Instrumental conditioning is a form of associative learning in which a voluntary response is strengthened or diminished by its consequences (i.e.: reward, punishment, etc.) (Thorndike 1911, Skinner 1966). In this kind of conditioning, the animal has to perform an operant response and learn the relationship between the response and the response-associated reward (Domjan 2005). *Pavlovian* or classical conditioning is another form of associative learning in which a stimulus initially incapable of evoking a response acquire the ability to do so, through repeated pairing with another stimulus (Pavlov 1927). Notably, the neurocircuitry underlying the classical conditioning processes are distinct from those underlying the instrumental learning (Bardo and Bevins 2000). Moreover, DA has been implicated in the

association between actions and outcomes in instrumental learning tasks (Schultz 1998, Dayan and Balleine 2002, Robinson *et al.* 2006), while in classical conditioning tests DA has been implicated in associations between neutral stimuli and rewards (Wilkinson *et al.* 1998, Dickinson *et al.* 2000, Di Ciano *et al.* 2001, Dalley *et al.* 2005). However, the role of DA in the distinct learning processes is not completely clear. For example, slightly hyperdopaminergic mice (DA levels elevated 70% above controls) display normal instrumental learning (Yin *et al.* 2006), and DA-depleted mice can acquire CPP for morphine reward (Hnasko *et al.* 2005), although they do not perform tasks that require intentional movement (Szczypka *et al.* 1999, Hnasko *et al.* 2006), such as operant responding. A recent report demonstrated that DA projections to the NAc (mediated by D1R) are critical for operant conditioning (Steinberg *et al.* 2014). Moreover, impaired D1R signaling in the NAc decreases lever pressing for food reinforcers (Nakajima 1989, Smith-Roe and Kelley 2000, Gore and Zweifel 2013). Likewise, reducing DA in the NAc using intra-NAc injections of either 6-OHDA or D1R antagonist, substantially decreased lever pressing for palatable food, without causing a general reduction in food motivation, as rats remained directed toward the acquisition and consumption of food when it is freely available (Salamone *et al.* 1991, Nowend *et al.* 2001). These findings suggest that diminishing DA in the NAc may impair the ability of animals to overcome work-related response costs in order to obtain food in a instrumental responding task (Nowend *et al.* 2001). Consistent with this idea, we observed decreased D1R protein expression and decreased DA release in the NAc of STAT3$^{DAT\text{-}KO}$ male mice, as compared to controls. Based on this result, we speculate that the significant impairment in food-motivated learning in a FR1 schedule of reinforcement observed in our model might be due to an impaired D1R transmission within the NAc of STAT3$^{DAT\text{-}KO}$ mice. Notably, in spite of their poor performance in a food-motivated learning task, STAT3$^{DAT\text{-}KO}$ male

mice acquired CPP for running reward, suggesting that their learning impairment is specific to the appetitive-operant conditioning task. The ability of $STAT3^{DAT\text{-}KO}$ mice to demonstrate associative learning in a CPP task but not in a simple operant conditioning task (FR1) underscores the point that there are multiple subtypes of reward-related learning, which are differently affected by LepRb-STAT3 signaling in DA neurons.

In view of the well-described role of mesolimbic DA system in physical activity and the reinforcing effects of running exercise, we measured voluntary wheel running in a separate cohort of mice. Voluntary running wheel exercise was shown to reduce the preference of rodents for palatable food, in addition to influence STAT3 activation selectively in the VTA (Scarpace *et al.* 2010, Shapiro *et al.* 2011). Furthermore, a single leptin injection into the VTA suppresses voluntary running exercise (Verhagen *et al.* 2011). Our results demonstrated that $STAT3^{DAT\text{-}KO}$ male mice exercise significantly more than their respective controls, suggesting that the ability of leptin in the VTA to suppress voluntary wheel running rely on STAT3 activation in DA neurons. Similarly to food, voluntary exercise can also be rewarding. As previously mentioned in this thesis, regular physical exercise can produce a rewarding effect in humans often referred to as "runner's high" (Panksepp *et al.* 2002, Dietrich and McDaniel 2004), and rats will work to have access to a running wheel (Belke 2006, Belke 2006, Belke and Christie-Fougere 2006) and spend more time in a place previously associated with the aftereffects of wheel running (Lett *et al.* 2000, Lett *et al.* 2001). To test the hypothesis whether the enhanced voluntary exercise in $STAT3^{DAT\text{-}KO}$ mice reflects an increase in the rewarding aftereffects of wheel running, we assessed the reward value of running in $STAT3^{DAT\text{-}KO}$ and control mice using a CPP paradigm. In the CPP paradigm, the reinforcing effects of objects and/or behaviors are evaluated by measuring the amount of time the animal spends in an environment previously associated with

that object/behavior (Tzschentke 2007). As the most striking finding of this study, we observed that STAT3^{DAT-KO} mice spend more time in a place previously associated with the aftereffects of wheel running, relative to controls. Moreover, a single intra-VTA leptin injection one hour prior to the CPP test impaired running CPP acquisition to a greater extent in the control group, firstly suggesting that STAT3^{DAT-KO} mice exercise more because the rewarding value of wheel running is much higher in these mice as compared to controls. Most importantly, the increased running reward observed in our model is modulated by LepRb-STAT3 signaling in DA neurons, and not by other activators for STAT3 such as CNTF or IL-6. In fact, given that LepRb-deficient *db/db* mice does not show STAT3 activation in the VTA after leptin administration (Fulton *et al.* 2006), and intra-VTA leptin decreases running reward in control mice but not in STAT3^{DAT-KO}, it is very unlikely that other STAT3 activator is involved in mediating the phenotypic characteristics of STAT3^{DAT-KO} mice. Very little is known about the neural controls of voluntary running and its rewarding effect, and we are the first to show that leptin modulates the rewarding aftereffects of running via LepRb-STAT3 signaling in DA neurons.

Besides the above-mentioned reduced D1R protein expression in NAc of STAT3^{DAT-KO} mice, the observed behavioral changes are accompanied by decreases in DA biosynthesis (decreased TH protein expression) in the NAc, which is consistent with the effects of central leptin to increase TH expression (Fulton *et al.* 2006, Leinninger *et al.* 2009), and suggests that STAT3 signaling in DA neurons is involved. Interestingly, our findings matches with those by Knab and coworkers, demonstrating that both D1R and TH expression were significantly downregulated in the NAc of mice bred for high wheel running exercise (Knab *et al.* 2009), and thus suggest that D1R as well as TH may be associated with the regulation of physical activity in STAT3^{DAT-KO} male mice. Moreover, we measured evoked DA release in the NAc core using fast scan cyclic voltammetry

in acute brain slice preparations and found that electrically stimulated DA release was reduced in STAT3$^{DAT\text{-}KO}$ male mice. This is in agreement with a previous report from Fulton and coworkers demonstrating that electrically evoked release of DA in the NAc is drastically reduced in *ob/ob* mice (Fulton *et al.* 2006), and suggest that diminished evoked DA release in STAT3$^{DAT\text{-}KO}$ male mice is induced by loss of DA-specific STAT3 signaling and could be partially related to a reduction in DA available for release, potentially due to diminished synthesis reflected by the observed reduced TH protein expression. Of note, there are no differences in DAT and D2R protein expression in the VTA, NAc, DLS and CeA.

As an alternative means to assess mesoaccumbens DA function, we investigated AMPH-induced locomotor sensitization in STAT3$^{DAT\text{-}KO}$ and control mice. Of note, one of the primary actions of AMPH is to increase dopaminergic concentration in the synaptic cleft by inhibiting DA reuptake and promoting reverse transport, resulting in efflux of DA via the DAT (Besson *et al.* 1971, Von Voigtlander and Moore 1973, Hoebel *et al.* 1983, Sulzer *et al.* 2005) - the resultant dramatic increase in extracellular DA has been suggested to be of major importance for AMPH-induced psychomotor activation (Kelly *et al.* 1975, Pijnenburg *et al.* 1975, Sulzer *et al.* 2005). Consistent with previous reports showing that leptin increases AMPH-induced locomotion in rodents (Hao *et al.* 2004, Fulton *et al.* 2006), a sensitized locomotor response was observed in control mice receiving continuous treatment with leptin or vehicle delivered via a 14-days subcutaneous mini-osmotic pump implants. Interestingly however, subcutaneous infusion of leptin also enhances the locomotor responses to AMPH in STAT3$^{DAT\text{-}KO}$ mice suggesting that LepRb-STAT3 signaling is important but not necessary for the actions of leptin to increase AMPH sensitization. Accordingly, Leinninger and coworkers reported that there is a population of LepRb neurons in the LHA expressing the neuropeptide NT, which is activated by leptin and modulates the

mesolimbic DA system (Leinninger *et al.* 2011). Most importantly, these authors observed that leptin signaling in NT neurons participates in the action of AMPH to stimulate locomotor activity (Leinninger *et al.* 2011). Thus, the fact that STAT3$^{DAT\text{-}KO}$ male mice show increased AMPH-induced locomotor response when treated with leptin, could well be related to leptin's action via an alternative signaling mechanism, such as the NT neurons in the LHA, to decrease DAT activity and consequently increase AMPH sensitization. Experiments needs to be conducted to test this hypothesis.

Taken together, our findings suggest an important role for leptin in attenuating ambulatory activity, voluntary exercise and running reward in male mice, that are mediated via LepRb-STAT3 signaling in midbrain DA neurons and changes in DA tone. We posit that leptin acts in mesolimbic brain reward circuitry as a signal of plenty to dampen the motivational and rewarding effects of several behaviors that serve to replenish energy stores. Consistent with this idea, our findings further suggest that LepRb-STAT3 signaling in DA neurons constitute a potential mechanism involved in hypoleptinemia-induced hyperactivity. Based on the fact that STAT3$^{DAT\text{-}KO}$ male mice show reduced food intake and reduced body fat mass due to increased locomotion and wheel running hyperactivity, these transgenic mice could potentially serve as a useful model for the study of activity-based anorexia (ABA).

9.2. Loss of STAT3 signaling in DA neurons induces an anxiogenic phenotype in female mice

It is well known that DA signaling in the VTA is implicated in anxiety-like behavior. Previous studies demonstrated that electrical stimulation of VTA neurons induces anxiogenic responses, while lesions of the VTA or inhibition of DA signaling within this region by stimulation of the inhibitory D2R have anxiolytic effects (Borowski and Kokkinidis 1996, Munro and Kokkinidis 1997, Gifkins *et al.* 2002). As mentioned previously, the VTA is known to project to the amygdala, among other areas. The amygdala is a subcortical structure in the limbic system which has long been highlighted as a major constituent of the neural circuits processing emotional behavior (Weiskrantz 1956, Aggleton 1993, LeDoux 2000). In regards to its anatomical organization, the amygdala consists of a group of anatomically and functionally distinct nuclei (Amaral and Insausti 1992, Saygin *et al.* 2011). Among these nuclei, the CeA is strongly modulated by DA and, most importantly, it is located in a strategic position to mediate many aspects of anxiety-like behavior, given that CeA neurons project to sites involved in mediating different aspects of mood and emotion (Amaral and Insausti 1992, Davis 1992). Consistent with these observations, DA signaling within the CeA is enhanced in response to a variety of emotional stimuli (Inglis and Moghaddam 1999, Torres *et al.* 2002) and D1R signaling in the CeA has been highly associated with anxiety behavior (Guarraci *et al.* 1999, de la Mora *et al.* 2005). In addition, leptin modulates DA signaling within the CeA via D1R (Liu *et al.* 2011) and play an important role in emotional behavior (Leshan *et al.* 2006, Scott *et al.* 2009, Liu *et al.* 2010). The anxiolytic and antidepressant properties of leptin were already described (Asakawa *et al.* 2003, Lu *et al.* 2006, Liu *et al.* 2010), however, the specific LepRb signaling pathway and brain sites has, until now, remained poorly understood.

As the most striking finding of this study, we observed that STAT3^{DAT-KO} female mice exhibited an anxiogenic phenotype, which was consistently observed in two widely used tests of anxiety: the open field test (OFT) and the elevated-plus maze (EPM). Of note, both OFT and EPM tests have proven to be efficient to detect anxiety-like behavior in studies other than those testing anxiolytic drugs (Teegarden and Bale 2007, Liu *et al.* 2011, Sharma *et al.* 2013).

Importantly, the anxiogenic behavior is not the result of general hypolocomotion or abnormalities in feeding activity as STAT3^{DAT-KO} female mice showed normal feeding behavior and locomotion when compared to littermate controls ***(Figures 10 and 11 respectively, Appendix II)***. Given that symptoms of anxiety and depression often co-occur (Sartorius *et al.* 1996), we assessed depressive-like behavior in STAT3^{DAT-KO} mice by determining immobility time in the forced-swim and tail suspension tests. In agreement with findings that LepRb signaling in DA neurons does not modulate behavioral despair in mice (Liu *et al.* 2011), depressive-like behavior is similar between genotypes. These findings suggest that leptin acts via distinct neural pathways to modulate anxiety and depression in mice. In fact, there is literature strongly supporting the involvement of LepRb signaling in the hippocampus in the modulation of depression-like behavior (Lu *et al.* 2006, Yamada *et al.* 2011, Garza *et al.* 2012, Guo *et al.* 2013). Taken together, our results points to the conclusion that STAT3 signaling in DA neurons does not affect DA signaling in brain structures involved in depression-like behavior (i.e.: hippocampus) or, alternatively, leptin's antidepressant-like effects is not mediated by STAT3 signaling in DA neurons.

Evidence from our immunoblotting studies on brain nuclei of STAT3^{DAT-KO} female mice demonstrates that DAT protein expression is reduced, while threonine-34 phosphorylation of DARPP32 is increased in the CeA of STAT3^{DAT-KO} as compared to controls. Briefly, D1R

signaling activates cAMP-dependent protein kinase (PKA), which will, in turn, directly stimulate DARPP32-Thr 34 phosphorylation (Nishi *et al.* 2000). These results suggest an increased DA signaling via D1R due to reduced DA reuptake, elicited by reduced DAT function, in midbrain DA neurons that projects to the CeA. Moreover, it represents a potential mechanism by which increased D1R signaling within the CeA mediates the anxiogenic-like behavior in $STAT3^{DAT\text{-}KO}$ female mice. Consistent with this hypothesis and observations in DA-specific LepRb KO mice (Liu *et al.* 2011), we found that pharmacological blockage of D1R signaling in the CeA normalized anxiogenic behavior in $STAT3^{DAT\text{-}KO}$ mice, suggesting that lack of STAT3 activation in DA neurons results in increased anxiety by somehow increasing D1R signaling.

As discussed in *Article 2*, our studies complement those of Liu and collaborators (Liu *et al.* 2011) and indicate that selective inactivation of STAT3 (tyr705) in DA neurons results in an anxiogenic phenotype, mediated by increased D1R signaling in the CeA of female but not male mice. Furthermore, STAT3 signaling in DA neurons does not affect despair-like behavior, suggesting that leptin's antidepressant-like action is mediated by other brain circuits or even other neurotransmitters (Harvey 2007, Guo *et al.* 2013). Intriguingly, in contrast to a previous study suggesting that lack of LepRb signaling in DA neurons increases anxiety in male mice (Liu *et al.* 2011), we found that loss of STAT3 activation in DA neurons did not affect anxiety-like behavior in $STAT3^{DAT\text{-}KO}$ male mice. There are a few hypothesis to explain the sexual dimorphism observed in our mice model. First of all, there is literature showing that females are much more prone to be afflicted by mood disorders than males (Kessler *et al.* 2005, Bekker and van Mens-Verhulst 2007), suggesting that gonadal hormones, such as estrogen and testosterone, may play an essential role in the etiology of anxiety and depression. Likewise, an important role for estrogen in augmenting both pre- and postsynaptic dopaminergic function was already

described (Morissette *et al.* 1990, Levesque and Di Paolo 1991, Hu *et al.* 2006, Becker and Hu 2008). Moreover, estrogens are known to regulate circulating leptin levels and leptin sensitivity (Rosenbaum *et al.* 1996, Shimizu *et al.* 1997, Clegg *et al.* 2006) and to improve mood (Joffe and Cohen 1998); while testosterone or androgens suppress leptin concentration (Hislop *et al.* 1999) and have anxiolytic and antidepressant benefits (Wang *et al.* 1996, Hermans *et al.* 2006, DiBlasio *et al.* 2008). It was already reported that there are differences in estrogen levels between males and females, with females showing higher levels than males (Clegg *et al.* 2006). In addition, leptinemia also varies between males and females (Maffei *et al.* 1995, Castracane *et al.* 1998), and female rats exhibits increased sensitivity to the central effects of leptin as compared to males (Clegg *et al.* 2003). Most importantly, estrogen receptors (ERs) colocalize with TH positive cells in mesolimbic regions (Kritzer 1997, Creutz and Kritzer 2002), including the amygdala (Asmus and Newman 1993, Cavanaugh and Lonstein 2010, Cavanaugh and Lonstein 2010), suggesting a potential role for estrogens on TH cell activity. In agreement with these findings, circulating levels of both estrogen receptors (ERα and ERβ) are correlated with DA hyperactivity within the mesolimbic system (Thompson and Moss 1997). Of note, estrogen modulates STAT3 in the hypothalamus (Gao and Horvath 2008) and targets ERβ in the VTA (Creutz and Kritzer 2002). Furthermore, a dense population of estrogen-expressing neurons has already been described in limbic regions, such as the CeA, in female rats (Pfaff and Keiner 1973). Given the growing literature suggesting marked sexual dimorphisms in brain wiring, morphology and chemistry (De Vries and Boyle 1998, Cosgrove *et al.* 2007), we speculate that sex differences observed in our studies may involve a difference in the number of ERs in the CeA of males *versus* females; an altered sensitivity to the anxiolytic actions of estrogen; a

different effect of estrogen in male and female brain or even due to estrogen action to increase DA reuptake within the CeA. However, these hypotheses awaits further experimentation.

Besides suggesting that leptin regulates feeding or emotion-related behavior by distinct neuronal targets and in a sex-specific fashion, the results presented on this study unravel the signaling pathway by which leptin acts on DA neurons to modulate anxiety-like behavior. Importantly our studies contribute to the mechanistic understanding of the role of leptin in anxiogenic behavior and provide insights into novel therapeutic approaches for the treatment of anxiety disorders ***(For a graphic representation of our main conclusion, see Figure 5)***.

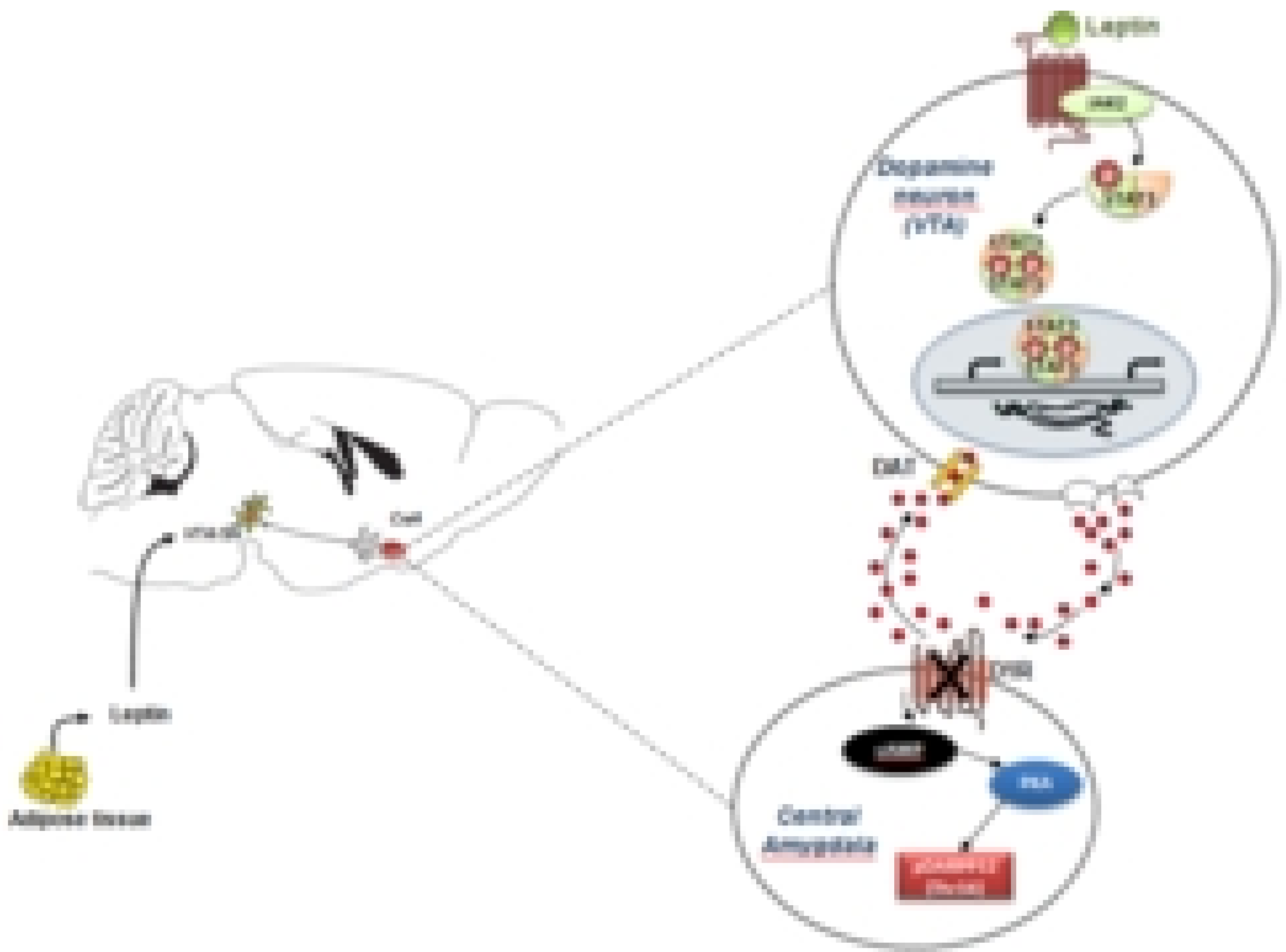

Figure 5: ***The anxiolytic actions of leptin in female mice are mediated by increased LepRb-STAT3 signaling in midbrain DA neurons that target the CeA to decrease D1R signaling, perhaps via increased DA reuptake.***

10. General limitations

Limitations of this study include:

- we cannot totally exclude the possibility of developmental compensation that might have occurred in $STAT3^{DAT\text{-}KO}$ mice;

- we cannot affirm that all the phenotype of $STAT3^{DAT\text{-}KO}$ mice was caused by lack of LepRb-STAT3 signaling in DA neurons, as not all experiments were performed in leptin-treated mice;

- we did not assess DA release in the CeA of $STAT3^{DAT\text{-}KO}$ mice, in order to better understand the mechanisms underlying the observed anxiogenic phenotype in our model;

- we did not investigate whether estrogen and leptin signaling interact in the VTA.

- the estrous cycle was not monitored or controlled in our study. Thus, the contribution of the hormonal milieu to the gender differences reported cannot be determined.

11. Perspectives

The identification of a major role for STAT3 signaling in DA neurons in the modulation of locomotion, running reward, operant learning, DA tone and anxiety-like behavior in addition to the sexual dimorphic phenotype observed in STAT3$^{DAT\text{-}KO}$ mice will undoubtedly lead to a great amount of future research. The following are a select few studies that may shed further insight on STAT3 signaling in midbrain DA neurons.

- *Which DA-relevant genes are directly modulated by STAT3?*

To address this question, future studies can evaluate the direct binding of STAT3 to promoters using pSTAT3 chromatin immunoprecipitation.

- *Which are the LepRb signaling pathways mediating the effects of leptin in feeding behavior?*

As mentioned in previous sections, leptin's effect in eating behavior is well described (Fulton 2010, Narayanan *et al.* 2010, Fernandes *et al.* 2013). However, we found that DA specific STAT3 activation does not mediate the effects of leptin on any aspect of feeding behavior, suggesting that either GABA neurons or other LepRb signaling pathway is involved. To further our understanding of the LepRb signaling pathways mediating the effects of leptin in feeding behavior, it would be interesting to perform experiments involving the use of viral-mediated knockdown of STAT3 in the VTA by administering AAV delivery of Cre-recombinase directly into the VTA of STAT3$^{lox/lox}$ mice, targeting both DA and GABA neurons. Alternatively, if this KO model also does not show any effect on feeding behavior, it would be relevant to use

pharmacological manipulations to modulate the activity of LepRb relevant signaling pathways (i.e.: PI3-K, MAPK and AMPK) in the VTA. These experiments will allow a better understanding of the contribution of specific LepRb signaling pathways in the actions of leptin in the midbrain to impact feeding behavior.

- *Which signaling molecules are involved in leptin-induced increased AMPH sensitization in STAT3 $^{DAT\text{-}KO}$ mice?*

Leptin has been shown to play important roles in the control of the mesolimbic DA system, including in the behavioral responses to AMPH (Sulzer *et al.* 1995, Figlewicz *et al.* 1998, Kahlig *et al.* 2005, Fulton *et al.* 2006). Our data demonstrated that leptin peripheral administration induced locomotor sensitization to AMPH in STAT3$^{DAT\text{-}KO}$ mice. This is suggestive that leptin's effect to enhance AMPH sensitization (Fulton *et al.* 2006) does not only involve STAT3 signaling in DA neurons. It was already reported that:

1) VTA NT signaling contributes to AMPH psychomotor activation (Panayi *et al.* 2005);

2) leptin action via NT neurons modulates the locomotor stimulating effects of AMPH (Leinninger *et al.* 2011);

3) LepRb neurons expressing NT projects to the VTA (Leinninger *et al.* 2011)

Thus, we hypothesize that leptin may be acting via NT neurons to induce AMPH sensitization in STAT3$^{DAT\text{-}KO}$ mice. As a means to test this hypothesis, I propose to determine whether an intra-VTA injection of the NT receptor antagonist (SR142948A) would prevent leptin-induced AMPH

sensitization in $STAT3^{DAT-KO}$ mice. This experiment will determine whether LepRb-NT signaling mediates leptin action to promote AMPH sensitization.

- *What is the contribution of estrogen signaling for the sexual dimorphism observed in this study?*

The answer to this question could be found by determining whether central estradiol administration activates STAT3 in DA neurons. If it is the case, it would be very interesting and informative to investigate whether ovariectomized $STAT3^{DAT-KO}$ mice results in a behavioral phenotype similar to that observed in $STAT3^{DAT-KO}$ male mice. These studies could open up a compelling line of research on the crosstalk between leptin and estrogen signaling in DA neurons.

12. Conclusion

The salient findings of this thesis are:

I. Our studies with STAT3^{DAT-KO} mice suggest that the influence of leptin in the midbrain to decrease free-feeding intake does not require STAT3 activation in DA neurons. Alternatively, previous data suggest that JAK2 (Morton *et al.* 2009)or ERK1/2-MAPK signaling (Trinko *et al.* 2011) is likely involved in the anorectic responses of leptin in the VTA. Beyond that, we found that LepRb-STAT3 signaling in DA neurons does not modulate feeding behavior.

II. Based on our findings that LepRb-STAT3 signaling in DA neurons mediates the effect of leptin in the midbrain to decrease locomotor activity, voluntary exercise and running reward in male mice, we hypothesize that leptin acts in mesolimbic brain reward circuitry as a satiety signal to reduce the motivational and rewarding value of behaviors that contribute to replenish energy stores. Consistent with this idea, lack of STAT3 activation in midbrain DA neurons reduced both DA biosynthesis and NAc evoked DA release.

III. Loss of STAT3 signaling in DA neurons increases anxiety in female mice without affecting depressive-like behavior. Furthermore, the anxiolytic action of leptin in female mice is mediated by increased LepRb-STAT3 signaling in midbrain DA neurons that target the CeA to decrease D1R signaling, perhaps via increased DA reuptake.

Appendices

The aim of this section is to describe relevant unpublished data obtained from STAT3$^{DAT\text{-}KO}$ *mice which will hopefully contribute for a broader understanding of the function of STAT3 signaling in DA neurons.*

13. Appendices

13.1. Appendix I

Unpublished results on the role for STAT3 signaling in DA neurons in the modulation of feeding behavior in male mice

In addition to the chow intake studies described in *Article 1*, we also conducted experiments to check long-term HF intake and body weight of control and in STAT3$^{DAT\text{-}KO}$ mice. In agreement with what was observed in the chow-fed groups, there are no differences in HF consumption and/or body weight between genotypes ***(Figure 6A-B, Appendix I)***. Notably, besides the hypothalamus, the VTA has been suggested as another susceptible site for leptin resistance (Matheny *et al.* 2011, Bruijnzeel *et al.* 2013), given the observations that leptin-induced STAT3 phosphorylation is impaired in this region in long-term HF-fed animals (Matheny *et al.* 2011). Thus, a possible hypothesis to explain why food intake and body weights are not different between HF-fed STAT3$^{DAT\text{-}KO}$ and control mice could be that the long term exposure to this palatable food triggers leptin resistance in the VTA of *control* mice, by increasing SOCS 3 expression. This way, VTA leptin-resistant *control* mice would resemble the phenotype of STAT3$^{DAT\text{-}KO}$ mice in which concerns to HF intake and body weight. In another words, we hypothesize that in a HF condition LepRb-STAT3 signaling is also disrupted in the VTA of control mice, so that both STAT3$^{DAT\text{-}KO}$ and control would exhibit a similar phenotype. This is a very speculative explanation, which would require further experimentation.

In regards to other aspects of feeding behavior, we also failed to observe differences between genotypes in FAA ***(Figure 7A-B, Appendix I)*** restriction-induced feeding ***(Figure 8, Appendix I)*** and food preference ***(Figure 9, Appendix I)*** tests. FAA is defined as a behavior which includes increased locomotion prior to receiving a daily scheduled meal (Merkestein *et al.* 2012).

As shown in *Figure 7*, STAT3 signaling in midbrain dopaminergic neurons does not affect FAA in mice, when using a food-restriction protocol. Similarly, lack of leptin in *ob/ob* mice failed to affect critically the expression of FAA (Gunapala *et al.* 2011). On the other hand Ribeiro and coworkers observed that leptin administration abolishes FAA in *ob/ob* mice (Ribeiro *et al.* 2011). Moreover, leptin receptor-deficient *Zucker* rats exhibited enhanced FAA after 22 days of food restriction (Mistlberger and Marchant 1999). The apparent inconsistency regarding leptin's effect on the expression of FAA is likely related to different experimental conditions, including the duration of the food restriction protocol (long *versus* short duration) and also to the type of restriction used (caloric restriction *versus* food intake restriction). To access restriction-induced feeding in $STAT3^{DAT-KO}$ mice, we used a relatively short-term food restriction protocol (12 days) during which the animals were habituated to have a limited access to standard chow daily (4h only), while most of the studies suggesting that leptin reduces FAA used a long-term restriction protocol (22-28 days). Furthermore, our findings suggest that locomotor behavior and FAA are regulated independently by LepRb-STAT3 signaling in DA neurons.

We also failed to observe differences in chow consumption between $STAT3^{DAT-KO}$ and control mice, following a 3-days food restriction-induced feeding, which is consistent with our previous observation that there are no differences in regular chow consumption between genotypes. Given food-restriction is known to induce stress (Ilott *et al.* 2014), and leptin has been suggested to affect stress-induced eating (Appelhans 2010, Tomiyama *et al.* 2012), a possible explanation for the lack of difference in restriction-induced feeding between groups could be that leptin does not modulate this behavior via LepRb-STAT3 signaling in midbrain DA neurons. Alternatively, Tomiyama and coworkers reported that the influence of leptin in stress-induced eating in humans is specific for sweetened HF diet, as they failed to find a relationship between leptin and stress-

induced feeding in other food category (Tomiyama *et al.* 2012). Consistent with these findings, other studies in rodents and humans suggested that stress affects sweet food intake to a much greater extent than it affects the intake of regular chow or salty foods (Kawai *et al.* 2000, Epel *et al.* 2001, Dallman *et al.* 2003, Habhab *et al.* 2009). Therefore, the inconsistency between our results and these findings could be due to the use of different protocols and also to the fact that we used standard chow in our experiments, instead of opting for a more palatable diet.

A role for DA in the modulation of food choice was previously described (Cooper and Al-Naser 2006). In addition, leptin has been suggested to modulate food preference in humans and rodents (Wetzler *et al.* 2004, Licinio *et al.* 2007, Tomiyama *et al.* 2012), although the molecular mechanisms and cell types involved remain unknown. Moreover, to our knowledge, the effects of STAT3 signaling in DA neurons have not been previously reported in any form of food-preference situation. Thus, we assessed food choice (high-fat *versus* low-fat diet) in control and $STAT3^{DAT\text{-}KO}$ mice and we failed to observe differences between genotypes, further suggesting that leptin's modulation of food preference does not involve STAT3 signaling in DA neurons.

Along with the findings presented on *Article 1*, these results strengthen our conclusion that STAT3 signaling in DA neurons does not influence any aspect of feeding behavior.

Methods

Long-term HF intake and body weight

STAT3$^{DAT\text{-}KO}$ and control mice were housed individually and had free access to a HF diet (D12231; Research Diets, Inc., New Brunswick, NJ, USA) containing 58% kcal from fat; 16.4% kcal from protein and 25.5% kcal from carbohydrates and tap water throughout the experiment (8 to 23 weeks of age). The animal room was kept at 22–24°C in a 12:12 h light–dark cycle. Body weight and HF intake were assessed three times a week at 10am (onset of the dark period). In addition to the amount of food consumption, weekly HF caloric intake for each mouse was normalized by its respective body weight.

Food anticipatory activity

Mice were habituated to have a 4 hour-limited access to standard chow (Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Indianapolis, Indiana, USA) daily (12-4pm) during 19 days in a novel entrainment cage (CLAMS metabolic chambers). On day 20, mice were placed in their entrainment cage 4h prior to food access (8am) and locomotor activity was measured using an automated comprehensive laboratory animal monitoring system (CLAMS, Columbus Instruments, Columbus, OH, USA). Locomotor activity was defined as successive linear infrared beam breaks recorded in 20min bins (breaks/20min).

Food restriction-induced feeding

All mice were housed individually with free access to standard chow and water. Food intake was measured for 3 consecutive days. After that (on the following 3 days) STAT3$^{DAT\text{-}KO}$ and control mice were 50% food restricted. On day 4 all mice were given free access to standard chow and food consumption was measured after 2, 6, 12 and 24h.

Food preference test

Food choice was assessed in a food preference test for the HF diet (D12231; Research Diets, Inc., New Brunswick, NJ) containing 58% Kcal from fat; 16.4% Kcal from protein and 25.5% Kcal from carbohydrates, or ingredient-matched LF diet (D12328; Research Diets, Inc., New Brunswick, NJ), containing 10.5% Kcal from fat; 16.4% Kcal from protein and 73.1% Kcal from carbohydrates. All mice were single housed with free access to tap water. In each cage two cups were provided in opposite corners one filled with HF diet and the other filled with LF diet. The location of the cups was switched every 24 hour and food choice was assessed for 3 consecutive days by measuring the amount of diets consumed. The results were presented as a ratio of HF preference.

Statistical analyses

Data were analyzed using GraphPad 5 and presented as means and respective standard errors. A two-way ANOVA with Bonferoni post-hoc tests was used to compare STAT3$^{DAT\text{-}KO}$ and control mice body weight, HF caloric intake, chow intake in a food restriction-induced feeding test, FAA and food preference. Criterion for significance was set to $p \leq 0.05$ in all comparisons.

Figure legends

Figure 6. ***Long-term high-fat(HF) diet intake and body weight is not different between STAT3$^{DAT\text{-}KO}$ and control mice.*** (A) Cumulative HF caloric intake of STAT3$^{DAT\text{-}KO}$ and control mice. n= 8-11/group. (B) Long-term body weight in STAT3$^{DAT\text{-}KO}$ and control mice. n= 8-11/group.

Figure 7. ***Food anticipatory activity (FAA) is not different between STAT3$^{DAT\text{-}KO}$ male mice and littermate controls****.* (A) 4h-locomotor activity prior to food access is not different in control and STAT3$^{DAT\text{-}KO}$ mice. n= 5/group. (B) Locomotor activity is not different between control and STAT3$^{DAT\text{-}KO}$ mice 60 minutes prior to access of food (n=5/group).

Figure 8. ***Food restriction-induced feeding is not different between STAT3$^{DAT\text{-}KO}$ male mice and littermate controls****.* (A) Protocol schematics. (B) Free-access to food after three days of 50% food restriction did not alter significantly regular chow consumption between genotypes.n=7/group.

Figure 9: ***Food preference in STAT3$^{DAT\text{-}KO}$ mice.*** HF- versus LF-diet preference is similar between STAT3$^{DAT\text{-}KO}$ and littermate controls. n=10-14/group.

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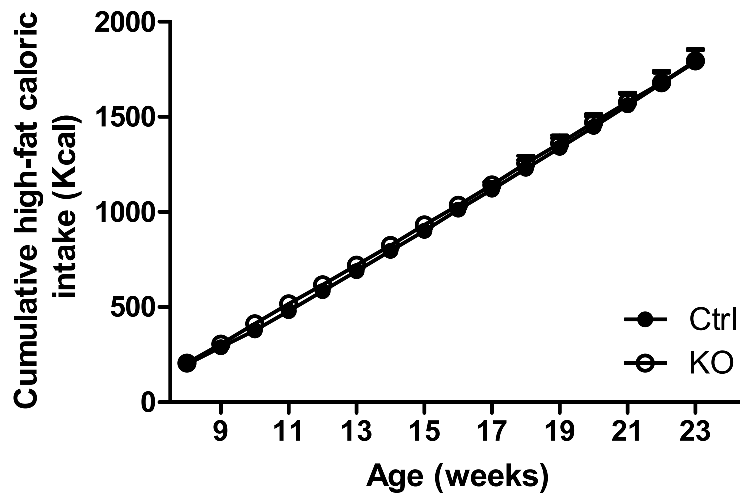

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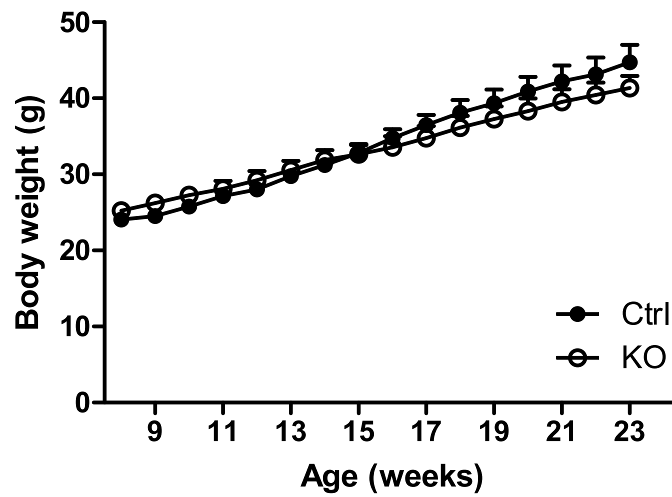

Figure 6

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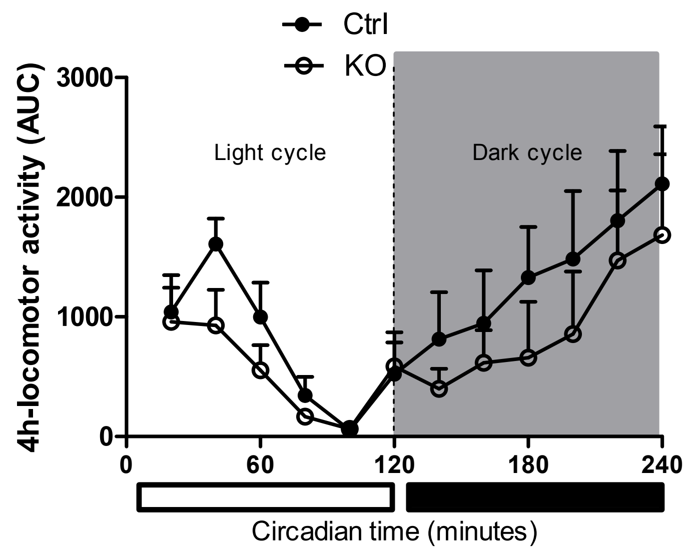

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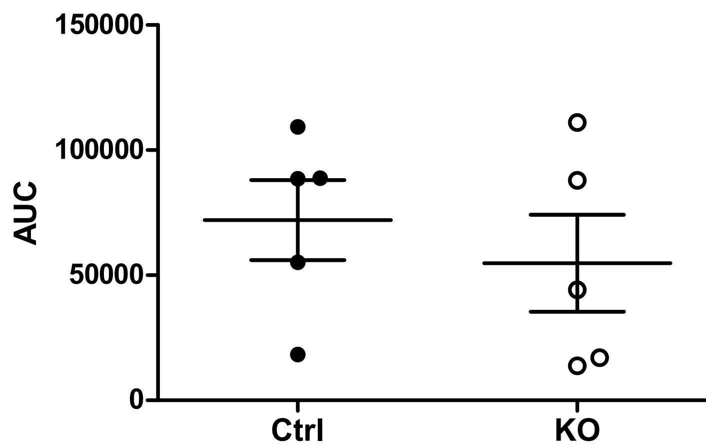


Figure 7

A)

B)

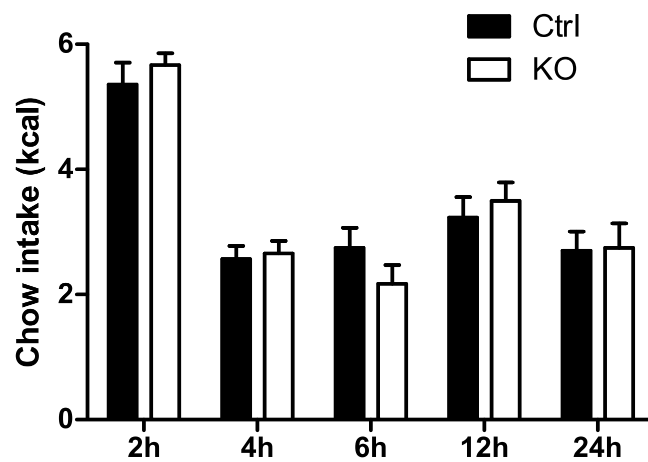


Figure 8

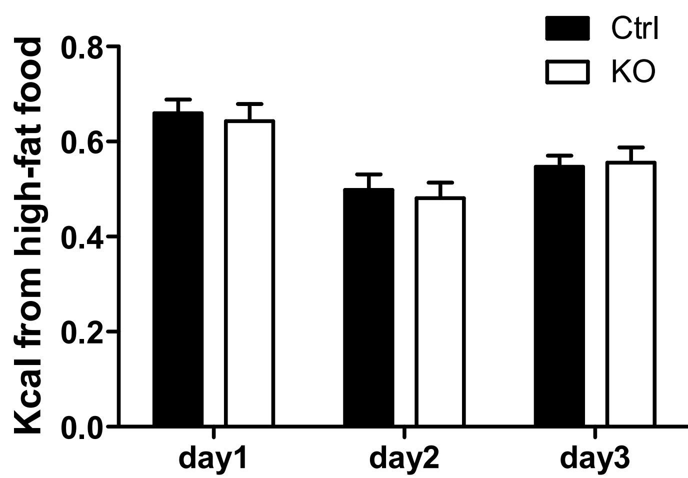


Figure 9

13.2. Appendix II

Unpublished results on the role for STAT3 signaling in DA neurons in the modulation of feeding behavior and learning in female mice

In agreement with our findings in STAT3$^{DAT\text{-}KO}$ male mice, our studies in female mice also demonstrated that STAT3 signaling in DA neurons does not influence food intake and body weight in chow-fed animals ***(Figure 10A, Appendix II)***. Likewise, food preference (HF *versus* LF diet) as well as HF diet intake was also unaltered in STAT3$^{DAT\text{-}KO}$ female mice ***(Figure 10B-C, Appendix II)***. However, we found that gender significantly affects general locomotion in STAT3$^{DAT\text{-}KO}$ mice as, opposed to what was observed in males, ambulatory activity and voluntary wheel running exercise is similar between STAT3$^{DAT\text{-}KO}$ and control female mice ***(Figure 11A-B, Appendix II).*** The reason for the gender difference in locomotor behavior is not known although, we hypothesize that estrogen signaling in DA neurons could be involved, as it is well described that there are sex differences in dopaminergic function in the brain (Kuhn *et al.* 2010). Furthermore, circulating leptin levels are modulated by different cytokines and hormones (including estrogen) that are also under the influence of gender (Considine 2001, Janeckova 2001). Of note, leptin levels are higher in females than in males (Frederich *et al.* 1995, Considine 2001, Janeckova 2001), also suggestive of a possible sexual dimorphism of LepRb signaling.

Similar to what was observed in STAT3$^{DAT\text{-}KO}$ male mice, lack of STAT3 signaling in DA neurons also suppresses lever pressing for food in females - STAT3$^{DAT\text{-}KO}$ female mice showed severe impairment in learning a FR1 schedule of reinforcement in an appetitive operant conditioning task ***(Figure 12A-B, Appendix II)***. There is data implicating DA signaling in the dorsolateral striatum (DLS) in the modulation of learning, including operant learning (Frank *et al.* 2004, Yin *et al.* 2006, Balleine *et al.* 2007, Balleine and O'Doherty 2010). Consistent with

this idea, we found that D1R protein expression is downregulated in the DLS of STAT3$^{DAT\text{-}KO}$ female mice as compared to controls, which we hypothesize may be related to the severe impairment in learning a FR1 schedule of reinforcement for food pellet reward ***(Figure 12C, Appendix II)***. Furthermore, STAT3$^{DAT\text{-}KO}$ female mice exhibited normal performance in a CPP for palatable food (sweetened HF pellets), strengthening our hypothesis that loss of STAT3 in DA neurons mediates leptin's effect in appetitive operant learning, but not in a classical conditioning task ***(Figure 13, Appendix II)***. Of note, the learning impairment observed in STAT3$^{DAT\text{-}KO}$ mice is not related to locomotor deficits or loss of appetite, as these mice exhibited normal ambulatory activity and feeding behavior as compared to controls. Although our findings suggest that STAT3 signaling in DA neurons is critical for operant learning, more experiments needs to be conducted to shed light on the detailed mechanisms underlying the effects of leptin signaling in different forms learning.

Methods

Long-term food intake and body weight

STAT3^{DAT-KO} and control mice were housed individually with free access to standard chow (Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Indianapolis, Indiana, USA) and tap water throughout the experiment (8 to 23 weeks of age). The animal room was kept at 22–24°C in a 12:12 h light–dark cycle. Body weight and food intake were assessed three times a week at the onset of the dark period.

Food preference test

Food choice was assessed in a food preference test for the HF diet (D12231; Research Diets, Inc., New Brunswick, NJ) containing 58% Kcal from fat; 16.4% Kcal from protein and 25.5% Kcal from carbohydrates, or ingredient-matched LF diet (D12328; Research Diets, Inc., New Brunswick, NJ), containing 10.5% Kcal from fat; 16.4% Kcal from protein and 73.1% Kcal from carbohydrates. All mice were single housed with free access to tap water. In each cage two cups were provided in opposite corners one filled with HF diet and the other filled with LF diet. The location of the cups was switched every 24 hour and food choice was assessed for 3 consecutive days by measuring the amount of diets consumed. The results were presented as a ratio of HF preference.

Long-term HF intake

STAT3^{DAT-KO} and control mice were housed individually and had free access to a HF diet (D12231; Research Diets, Inc., New Brunswick, NJ, USA) containing 58% kcal from fat; 16.4% kcal from protein and 25.5% kcal from carbohydrates and tap water throughout the experiment

(8 to 23 weeks of age). The animal room was kept at 22–24°C in a 12:12 h light–dark cycle. Food intake was assessed three times a week at the onset of the dark period.

Ambulatory activity

To access 24-hour spontaneous locomotor activity $STAT3^{DAT-KO}$ and littermate controls were individually placed into metabolic cages (Accuscan Instruments Inc., Columbus, OH, USA) consisting of 16 light beams arrays in x, y and z axes. Distance travelled (horizontal activity) was measured by nearby computer-controlled software.

Voluntary wheel running activity

To record daily running wheel behavior, animals were single housed into standard rat cages (26,03cm width x 48,26cm depth x 20,32cm height) containing plastic wireless running wheels (one wheel revolution=36.9 cm, Med Associates Inc., St. Albans, VT, USA). Wheel running activity was continuously monitored through wireless transmitters and recorded using the Wireless Running Wheel Manager Data Acquisition Software (Med Associates Inc., St. Albans, VT, USA). Data were exported from the wheel manager database for further analysis. The parameter evaluated was the total wheel running activity (revolutions *per* 24 h).

Appetitive food-motivated operant learning

Fixed ratio 1 (1:1) schedule of reinforcement

We used mouse operant chambers (Med Associates Inc., St. Albans, VT, USA) equipped with two levers and a pellet dispenser delivering 20mg of sucrose pellets (Bio-Serv, Frenchtown, NJ, USA). During the operant conditioning sessions, the mouse is expected to press the designated

active lever (triggering food reward) to get a reinforcer. The allocation of right and left levers were counterbalanced between mice. All mice were trained in 60 min sessions to press the active lever on a continuous reinforcement schedule (FR1), in which every lever press is reinforced with a 20 mg sucrose pellet. All training was conducted in the dark cycle and the animals were 50% food restricted during the operant training. Operant training was carried out over 10 days.

Conditioned Place Preference for sweetened HF food

The place preference apparatus (Med Associates Inc., St. Albans, VT, USA) consisted of three chambers with overall inside dimensions of: 46.5cm length x 12.7cm width x 12.5cm height. The two choice compartments consist of a black compartment with a stainless steel grid rod floor and a white compartment with a spaced stainless steel mesh floor. These two chambers are separated by a small center compartment that is 11.7 cm long with a neutral gray finish and a PVC floor. *Preference test*: On day 0 each experimental mouse was confined to both the black and white compartment of the CPP apparatus for 5 minutes to allow habituation to each chamber. On this same day, animals were allowed to move freely within the apparatus for 15 minutes, during which the amount of time spent in each compartment was recorded. *Conditioning training*: In the following 14 days, mice were randomly assigned to a pairing compartment (black or white) associated with palatable food (sweetened HF pellets, containing 48.9% Kcal as fat, Bio-Serv, Frenchtown, NJ, USA) on even days or no food on even days. Mice were confined to the appropriate compartment for 30 min during the 14-days conditioning period. *CPP test:* On day 15, each mouse was placed back in the CPP apparatus and allowed to move freely across compartments for 15 min. The amount of time spent in each compartment was recorded and the proportion of time spent on the paired side was compared to that obtained during the pre-test.

Western immunoblotting

Mice were decapitated under Isoflurane anaesthesia. Brains were rapidly dissected and stored at -80°C. Frozen brains were sliced into 0.5mm coronal sections using a brain matrix. Coronal sections were mounted onto slides and maintained on dry ice. Nuclei were microdissected using brain tissue punches (Stoelting Inc., Wood Dale, IL). Bilateral punches of 1.0mm diameter were obtained from the DLS. Microdissected tissues were homogenized on ice in 100 ml of cell lysis buffer (100mM Tris, pH 7.5; 750mM NaCl; 5mM Na_2EDTA; 5mM EGTA, pH 7.5; 5% Triton x-100; 12.5mM sodium pyrophosphate; 5mM beta-glycerophosphate; 1mM, 5mM Na_3VO_4; 5µg/ml leupeptin) with added protease (PMSF 1mM) and phosphotase inhibitors (Sigma phosphatase inhibitor cocktails I and II) in 1.5 ml tubes using a motorized pestle, and after that centrifuged for 15 min at 14000 g. Protein concentrations were measured using BCA protein assay (Pierce Biotechnology, IL, USA). Protein samples were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to a nitrocellulose membrane (EMD Millipore Corporation, Billerica, MA, USA). Non-specific binding sites were blocked in TBS 5% low-fat milk and 0.1% Tween-20 or 5% BSA. Membranes were rinsed in buffer (0.1% Tween-20 in TBS) and then incubated with anti-D1R (1:1000; Millipore, EMD Millipore Corporation, Billerica, MA, USA) followed by anti-rabbit IgG horseradish peroxidase-conjugate (1:5000). After rinsing with buffer, immunocomplexes were visualized by chemiluminescence using the western lighting plus ECL kit (PerkinElmer, Waltham, MA, USA). Protein size was compared by using precision plus protein ladder (Bio-Rad, Bedford, MA, USA). The film signals were digitally scanned and then density quantified using ImageJ software. GAPDH (1:10000, Cell

Signaling, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as an internal control, such that data were standardized according to GAPDH values.

Statistical analyses

Data were analyzed using GraphPad 5 and presented as means and respective standard errors. A two-way ANOVA with Bonferoni post-hoc tests was used to compare control and $STAT3^{DAT\text{-}KO}$ mice body weight, food intake (chow and HF diet), food preference, food motivation and voluntary exercise. Unpaired t-tests were used to compare control and $STAT3^{DAT\text{-}KO}$ mice locomotor activity, food CPP and D1R protein expression. Criterion for significance was set to $p \leq 0.05$ in all comparisons.

Figure legends

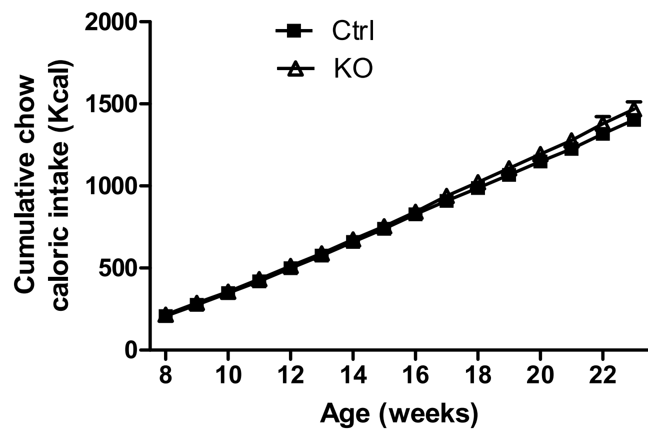
Figure 10. ***Caloric intake, body weight, and food preference is not different in STAT3^{DAT-KO} female mice as compared to controls***. (A) Average long-term caloric intake and body weight of STAT3^{DAT-KO} female mice and littermate controls on regular chow diet. n=8-9/group (B) Food-preference test in STAT3^{DAT-KO} and control mice. n=11-12/group. (C) Average long-term high-fat caloric intake of STAT3^{DAT-KO} female mice and littermate controls. n=12-13/group.

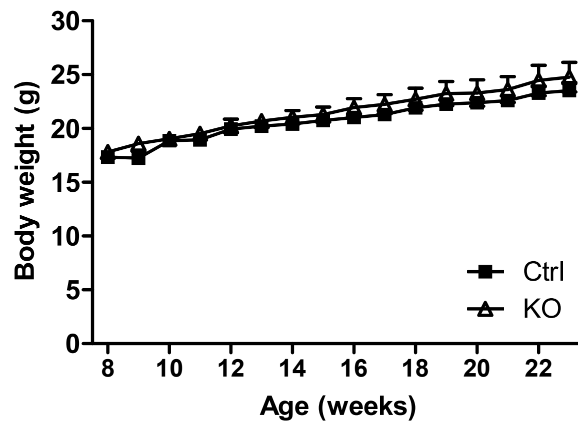
Figure 11. ***Locomotor activity and voluntary exercise is not different in STAT3^{DAT-KO} female mice as compared to controls***. (A) Dark (active) phase locomotor activity expressed as total distance travelled during the test in STAT3^{DAT-KO} female mice as compared to controls. n=7-9/group. (B) Voluntary wheel running exercise in female mice, expressed as average number of wheel revolutions per week. n=10 mice/group.

Figure 12. ***Operant conditioning task and D1R protein expression in STAT3^{DAT-KO} and control mice***. (A) Total lever press. n= 8-10/group ***$p<0.001$ (B) Percentage of correct over incorrect responses by day 10. n= 8-10/group. **$p<0.01$ (C) Significant decrease in D1R protein levels in the dorsolateral striatum (DLS) of STAT3^{DAT-KO} mice as compared to controls. n=4-7/group. **$p<0.01$.

Figure 13. ***STAT3^{DAT-KO} female mice acquired CPP for a sweetened high-fat food***. (A) CPP protocol schematics (B) Preference for the food-paired side of the CPP apparatus after minus before the CPP test. n=11/group.

A)





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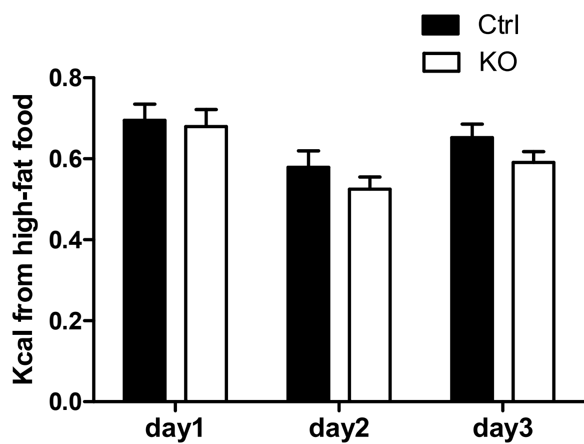

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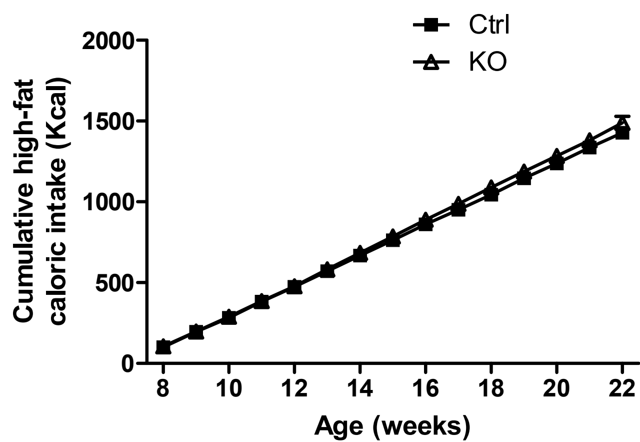

Figure 10

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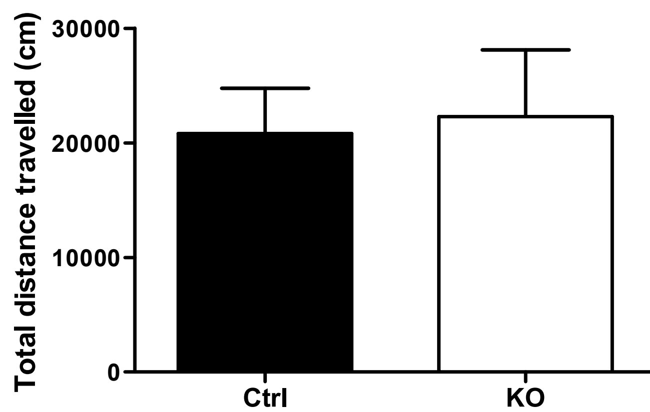

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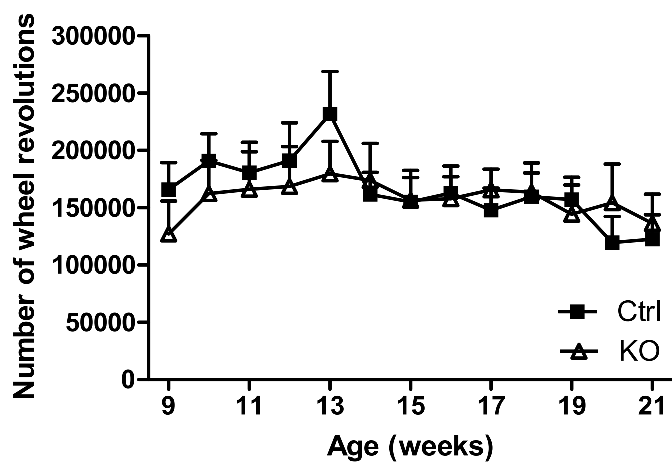

Figure 11

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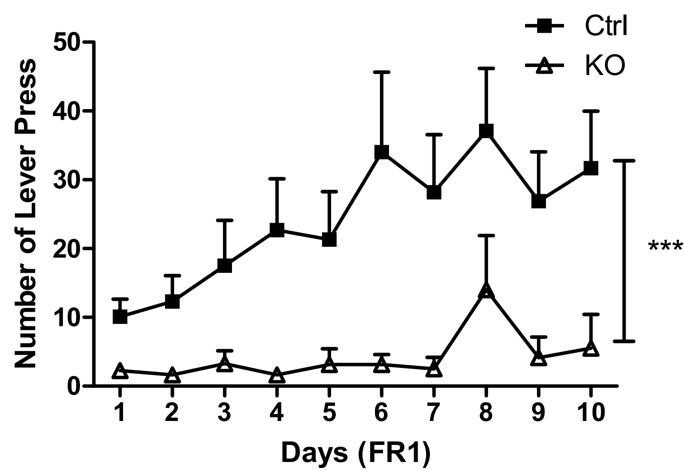

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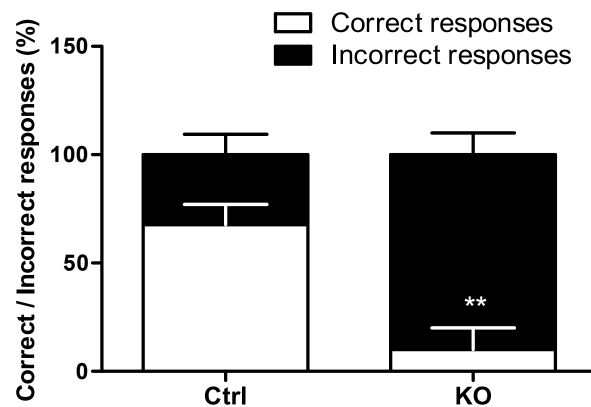

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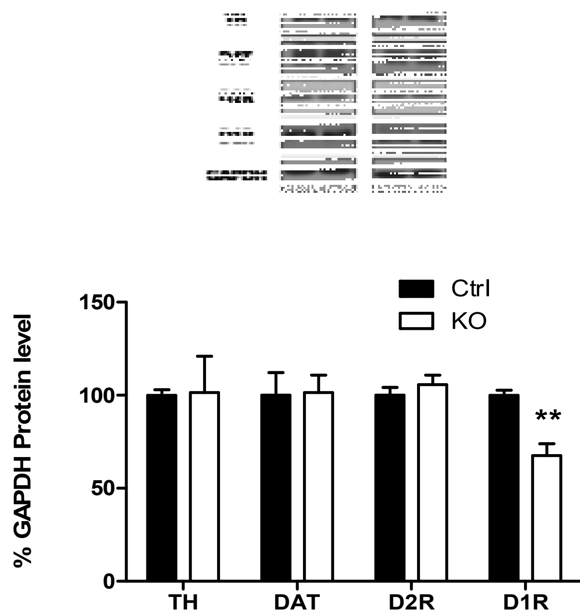

Figure 12

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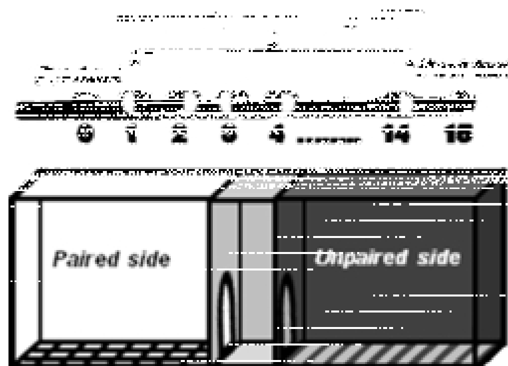

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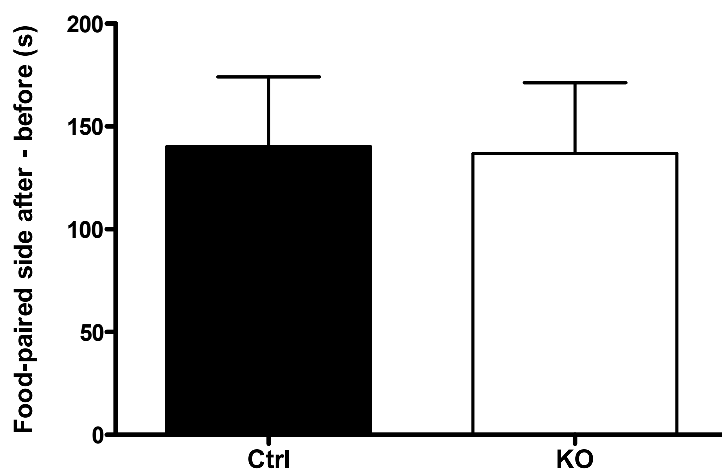

Figure 13

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