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Atypical Antipsychotic-Induced Weight Gain: A Look at Leptin Signaling

Jessica Y. Davie

Atypical Antipsychotic-Induced Weight Gain: A Look at Leptin Signaling

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Jessica Y. Davie

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The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

ABSTRACT AND KEYWORDS

Atypical antipsychotic drugs are associated with increased weight gain, which often leads to patient non-compliance and decreased quality of life among individuals with schizophrenia. It is proposed that atypical antipsychotics facilitate metabolic disturbances by disrupting hypothalamic control of feeding and energy homeostasis. The first aim of this thesis focused on the development of a reliable animal model of antipsychotic-induced weight gain. Female Sprague Dawley rats received daily oral administrations of olanzapine for 14 days. After sub-chronic treatment, these animals were sacrificed to analyze the expression of specific hypothalamic peptides involved in leptin signaling pathways, specifically phosphorylated signal transducer and activator of transcription (pSTAT3), pro-opiomelanocortin (POMC) and Agouti-related peptide (AgRP). Immunohistochemistry was used to localize and quantify the number of pSTAT3-immunoreactive neurons in the ventromedial nucleus (VMH) and arcuate nucleus (ARC) of the hypothalamus. POMC and AgRP immunoreactivity was quantified in the ARC. Results show that sub-chronic administration of olanzapine in female rats is associated with decreased pSTAT3 in the VMH and reduced POMC in the ARC. These findings implicate a possible mechanism of antipsychotic-induced weight gain by dampening anorexigenic signals via reduced pSTAT3 and POMC expression.

KEYWORDS

Antipsychotic, atypical, olanzapine, weight gain, rodent model, leptin, POMC, AgRP, STAT3

CO-AUTHORSHIP STATEMENT

The manuscript found in chapter 2 of this thesis is co-authored by:

Dr. Raj Rajakumar, PhD: As my supervisor, Raj developed this project, provided the laboratory, equipment, and supplies needed to complete this project. Funding was provided by the Ontario Mental Health Foundation.

Dr. Kem Rogers, PhD: As my secondary supervisor, Kem provided the microscope equipment and expertise. He also assisted with the direction and troubleshooting of this study.

Jessica Davie, MSc: As the first author of this paper, I was responsible for all experimental work and data analysis, preparation of figures and writing of the manuscript.

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LIST OF ABBREVIATIONS, SYMBOLS, NOMENCLATURE

3V	Third ventricle
5-HT	5-hydroxytryptamine
5-HT_{1A}	5-hydroxytryptamine-1A receptor subtype
5- HT_{2A}	5-hydroxytryptamine-2A receptor subtype
5-HT_{2C}	5-hydroxytryptamine-2C receptor subtype
5-HT_6	5-hydroxytryptamine-6 receptor subtype
αMSH	Alpha Melanocyte Stimulating Hormone
β	Beta
γ	Gamma
ABC	Avidin-Biotin Complex
AgRP	Agouti-Related Peptide
AMPK	Adenosine Monophosphate-activated Protein Kinase
ARC	Arcuate Nucleus
C	Control
CART	Cocain and Amphetamine Regulated Transcript
D2	Dopamine Receptor 2
DAB	3, 3'-Diaminobenzidine Tetrahydrochloride
DMH	Dorsomedial Hypothalamus
DNA	Deoxyribonucleic Acid
EDTA	Ethylene-diamine-tetraacetic Acid
EIA	Enzyme Immunoassay

EPS	Extrapyramidal Symptoms
GABA	Gamma Amino Butyric Acid
H	Haloperidol
H1	Histamine Receptor 1
HCRT	Hypocretin
i.m.	Intramuscular
i.p.	Intraperitoneal
JAK2	Janus Tyrosine Kinase 2
LbR	Leptin Receptor
LEP	Leptin
MC3R	Melanocortin Receptor 3
MC4R	Melanocortin Receptor 4
MCH	Melanin Concentrating Hormone
MCHR1	Melanin Concentrating Hormone Receptor 1
mRNA	Messenger Ribonucleic Acid
NMDA	N-methyl-D-aspartic acid
NPY	Neuropeptide Y
O	Olanzapine
Ob	Obese
P	Phosphate
PB	Phosphate Buffer
PFLH	Perifornical Area/Lateral Hypothalamus
p.o.	oral

POMC	Pro-opiomelanocortin
pSTAT3	Phosphorylated Signal Transducer and Activator of Transcription 3
PVN	Paraventricular Nucleus
STAT3	Signal Transducer and Activator of Transcription 3
Veh	Vehicle
VMH	Ventromedial Hypothalamus
Y1	Neuropeptide Y Receptor

Omit page

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Schizophrenia

Schizophrenia is a long-term brain disease disrupting normal mental function and social behaviour (1). The term schizophrenia encompasses a wide spectrum of conditions grouped into positive, negative and cognitive symptoms. Positive symptoms include hallucinations, delusions, and disorganized thought (1-3). A loss of motivation and sense of pleasure, a lack of interest and initiative, and social withdrawal are examples of negative symptoms (1, 3). Schizophrenic patients also have impaired cognitive functions such as memory, attention, fluency of speech, processing speed and executive function (4-11).

The incidence of schizophrenia in the general population is 1% (12). The exact cause of schizophrenia is still unknown, however, the pathogenesis appears to rely on a combination of genetic, developmental and environmental factors (3). Some theories have been proposed to explain the neuropathology of schizophrenia. For example, the dopamine theory was proposed on the basis that antipsychotic drugs treat psychosis by antagonizing dopamine D2 receptors and administration of dopamine receptor agonists induces psychosis in healthy patients (1, 3). Clinical studies have also shown evidence for dopamine hyperactivity in the meso-limbic system of patients with schizophrenia (1). Antipsychotic drugs are not effective in treating all schizophrenia symptoms, suggesting the involvement of other neurochemical systems. Glutamate and

NMDA receptor hypofunction have also been implicated in theories of schizophrenia. Antagonism of NMDA receptors produces psychotic symptoms and cognitive dysfunction in healthy controls (13).

Several risk factors have been identified that may predispose someone to schizophrenia, these include; genetics, socioeconomic status, maternal infections and season and location of birth (3). Schizophrenia occurs at equal rates in both males and females, however, the onset of schizophrenia occurs at a younger age for males, during late teenage years and early 20's (3, 14). In females, the onset of the disease on average, occurs five to seven years later than in males (15, 16). To help manage their symptoms, patients with this disease can be treated with psychosocial interventions including but not limited to, family therapy and psychiatric rehabilitation. Pharmacological agents such as antipsychotic drugs are also widely prescribed (3, 17, 18).

Schizophrenia and Weight Gain

Patients with schizophrenia have a significantly greater mortality rate from both natural and unnatural causes compared with the general population (19). The greatest risk factor aside from suicide is cardiovascular disease, accounting for 30-35% of deaths (20). Patients with schizophrenia commonly lead an unhealthy lifestyle due to poor diet, lack of exercise, smoking, substance abuse and chronic social stress. An important risk factor for cardiovascular disease is metabolic syndrome, which includes abdominal obesity, dyslipidemia, high blood pressure

and elevated fasting blood glucose levels. Studies suggest that the incidence of metabolic syndrome in patients with schizophrenia has risen by up to four times than its incidence in the general public in recent years (20). This increased incidence of metabolic syndrome has been correlated to the widespread use of atypical antipsychotics (21, 22).

Antipsychotic Drugs

Antipsychotic medication is the main form of pharmacological treatment of schizophrenia. These agents are effective in treating psychosis by ameliorating positive symptoms primarily by antagonizing the D2 family of dopamine receptors. In addition to dopamine receptor blockade, these drugs interact with varying intensity to subtypes of serotonin, histamine, adrenergic and muscarinic acetylcholine receptors (23). As such, each antipsychotic drug has its own characteristic receptor binding profile.

Antipsychotic drugs have been divided into two major classes: typical and atypical. Typical antipsychotics, such as haloperidol, were the first generation of these drugs to be developed. Chronic use of typical antipsychotics is associated with extrapyramidal motor symptoms (EPS) in humans and catalepsy in rodents (24). A new class of neuroleptic pharmaceuticals was sought after due to the undesirable involuntary motor movements caused by typicals. The second-generation antipsychotics, or atypicals, are able to treat psychosis with causing

little or no EPS (22, 24). The lack of EPS is thought to be a result of their high ratio of serotonin 5-HT_{2A} receptor to dopamine D2 receptor antagonism (23). 5-HT_{2A} antagonists are known to propagate striatal dopamine transmission and also release dopamine in the prefrontal cortex and the hippocampus (24). As a result, extreme alteration of dopamine transmission in the striatum, caused by first generation antipsychotics, is attenuated.

Antipsychotics and Weight Gain

Ample evidence indicates that chronic use of atypical antipsychotics contributes to the development of metabolic abnormalities such as weight gain, diabetes and dyslipidemia (21). Consequently, although atypical antipsychotic drugs possess decreased propensity to cause EPS, the overall quality of life of patients with schizophrenia has not improved. Poor patient compliance (22) and development of metabolic syndrome are two major issues in treating psychosis with atypical drugs.

Clinical Studies

Clinical manifestations of antipsychotic induced weight gain have been well documented. Olanzapine and clozapine have the highest propensity to cause weight gain following chronic treatment with patients gaining up to 10kg in the first year (25-27). Other atypical drugs such as risperidone and quetiapine cause a moderate increase in weight gain, approximately 2-3kg over the first year (28,

29). Aripiprizole, ziprasidone and typical antipsychotics, such as haloperidol, cause very minimal weight gain (25, 26).

Weight gain induced by chronic olanzapine and clozapine treatment may be a result of multiple factors, such as an increase in appetite and food intake (30, 31), reduced energy expenditure and reduced resting metabolic rate (31-33). A number of studies have reported olanzapine and clozapine induced binge eating and food craving after just one week of treatment (34, 35). Furthermore, male adolescents receiving olanzapine were shown to have increased caloric intake and decreased physical activity levels, resulting in significant weight gain compared to patients treated with haloperidol (31). Other studies have reported significant increases in respiratory quotient and body fat, particularly central fat, in adults treated with olanzapine compared to controls (30, 36). A case series conducted by Procyshyn *et al.* revealed that clozapine treatment is associated with a significant decrease (10-16%) in resting metabolic rate in adults and was correlated with an increase in total body weight (32).

Significant increases in leptin and triglyceride levels have also been reported in patients following chronic treatment with clozapine and olanzapine (37-39). A recent meta-analysis of 22 studies evaluated the effects of antipsychotic drugs on circulating leptin levels in patients. Clozapine and olanzapine significantly increased serum leptin levels from baseline and healthy controls directly after commencement of treatment and remained elevated for several months (40).

Analysis of typical antipsychotic treatment on serum leptin levels revealed no such effect (40).

Only a few studies have reported serum ghrelin levels, another hormone important in regulating energy homeostasis, following chronic antipsychotic therapy and the results are generally inconclusive (41-43). A few studies have found that atypical antipsychotics decrease serum ghrelin levels from baseline following two to nine weeks of treatment, but that an increase in levels is seen following longer treatment periods extending 26 and 52 weeks (41-43).

Several studies have also found decreased serum adiponectin levels, changes in glucose metabolism, the presence of insulin resistance, and development of type II diabetes following chronic antipsychotic treatment (21, 44, 45). It is unclear whether this is a direct effect or a consequence of increased adiposity. A study conducted by Ebenbichler *et al.* observed significant increases in fasting serum insulin and glucose levels and evidence of insulin resistance in chronically olanzapine treated patients compared to healthy controls (46). These patients also showed a significant increase in body weight and body fat (46).

Rodent Studies

The results of rodent studies of antipsychotic-induced weight gain are somewhat inconsistent. It should be noted that different regimens of drug administration, doses, and routes of administration may account for some of the discrepancies

reported between these studies. Successful attempts of animal models have been reported, mainly in female rats, while clinical findings indicate significant weight gain in both sexes (47, 48). Some studies report that hyperphagia and increased meal size are responsible for weight gain in rodents treated chronically with olanzapine (47, 49, 50). A recent study by Davoodi *et al.* showed that 1mg/kg, p.o. of olanzapine, twice a day for seven days increased weight gain, total food intake, meal size and duration of feeding (49). They also found that the olanzapine-induced weight gain was prevented if rats were given a restricted amount of food to the level of vehicle treated controls (49). These results suggest that increased feeding might play a more important role compared to altered energy balance in olanzapine-induced weight gain.

In contrast to the data cited above, several reports have indicated that chronic olanzapine induced weight gain results from decreased energy expenditure and suppressed metabolic rates. Decreased locomotor activity and brown adipose tissue temperature in the absence of increased food intake following 24 days of olanzapine treatment were reported in a study conducted by Stephanidis *et al.* (51). In addition, a significant increase in fat deposition in visceral and subcutaneous tissue was seen following five weeks of olanzapine treatment compared to haloperidol or vehicle treated rats (52, 53). Furthermore, adipocytes from olanzapine treated rats had significantly reduced lipolytic activity, decreased levels of hormone-sensitive lipase and increased levels of fatty acid synthase (52).

Similar to human studies, increased circulating levels of leptin and insulin following olanzapine and clozapine but not haloperidol treatment have been reported in rodents (54-56). Rats receiving clozapine or olanzapine treatment also displayed increased levels of adiponectin and prolactin and evidence of insulin resistance (48, 56).

In summary, the atypical antipsychotic drugs such as clozapine and olanzapine, despite their decreased tendency to cause extrapyramidal motor side effects, promote profound increases in body weight and predispose metabolic disturbances. A combination of factors including central control of regulation of feeding and energy balance is likely to be responsible for atypical antipsychotic induced weight gain. Despite discrepancies, the rodent still provides a useful tool for investigating mechanisms underlying chronic atypical antipsychotic-induced weight gain.

Histamine Receptor Antagonism

One possible mechanism of antipsychotic induced weight gain may be via histamine H1 receptor antagonism in the hypothalamus. Both olanzapine and clozapine, responsible for substantial weight gain in patients and animals, are potent antagonists of the H1 receptor and possess significantly higher affinity for H1 receptors than that of risperidone and haloperidol (57). The histamine H1 receptor has been linked to feeding behaviour (58). This notion was confirmed by

studies that demonstrated a positive correlation between affinity of atypical antipsychotics for the histamine H1 receptor and their potency to cause weight gain (59, 60).

Injections of potent antihistamines into the third ventricle (intra-cerebral ventricular), ventromedial nucleus and paraventricular nucleus (intracerebral) of the hypothalamus significantly increased food intake compared to haloperidol and desipramine, which are weak H1 receptor antagonists (61-67). More recently an acute injection of olanzapine in male rats attenuated H1 receptor agonist-induced hypophagia, in contrast to ziprasidone, a relatively weak H1 receptor antagonist, which showed no such effect (68). Olanzapine treatment in rats has also been associated with significant decreases in H1 receptor mRNA expression in the arcuate and ventromedial nuclei compared to haloperidol and aripriprazole treated rats (69). Results from this study also revealed that H1 receptor binding density was significantly decreased in the ventromedial nucleus in olanzapine treated rats, a finding that was negatively correlated with body weight gain (69).

In addition to these findings, Kim and collaborators recently demonstrated that olanzapine and clozapine significantly enhance hypothalamic phospho-adenosine monophosphate-activated protein kinase (AMPK) activity, particularly in the arcuate and paraventricular nuclei. Phospho-AMPK activity in the hypothalamus has been correlated with increases in food intake and body weight

and is inhibited by leptin, insulin, high blood glucose levels and food intake (70). Furthermore, leptin-reduced phospho-AMPK activity was reversed by clozapine, an effect that was completely abolished in H1 receptor knockout mice (71).

In summary, olanzapine and clozapine are potent antagonists of the H1 receptor, compared to the typical antipsychotic, haloperidol, which only weakly antagonizes this receptor. Given the evidence that olanzapine and clozapine induce significant weight gain compared to haloperidol, these findings clearly suggest a relationship between olanzapine and clozapine H1 receptor antagonism and altered hypothalamic regulation of energy homeostasis.

Serotonin Receptor Antagonism

An alternate mechanism of antipsychotic induced weight gain has also been proposed. Serotonin (5-hydroxytryptamine) and its receptors, 5-HT_{1A} and 5-HT_{2C}, are linked to the regulation of appetite and body weight (72, 73). Serotonin has anorexigenic properties and acts to suppress food intake, increase metabolism and lipid oxidation and reduce body weight (73-75). As such, decreased 5-HT_{2C} receptor activity results in an increase in feeding, frequency and meal size, leading to obesity (76-79). Interactions between the 5-HT_{2C} receptor and the melanocortin system have also been found. Antagonism of melanocortin receptors 3 and 4 attenuates the anorexigenic effects of 5-HT_{2C} agonists (80). The anorexigenic effects, particularly suppression of carbohydrate feeding, produced by serotonin and its receptor subtypes were localized to the

hypothalamus, specifically the paraventricular, ventromedial and suprachiasmatic nuclei (81).

Olanzapine and clozapine bind with high affinity to a number of serotonin receptors including 5-HT_{2A}, 5-HT_{2C} and 5-HT_6 subtypes (82, 83). Haloperidol also binds to 5-HT_{2A} receptors with moderate affinity and has very low affinity for other subtypes (84). The 5-HT_{2C} receptor has been shown to be particularly important in antipsychotic induced weight gain. Clinical studies revealed that patients with a particular 5-HT_{2C} receptor polymorphism were protected from clozapine induced weight gain (85, 86). The mechanisms of this phenomenon are currently unknown however it is speculated that this polymorphism reduces receptor activity resulting in natural adaptive changes in other weight regulating systems to compensate for decreased serotonin signaling.

To summarize, olanzapine and clozapine have greater affinities for histamine H1 and serotonin 5-HT_{2C} receptors compared to haloperidol. Given the involvement of these receptor sub-types in food intake and energy regulation, it is plausible to hypothesize that olanzapine and clozapine antagonism of either of these receptors will lead to weight gain.

Energy Homeostasis

The hypothalamus regulates energy homeostasis by detecting and interpreting peripheral signals released from adipose tissue and digestive organs. These peripheral signals, such as leptin, ghrelin and insulin, are released into circulation to relay their message to the brain. As a result, the hypothalamus receives continuous information about the body's energy and nutritional status. When nutritional and energy supplies are low, the hypothalamus stimulates food intake and conserves energy until these stores are replenished. The signals are able to reach the ventral hypothalamus, via cerebral spinal fluid and at the median eminence, where the blood brain barrier is permeable to these hormones.

Hypothalamic Regulatory Peptides

The hypothalamus comprises multiple sub-nuclei with neurons specific to relaying signals involving changes in food intake and energy expenditure. An important nucleus in the hypothalamus involved in energy homeostasis is the arcuate nucleus (ARC), which lies dorsal to the median eminence and adjacent to the third ventricle. This area contains two main populations of neurons that respectively express either neuropeptide Y and Agouti-related protein (NPY/AgRP) or pro-opiomelanocortin and cocaine and amphetamine-regulated transcript (POMC/CART). These are thought to be first order neurons of the metabolic control system, receiving peripheral information of nutritional status.

POMC, a peptide that signals satiety, is localized in neurons in the ARC, and is the precursor to α-melanocyte stimulating hormone (αMSH) and β-endorphin. These neurons are stimulated by a number of peripheral hormones including leptin and insulin, as well as by gonadal steroids such as estradiol (87-90). A major target of POMC neurons is the paraventricular nucleus (PVN) of the hypothalamus. POMC axon terminals synapse with neurons of the PVN and release αMSH (91). αMSH is the endogenous ligand for melanocortin receptor sub-types 3 and 4 (MC3R, MC4R), which are expressed on neurons in the PVN. αMSH-MC4R binding propagates the anorexigenic signal to inhibit food intake and increase energy expenditure (92).

CART is also expressed in the arcuate nucleus and is co-localized with POMC (93). This peptide was first discovered following acute administrations of psychomotor stimulants (94). CART expression is stimulated by leptin, circulating lipids and a high fat diet (95-97). Like POMC, activation of CART decreases food intake and also stimulates energy expenditure (98).

NPY and AgRP are co-localized in a separate group of neurons found in the ARC. Both NPY and AgRP are highly responsive to states of energy depletion, and are activated to restore energy balance by increasing food intake and decreasing energy expenditure. NPY and AgRP are stimulated by ghrelin and corticosterone to restore glucose availability (99, 100). Conversely, leptin and insulin inhibit both peptides to avoid excessive food intake and inactivity (101,

102). These peptides parallel each other and produce similar and additive effects to ensure the body is able to restore energy sources and conserve them when they are low. When energy supplies and circulating leptin levels are low, NPY/AgRP are able to counteract the anorexigenic actions of POMC neurons (103). In particular, NPY/AgRP neurons co-expressed with γ-amino butyric acid (GABA) project to POMC neurons and can directly inhibit their activity (92). Furthermore, AgRP is an endogenous ligand for MC4R, and antagonizes these receptors, and therefore the stimulatory effect of αMSH leading to suppression of their basal activity (104).

The role of the ARC and melanocortin system in maintaining energy homeostasis has been well characterized. However, ablation of AgRP, POMC or leptin receptors from the ARC produces only modest alterations in body weight (105-108). This evidence suggests involvement of other regulating systems that are able to compensate for this loss, at least following acute changes.

In addition to the melanocortin system, a separate population of neurons exists in the perifornical area/lateral hypothalamus (PFLH) that synthesizes orexigenic peptides. The orexins A and B, also called hypocretins, project within the hypothalamus as well as other areas of the brain. They are able to respond to multiple signals such as food deprivation, ghrelin, insulin, triglycerides and glucocorticoids (109). Similar to peptides in the ARC, orexin containing neurons also express low levels of leptin receptors, however, this hormone is not known

to be a key regulator of orexin peptides (110). Stimulation of orexin neurons initiates food intake and increases metabolic rate, locomotor activity and sympathetic tone (111, 112).

An alternate population of neurons in the PFLH express another orexigenic peptide, melanin-concentrating hormone (MCH). The melanin concentrating hormone receptor, MCHR1, is widely expressed throughout the brain, in addition to the hypothalamus. MCH neurons also contain leptin receptors, but are not regulated by leptin to the same extent as ARC peptides (113-116). Many studies have found multiple factors that influence MCH expression such as fasting, food deprivation, decreased fat oxidation and insulin (90, 91, 117). Stimulation of MCH neurons sends strong signals to decrease energy expenditure, physical activity, fat oxidation and thermogenesis (118, 119).

Reciprocal connections have been mapped between the ARC and PFLH (120), suggesting that orexin and MCH neurons are able to interact with the melanocortin system. NPY mRNA in the arcuate nucleus is increased following MCH administrations, and a NPY (Y1) receptor antagonist inhibits MCH-induced feeding (120, 121). Other studies have found an additive effect of NPY and MCH on food intake, following injections of both peptides (122). In addition, orexin injections stimulate c-fos expression in NPY neurons and vice versa. These findings indicate that ARC and PFLH neurons communicate with each other to

ensure a correct energy balance is established under different physiological conditions.

Leptin Signalling

Leptin, a protein hormone product from the *ob* gene, is primarily produced by adipocytes in proportion to fat mass (123). Leptin receptors are abundant in the hypothalamus and found in the ARC, ventromedial nucleus (VMH), dorsomedial nucleus (DMH), PFLH and the ventral premammillary nucleus (123-127). Upon activation of a leptin receptor, a conformational change is initiated resulting in the activation of an intracellular signaling cascade involving Janus Tyrosine Kinase 2 (JAK2) (128-130). Signal transducer and activator or transcription 3 (STAT3) is recruited to the site and subsequently activated via phosphorylation by JAK2. Phosphorylated STAT3 dimerizes and translocates to the nucleus where it binds to DNA to regulate transcription of multiple genes, including the promotor sequence of the gene encoding POMC (123, 131-134) (Fig 1). Studies have shown that non-functional STAT3 in POMC neurons completely suppresses POMC expression resulting in significant weight gain (135). Leptin deficiency and non-functional leptin receptors unable to activate STAT3 signaling results in decreased expression of POMC and CART (95, 96, 136), and increased expression of AgRP and NPY in the ARC (137). Hyperphagia, obesity, diabetes and reduced energy expenditure develop under these conditions (136-138).

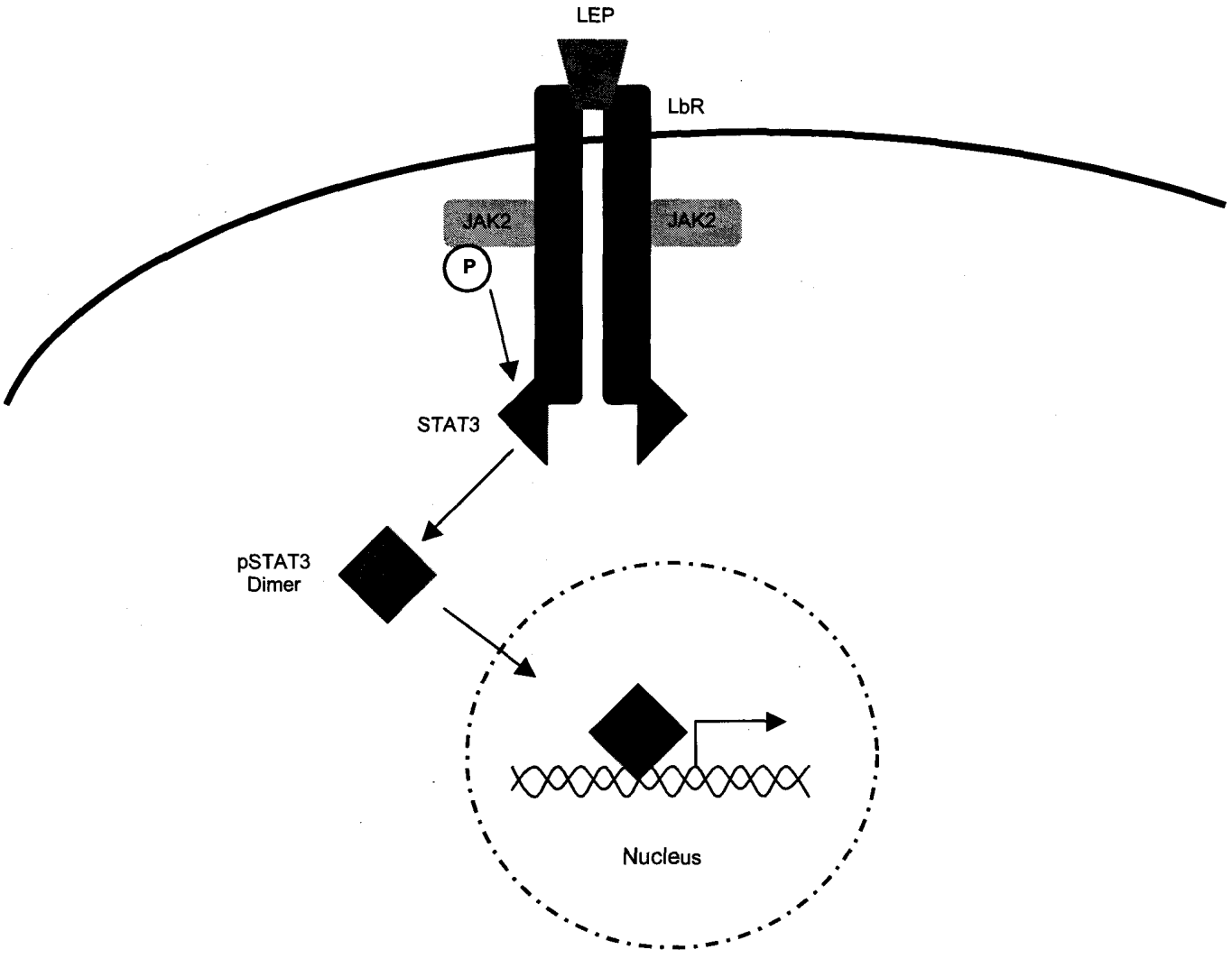

Fig 1.1. Leptin signaling via receptor activation and the JAK-STAT pathway. Binding of leptin to its receptor initiates of conformational change leading to activation of JAK2. JAK2 in turn activates STAT3 via phosphorylation. STAT3 dimerizes and relocates to the nucleus where it binds to specific DNA sequences and regulates transcription of multiple genes. *Abbreviations:* leptin (LEP), leptin receptor (LbR), Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), phosphorylated-signal transducer and activator of transcription (pSTAT3). *Modified from Elloitt et al. 2004.*

When a positive energy balance exists, serum leptin levels are high and POMC/CART neurons are activated to induce energy expenditure and inhibit food intake (96, 136). Concurrently, leptin inhibits the activation of NPY/AgRP expressing neurons (139, 140) (Fig.2).

In addition to the ARC, PVN and the PFLH, the VMH and DMH also play a role in energy homeostasis. The VMH was once termed the 'satiety center' of the brain, since lesions in this area produced rats that were hyperphagic. Since then, the VMH has received less attention and its exact function in energy homeostasis is still not clear. However, in addition to hyperphagia, VMH lesions in rodents lead to obesity, diabetes and elevated levels of corticosteroids (141-144). As previously stated, the VMH contains leptin receptors and are able to respond to leptin's anorexigenic signals. Loss of leptin receptors in the VMH results in significant weight gain, increased fat mass, dyslipidemia, hyperleptinemia, hyperinsulinemia and glucose intolerance (145).

In summary, the hypothalamic regulation of energy homeostasis is complex. It involves various peptides and nuclei that individually respond to a wide array of peripheral and central signals relating to energy status. Sub-nuclei are interconnected and able to communicate with each other to up-regulate or down-regulate peptide expression during different physiological states in order to maintain a suitable energy balance.

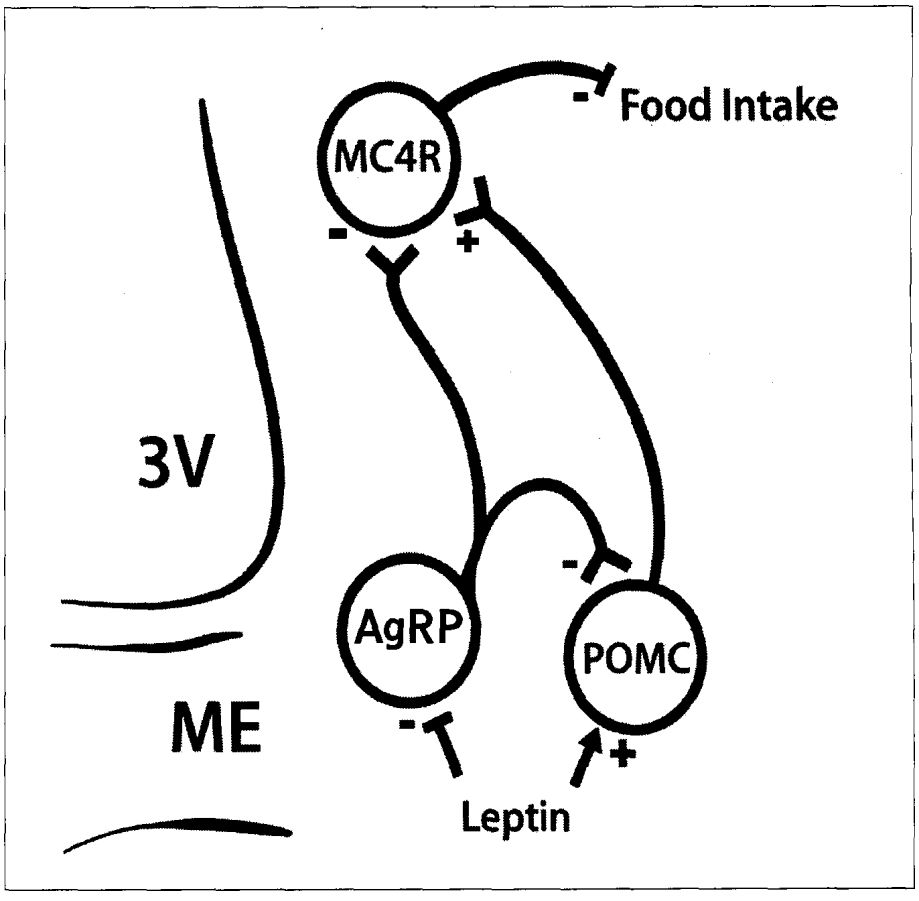

Fig 1.2 The melanocortin system. Leptin inhibits NPY/AgRP (AgRP) neurons and activates POMC/CART (POMC) neurons located in the arcuate nucleus. Stimulation of POMC neurons leads to activation of neurons containing melanocortin 4 receptors (MC4R). This activation leads to a decrease in food intake. *Abbreviations:* median eminence (ME), third ventricle (3V), neuropeptide Y (NPY), Agouti-related peptide (AgRP), pro-opiomelanocortin (POMC), cocaine and amphetamine regulated transcript (CART), melanocortin receptor 4 (MC4R). *Modified from Druce et al. 2004.*

Antipsychotic Drugs and Their Effects on the Hypothalamus

There is strong evidence supporting the hypothesis that atypical antipsychotic drugs alter normal hypothalamic function (146-149). However, rodent studies investigating the effects of chronic antipsychotics on hypothalamic peptides are inconclusive. One study showed that 14 days of chlorpromazine and haloperidol treatment in male Wistar rats significantly induced NPY-like immunoreactivity in the hypothalamus compared to sulpiride, clozapine or vehicle treated rats (146). Only after 28 days of treatment did clozapine treated rats show a significant increase in NPY-like immunoreactivity in the hypothalamus. Results of a more recent study by Kirk *et al.*, contradict these findings (147). Adult male Sprague Dawley rats receiving injections of clozapine for 21 days displayed significantly greater NPY-immunoreactive cell density in the ARC when compared to haloperidol and control treated rats. Both studies failed to record data on animal weights and consequently no connection between NPY expression and weight gain can be made (147). It should be noted however, that the former study measured NPY expression in the entire hypothalamus compared to the latter study, which specifically measured NPY levels in the arcuate nucleus only. NPY expression in the ARC is closely related to changes in energy homeostasis, whereas NPY in other hypothalamic areas may be involved in different functions. This observation may explain the discrepancy between the two studies.

Other studies have shown a relationship between antipsychotic treatment and increased orexinergic activity in the PFLH. Clozapine, olanzapine, risperidone

and chlorpromazine were able to significantly increase the percentage of orexin neurons expressing c-fos in the lateral hypothalamus of male Sprague Dawley rats compared to vehicle (148). These drugs are all associated with weight gain in patients and rats; however, this parameter was also not assessed in this study. Wallingford *et al.* was able to parallel these results in female Sprague Dawley rats receiving olanzapine. Significant increases in weight gain, food intake and percentage of orexin neurons expressing c-fos were observed (149). Following these investigations, Davoodi *et al.* set out to test whether olanzapine altered expression of other hypothalamic mRNA levels. Acute and sub-chronic oral administrations of olanzapine did not alter levels of hypothalamic NPY, HCRT, MCH and POMC mRNA when compared to vehicle (49).

In summary, evidence indicates that antipsychotic treatment may induce weight gain by altering the expression of hypothalamic peptides, both orexigenic and anorexigenic, including POMC, AgRP, NPY and orexin. However, it is not clear whether the propensities to cause weight gain of a particular antipsychotic agent can be correlated to changes they exert on these peptides. In addition, relative contribution of changes to the central control of food intake versus that of energy balance in antipsychotic-induced weight gain is not clear. More studies are needed to address these issues.

Rationale and Hypothesis

Increased prevalence of obesity among patients with schizophrenia has developed into a major risk factor for cardiovascular disease and type II diabetes. Treatment of atypical antipsychotic drugs is identified as one of the main culprits. In addition to the potential co-morbidity, antipsychotic-induced weight gain possesses a potential threat to patient compliance. An understanding of mechanisms underlying antipsychotic-induced weight gain is imperative for the development of rational treatment and preventive strategies for this side effect and to develop safer antipsychotic medications.

The first objective of this thesis was to personalize a procedure that would reliably produce an animal model with atypical antipsychotic-induced weight gain. Two antipsychotic agents were chosen: 1) olanzapine, an atypical agent, highly effective in treating positive symptoms, preferred by patients and clinicians, but, has a very high liability to cause weight gain after a few weeks of use in patients and after a few days of use in rodents; 2) haloperidol, a typical agent with low propensity to cause weight gain in patients. In addition to changes in weight, we also measured daily food intake to assess the relative contribution of food consumption towards weight gain in these animals. Based on our preliminary studies, it was hypothesized that a two week administration period of olanzapine will increase body weight compared to vehicle or haloperidol treated rats.

The second aim of this study was to determine whether antipsychotic-induced weight gain is associated with altered leptin signaling. Since increased circulating leptin levels are associated with antipsychotic-induced weight gain in patients, and a possible leptin resistance was proposed as a contributing factor to weight gain in these patients, it is anticipated that a similar phenomenon may occur in rodents as well. Animals were challenged with a single systemic injection of leptin prior to sacrifice and leptin signaling was quantified by assessing the number of phosphorylated-STAT3-containing cells in the ARC and VMH. The VMH is involved in controlling energy balance while the ARC contains neurons controlling the melanocortin system. In addition, fasting levels of serum leptin, insulin and glucose were measured prior to sacrifice, to determine whether leptin levels are increased as reported in patients, and to verify whether evidence of impairment of glucose homeostasis would occur in this model. It was hypothesized that impaired leptin signaling would be present in both the ARC and VMH along with increased serum leptin levels, suggesting leptin resistance in rats treated with olanzapine.

The third and final aim of the study was to investigate whether chronic administration of antipsychotic agents affects orexigenic and anorexigenic peptides in the ARC. Immunohistochemistry was used to localize and quantify the number of POMC-immunoreactive neurons as a measure of POMC protein expression and quantify the density of AgRP-immunoreactive fibers within the ARC and PVN as a measure of AgRP protein expression. Leptin directly

modulates the expression of POMC and AgRP via STAT3 phosphorylation. Measuring the expression of these peptides would provide information on whether altered leptin signaling, investigated in the ARC in aim 2, is translated further in changes in the expression of these downstream peptides. It was hypothesized that chronic olanzapine treatment would increase AgRP immunoreactivity in the hypothalamus and cause a reduction in POMC immunoreactivity.

This study is the first phase of a larger study with an overall aim of using a reliable rodent model of atypical antipsychotic-induced weight gain to employ in determining hypothalamic mechanisms underlying this metabolic disturbance. The expected results of this study will further characterize the animal model and provide further insight into the effects of chronic antipsychotic drug treatment on hypothalamic peptides and function. It will therefore facilitate in designing future studies to address molecular mechanisms associated with weight gain.

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CHAPTER 2: SUB-CHRONIC OLANZAPINE REDUCES EXPRESSION OF ANOREXIGENIC PEPTIDES IN THE RAT HYPOTHALAMUS.

INTRODUCTION

Antipsychotic drugs are the main form of pharmacological treatment of schizophrenia. Although the second generation or atypical drugs seemed to be superior to typicals, due to decreased propensity to cause EPS, they are associated with disrupting normal energy balance resulting in significant weight gain (1). This side effect increases a patient's risk for more severe complications such as metabolic syndrome, cardiovascular disease and type II diabetes (1). Consequently, treatment non-compliance and the overall quality of life of patients with schizophrenia have not improved (2, 3).

Atypical antipsychotic-induced weight gain in the clinical setting has been successfully characterized. The atypicals, clozapine and olanzapine are known to induce the most robust changes in body weight, with patients gaining up to 10kg in the first year of chronic treatment (4-6). Risperidone and quetiapine, cause a moderate increase in weight gain and aripiprizole, ziprasidone and typical antipsychotics, such as haloperidol, cause very minimal weight gain (4, 5, 7, 8). Despite multiple investigations, antipsychotic induced weight gain in rodents is ill defined. The inconsistencies reported between studies may be attributed to differences in animal species and sex as well as drug protocol, including dose and route of administration.

A combination of multiple factors such as modified feeding behaviour and decreases in energy expenditure are likely to be responsible for atypical antipsychotic-induced weight gain in patients and rats. Increases in food intake have been reported in both humans and rats receiving olanzapine treatment (9-11). Decreased physical activity levels and reduced metabolic rates have also been documented (11-14). Increased levels of circulating leptin and insulin have also been associated with olanzapine and clozapine treatment (15-24), and may be a direct result of increased adipose tissue (10, 25-27).

Atypical antipsychotics are able to significantly increase body weight in the presence of elevated adiposity signals, suggesting that these drugs interfere with central control systems that are responsible for relaying these signals. Neurons in the hypothalamus containing leptin receptors are able to interpret the peripheral signal that adequate amounts of fat tissue have been attained. As a result, these neurons act accordingly to maintain energy balance. Upon leptin receptor activation, phosphorylated signal transducer and activator of transcription 3 (pSTAT3) translocates to the nucleus to regulate the transcription of genes involved in energy homeostasis (28-32). Specifically, in the arcuate nucleus (ARC), pro-opiomelanocortin (POMC) containing neurons are able to respond to leptin by transmitting the signal, via α-melanocyte stimulating hormone (αMSH), to inhibit food intake and increase energy expenditure (33-35). A separate group of neurons in the ARC, co-localizing neuropeptide Y (NPY) and Agouti-related protein (AgRP), are inhibited by the actions of leptin. In the

absence of leptin, these neurons counteract and suppress the action of POMC neurons to stimulate food intake and decrease energy expenditure (36-38).

Contradictory data exists regarding the effects of antipsychotics on hypothalamic peptides. A few studies have found that clozapine treatment increased levels of NPY immunoreactivity in the hypothalamus (39, 40), while other studies have reported increased c-fos expression in orexin neurons (41, 42). A recent study found that sub-chronic olanzapine treatment had no effect on levels of orexin, NPY, POMC or melanin-concentrating hormone mRNA (9). Based on this evidence, it is possible that antipsychotic treatment alters expression of hypothalamic peptides, however, additional studies need to be conducted in greater detail.

In the current study, we used a rodent model of atypical antipsychotic-induced weight gain to investigate the effects of sub-chronic administration of olanzapine on food intake and adiposity signals. Comparison of olanzapine-fed animals with haloperidol and vehicle treated animals allowed us to determine if the observed effects were specific to atypical drugs. After 14 days of treatment, we examined leptin signaling in the ARC and ventromedial nucleus (VMH) by measuring the number of immunoreactive pSTAT3 neurons following a single leptin injection. We also analyzed basal levels of POMC and AgRP immunoreactivity in the ARC to determine if the expression of peptides regulated by leptin signaling is altered. We hypothesized that sub-chronic olanzapine treatment, but not haloperidol

would significantly elevate circulating levels of leptin and insulin and that leptin signaling in the hypothalamus would be impaired. Specifically, we expected to see decreased pSTAT3 expression in both the ARC and VMH, suppressed levels of POMC and increased levels of AgRP as a direct result of lowered leptin signaling. The results of this study support our hypothesis that sub-chronic olanzapine treatment significantly decreases pSTAT3 immunoreactivity in the VMH and POMC immunoreactivity in the ARC. However, no changes were found in AgRP and pSTAT3 expression in the ARC.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats, aged 20-28 weeks and weighing 400g to 500g were used for all experiments and were purchased from Charles River Laboratories, P.Q., Canada. Upon arrival, rats were separated into individual cages and allowed to adapt to the environment for 4 days prior to commencement of treatment. They were housed in controlled humidity and temperature on a 12 hour light/dark cycle (lights on at 7AM). Standard rat chow (LabDiet®) and tap water were provided *ad libitum.* All procedures were approved by the University of Western Ontario Animal Care Committee, and are in compliance with the Canadian and National Institute of Health Guides for Care and Use of Laboratory Animals.

Procedure

Before commencement of treatment, animals were weighed and divided equally into 3 groups with comparable means of weight. The experiment involved a 14 day treatment period, during which animals were treated orally with olanzapine (Zyprexa), haloperidol (Haldol) or vehicle. Olanzapine and haloperidol were purchased from LHSC Pharmacy in oral tablet form, 5mg and 0.5mg, respectively, and the vehicle consisted of chocolate chip cookie dough (Pillsbury; purchased from local supermarket).

In the first series of studies, randomly selected groups of rats (n=10, each) were given olanzapine (2.5mg/kg daily), haloperidol (1mg/kg daily) or vehicle as described above for 14 days. Antipsychotic doses were chosen based on previous literature and preliminary results obtained from a number of pilot studies conducted in the laboratory. Pharmacokinetic studies have determined that 2.5mg/kg olanzapine, given orally for 2 weeks produced clinically comparable levels of drugs in the plasma and brain, including the hypothalamus (43). This dose has also been found to achieve peak occupancy at the D2 dopamine receptor comparable to patients (44). Previous studies have reported that 1mg/kg haloperidol in rats produce clinically comparable plasma levels seen in patients (45-47). However, the haloperidol group showed loss of weight and decreased food intake throughout the experimental period compared to the vehicle group. Consequently, it was concluded that 1mg/kg haloperidol likely causes an extended period of sedation and catalepsy interfering with feeding. This issue was addressed by reducing the dose of haloperidol to 0.5mg/kg. This dose is also shown to produce clinically comparable plasma levels (45-47).

A second, similar experiment was conducted with a reduced haloperidol dose. 60 animals were randomly divided into three groups (n=20, each) receiving a) 2.5mg/kg/day olanzapine, b) 0.5mg/kg/day haloperidol or c) vehicle. With the exception of the new haloperidol dose, all other procedures remained the same. This experiment was conducted primarily to confirm the results observed in the

initial experiment. Weight was measured every third day and food intake was only measured in a sub-set of animals every other day.

For oral administration, drug tablets were crushed into powder form and mixed with 1.5g of chocolate chip cookie dough with half a chocolate chip in each. Control animals received the vehicle; 1.5g of chocolate chip cookie dough with half a chocolate chip. Drugs were placed into each animal cage between 1300 and 1400 hrs, following daily weight and food intake measurement, and all rats consumed the entire cookie dough mixture within five to ten minutes. Drugs were administered between 1300 and 1400 hrs as preliminary studies have indicated that about 80% of feeding occurred in rats between 1900 and 2300 hrs, and adult rats show reduced activity and evidence of sedation up to 4 hrs following oral administration of antipsychotics. Two hundred grams of regular rat chow pellets were placed in the food dispenser each day. The following day, the amount of food in the dispenser and any food within the cage was measured and subtracted from the initial 200 grams, to achieve the total amount of food consumed by the rat in 24 hours.

Leptin Challenge

Approximately 20 hours after the final drug administration, animals from each treatment group were randomly divided into two groups. Each group received either a single intraperitoneal injection of 1.0mg/kg of leptin or vehicle. Human recombinant leptin was purchased from Sigma-Aldrich Laboratories (L 4146) in

lyophilized powder form. The leptin was reconstituted in 0.5 ml of 0.2 μm-filtered 15 mM HCl and 0.3 ml of 0.2 μm-filtered 7.5 mM NaOH to reach a pH of approximately 5.2. The vehicle consisted of 0.5 ml of 0.2 μm-filtered 15 mM HCl and 0.3 ml of 0.2 μm-filtered 7.5 mM NaOH to reach a pH of approximately 5.2.

Perfusion

Ninety minutes following leptin and vehicle injections, animals were anaesthetized with an overdose of urethane (2ml/kg of 45% solution; i.p.) and sacrificed by transcardiac perfusion. The circulatory system was initially flushed with approximately 150ml of 0.9% NaCl (saline) solution containing 0.5% heparin. Following saline, animals were perfused with 500ml solution containing 4% paraformaldehyde in 0.05M phosphate buffer (pH7.4-7.6). Rat brains were carefully dissected and post fixed in the same formaldehyde solution for one hour and transferred to 24% sucrose solution in 0.1M phosphate buffer (pH7.4-7.6) at 4°C until the brain was completely saturated in the sucrose solution (at least 48hrs).

Blood Samples

Blood samples were collected from a separate group of animals after receiving 14 days of oral administrations of 2.5mg/kg/day of olanzapine (n=10), 0.5mg/kg/day of haloperidol (n=10) or vehicle (n=10). Animals were fasted for 6 hours as above prior to blood collection. Approximately 24 hours following the last drug administration, the animals were anesthetized and blood samples were

collected from the abdominal inferior vena cava (between 1500 and 1700 hrs). Blood glucose levels were measured immediately using a digital glucometer with strips (OneTouch Ultra). Blood samples collected for serum were allowed to sit at room temperature for one hour and then centrifuged at 1000xg for 20 minutes. Blood samples collected for plasma were mixed with 3% EDTA and spun immediately for 20 minutes at 1000xg. Serum and plasma samples were stored at -20°C.

Leptin and Insulin Assays

Serum and plasma collected from blood samples were sent to the laboratory of Dr. Murray Huff at the Robarts Research Institute for analysis of serum leptin and plasma insulin levels. The leptin EIA kit (11-LEPHU-E01) and the insulin EIA kit (80-INSRT-E01) from ALPCO Diagnostic were used. Protocols for each test are attached in the appendix.

Immunohistochemistry

The hypothalamus was blocked from formaldehyde fixed brains of animals treated for 14 days with 2.5mg/kg/day of olanzapine, 0.5 mg/kg/day of haloperidol or vehicle. The hypothalamus was sliced with a freezing microtome into three parallel series of 40μm thick coronal sections; every section was collected and stored in a cryopreservative solution containing ethylene glycol, glycerol and 0.1M phosphate buffer (pH7.4) (3:3:4) at -20°C until processed for immunohistochemistry.

Tissue sections used for staining were washed with 0.1M phosphate buffer (PB) (pH 7.4-7.6) eight times for 15 minutes each to remove any cryopreservative solution, and four times for five minutes between each of the following steps. Following the first wash, sections were treated with 1% H_2O_2 in PB for 10 minutes to block endogenous peroxidase activity. To visualize pSTAT3 immunolabeling, a separate initial step was carried out, using a solution of methanol and 30% H_2O_2 (9:2) in place of 1% H_2O_2 in PB. Non-specific blockade was carried out by 2% normal goat serum (Sigma) and 2% bovine serum albumin (Sigma) in PB containing 0.1% triton-X for one hour at room temperature. For immunohistochemical staining, serial tissue sections from the rostral, middle and caudal hypothalamus of leptin and vehicle injected rats were incubated separately with rabbit anti-STAT3 (1:1000, Cell Signalling; Cat. #9132) and rabbit anti-pSTAT3 antibody (1:1000, Cell Signalling; Cat. #9131) for 20 and 24 hours, respectively, at room temperature on a rotator.

A separate series of tissue sections from vehicle injected animals were incubated with rabbit anti-POMC antibody (1:40,000, Phoenix Pharmaceuticals; Cat. #H 029 30) or guinea pig anti-AgRP antibody (1:1000, Antibodies Australia; Cat. #AS506) for 20 hours at room temperature.

Following incubation with primary antibodies, tissues were rinsed and incubated for an hour at room temperature with biotinylated goat anti-rabbit antibody (1:500; Vector Laboratories, Cat. #BA-1000) for STAT3, pSTAT3 and POMC and

biotinylated goat anti-guinea pig antibody (1:500; Vector Laboratories, Cat. #BA-7000) for AgRP. After incubation with the secondary antibody, tissues were rinsed and incubated with avidin-biotin complex (ABC; 10μl of A, 10μl of B in 1ml PB; Elite Peroxidase Kit, Vector Laboratories) for an hour at room temperature on a rotator. Sections were rinsed in PB, and incubated for 10 minutes in a solution containing 0.2% 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.001% H_2O_2 in PB. Sections were rinsed in PB and mounted on slides, air dried overnight, dehydrated in graded series of ethanol, cleared in xylene and cover-slipped using Entalan (Merck) mounting medium.

Non-specific binding of the primary and secondary antibodies was carried out by omitting incubation with a primary antibody (POMC, pSTAT3, STAT3 for biotinylated goat anti-rabbit secondary antibody and AgRP for biotinylated goat anti-guinea pig secondary antibody) using the above immunohistochemistry protocol (Fig A1). All staining was eliminated. Controls for POMC and AgRP primary antibodies have previously been tested in the rodent and ewe (48, 49). Briefly, POMC and AgRP primary antibodies were initially pre-absorbed with their respective antigens prior to tissue incubation. Specificity of the pSTAT3 antibody was previously determined by preabsorption with its antigen prior to immunohistochemical staining (50).

Quantitative Analyses of Immunostaining

Three sections each from the rostral, middle and caudal areas of the ARC and VMH from each animal were analyzed using Zeiss Axioplan 2 microscope using bright-field illumination. Appropriate rostrocaudal planes were identified using the Rat Brain Atlas (Paxinos and Watson; 5th edition). Sections were matched based on the location and size of the following landmarks: optic tract, fornix, ARC, VMH, PVN, and third ventricle. The following sections in relation to bregma were identified in each animal and photographed; -1.72, -1.80, -2.04, -2.28, -2.52, -2.76, -2.92, -3.12 and -3.36 (Fig A2). Images from all treatment groups were randomly relabeled with numbers to prevent biased analysis. The number of cells immunolabeled with POMC in the arcuate nucleus was counted by an investigator who was blinded to the nature of the animal. Similarly, the number of cells immunolabeled with pSTAT3 and STAT3 in the arcuate nucleus and ventromedial nucleus in both vehicle and leptin injected animals was counted.

For AgRP stained sections, only fibres were immunolabeled with DAB, due to the rapid transport of this protein from the soma. Nine sections from the paraventricular and arcuate nuclei were identified as described abovel: -1.44, -1.72, and -2.04 from the PVN, -1.72, -2.04, -2.28, -2.52, -2.92 and -3.12 from the ARC (Fig A2). Images from each of these sections were captured using AxioCam camera and AxiocamVision 4.3 software at the same magnification and camera settings. AgRP fibre density was measured using Image J© software (NIH, Bethesda, USA). A standard area at each brain level was selected and matched

in all brains. The same threshold was used for each image and the total percentage area of fibre density was recorded. Olanzapine and haloperidol measurements were normalized to that of vehicle treated animals.

Statistical Analysis

All analyses were performed using SPSS software. The data are expressed as mean +/- standard error (SE). Weight and food intake data were analyzed using repeated measures analysis of variance (ANOVA). Leptin and insulin assay and glucose data were compared using a one-way analysis of variance. Cell counts were analyzed with a two-way analysis of variance. All ANOVAs were followed by Tukey HSD *post hoc* tests for comparison between groups. Following a repeated measures ANOVA, the Tukey test was computed manually to determine significance at specific time points. A value of $p<0.05$ was considered statistically significant and is indicated by an asterisk or number sign in the figures.

RESULTS

Sub-chronic olanzapine significantly increases weight gain.

Sub-chronic olanzapine treatment via oral administration increased weight from baseline by approximately 6% on average ($p<0.01$). Animals receiving oral administrations of olanzapine gained significantly more weight than haloperidol treated (1.0mg/kg/day) ($p<0.01$) or control ($p<0.05$) rats (Fig. 2.1A). The olanzapine treated rats gained significantly more weight as early as the second day compared to the haloperidol group and as early as the fourth day of treatment compared to control rats ($p<0.01$) (Fig. 2.1A). The weight gain remained significantly increased for the rest of the treatment period. There was no significant difference in weight gain between the haloperidol and control groups. However, the haloperidol treated rats lost weight during the first three days of treatment leading to a significant difference in weight gain compared to control rats until day 9 ($p<0.01$) (Fig. 2.1A). There was no significant difference in weight change between haloperidol and vehicle treated rats from day 9 to day 14 (Fig. 2.1A).

There was a transient increase in food intake following olanzapine treatment. The olanzapine group increased their food intake on day 4 and days 6-12 compared to control ($p<0.01$) (Fig.2.1B). However, overall, food intake in olanzapine treated rats was not significantly different from the control rats. Food intake was significantly decreased in haloperidol (1.0mg/kg/day) treated rats compared to olanzapine animals but not control animals. There was a significant difference in

food intake; throughout the treatment period compared to the olanzapine group and from day 2 to day 6 compared to the control ($p<0.01$) (Fig. 2.1B).

Repeating this protocol produced the same results in the olanzapine and control groups but not haloperidol (Fig. 2.2A). Olanzapine significantly increased weight gain compared to haloperidol ($p<0.05$) (0.5mg/kg/day) and control ($p<0.01$). Animals treated for 14 days with 0.5mg/kg/day of haloperidol did not lose weight as observed in rats treated with 1.0mg/kg/day of haloperidol and was not significantly different from the control group. The olanzapine treated animals gained significantly more weight than the control and haloperidol animals following three days of treatment. Also, the haloperidol group had a significant increase in weight gain compared to the control group following seven days of treatment and gained on average approximately 2.5% of their initial weight ($p<0.01$) (Fig. 2.2A). Overall, this was not significant from the control group.

There was no overall difference in food intake between any of the treatment groups. However, a significant increase in food intake in olanzapine treated rats was observed at; three days of treatment compared to haloperidol and seven and nine days of treatment compared to haloperidol and control treated rats ($p<0.01$) (Fig. 2.2B).

Analysis of serum shows that sub-chronic olanzapine treatment significantly increases leptin in rats treated for 14 days compared to haloperidol and vehicle

treated animals (Table 2.1). No differences in plasma insulin or blood glucose levels were found (Table 2.1).

Table 2.1 *Blood sample data in female rats treated for 14 days.*

	Control	Haloperidol	Olanzapine	p
Leptin (ng/ml)	0.81±0.13	0.89±0.21	1.75±0.38 *#	0.029
Insulin (ng/ml)	1.24±0.22	1.17±0.20	1.57±0.35	ns
Glucose (nmol)	9.10±0.96	7.32±0.57	7.70±0.42	ns

Data are presented as mean ± SE. ns = no significant difference between groups. *($p<0.05$) designates a significant difference compared to the control group, # ($p<0.05$) designates a significant difference compared to the haloperidol group.

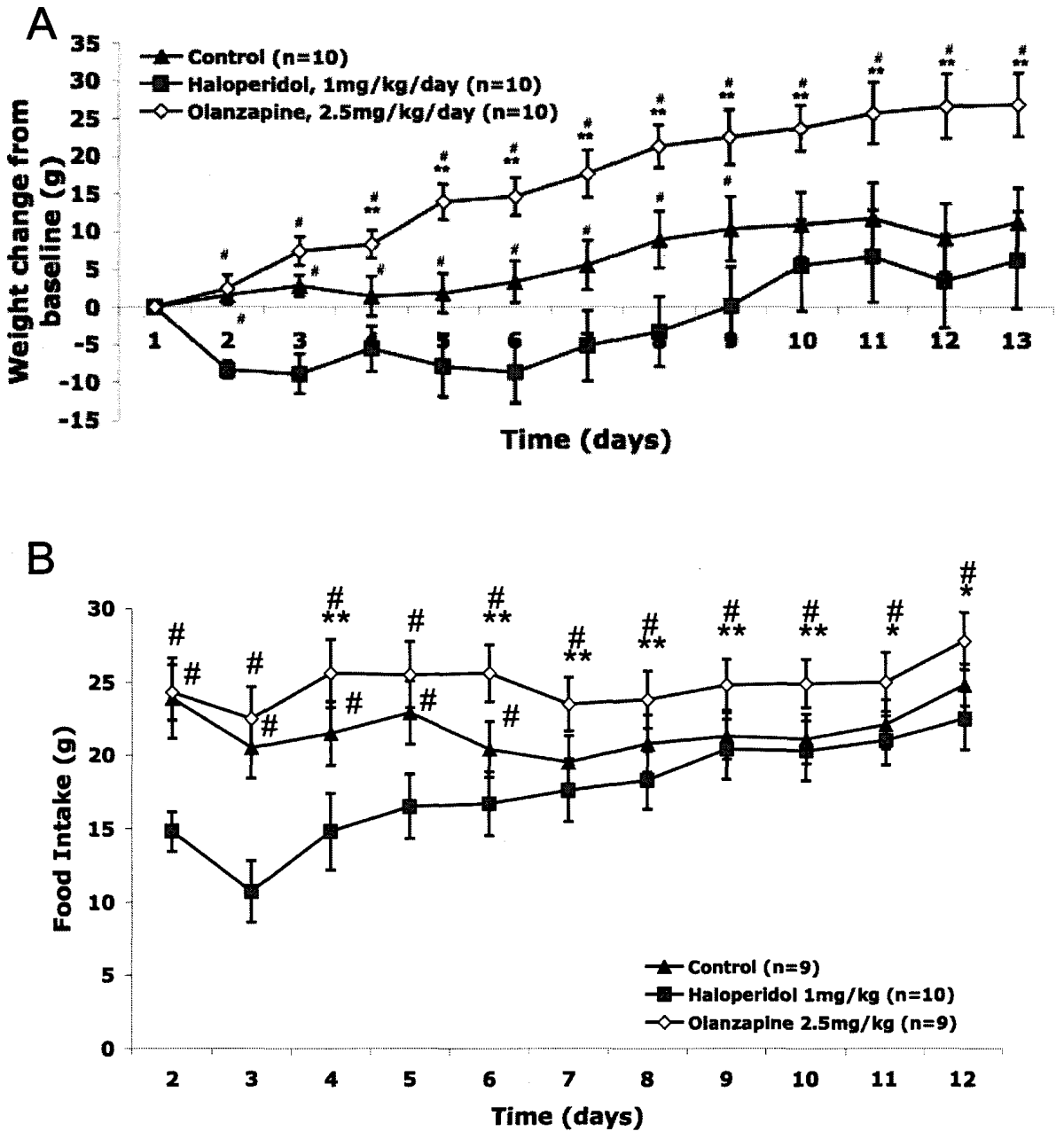

Fig. 2.1 Effect of sub-chronic olanzapine and haloperidol (1.0mg/kg/day) on mean body weight change from baseline (a) and mean daily food intake (b) over the 14 day treatment period. Data is presented as mean ± SE. ** ($p<0.01$), *($p<0.05$) designates a significant difference compared to the control group, # ($p<0.01$) designates a significant difference compared to the haloperidol group.

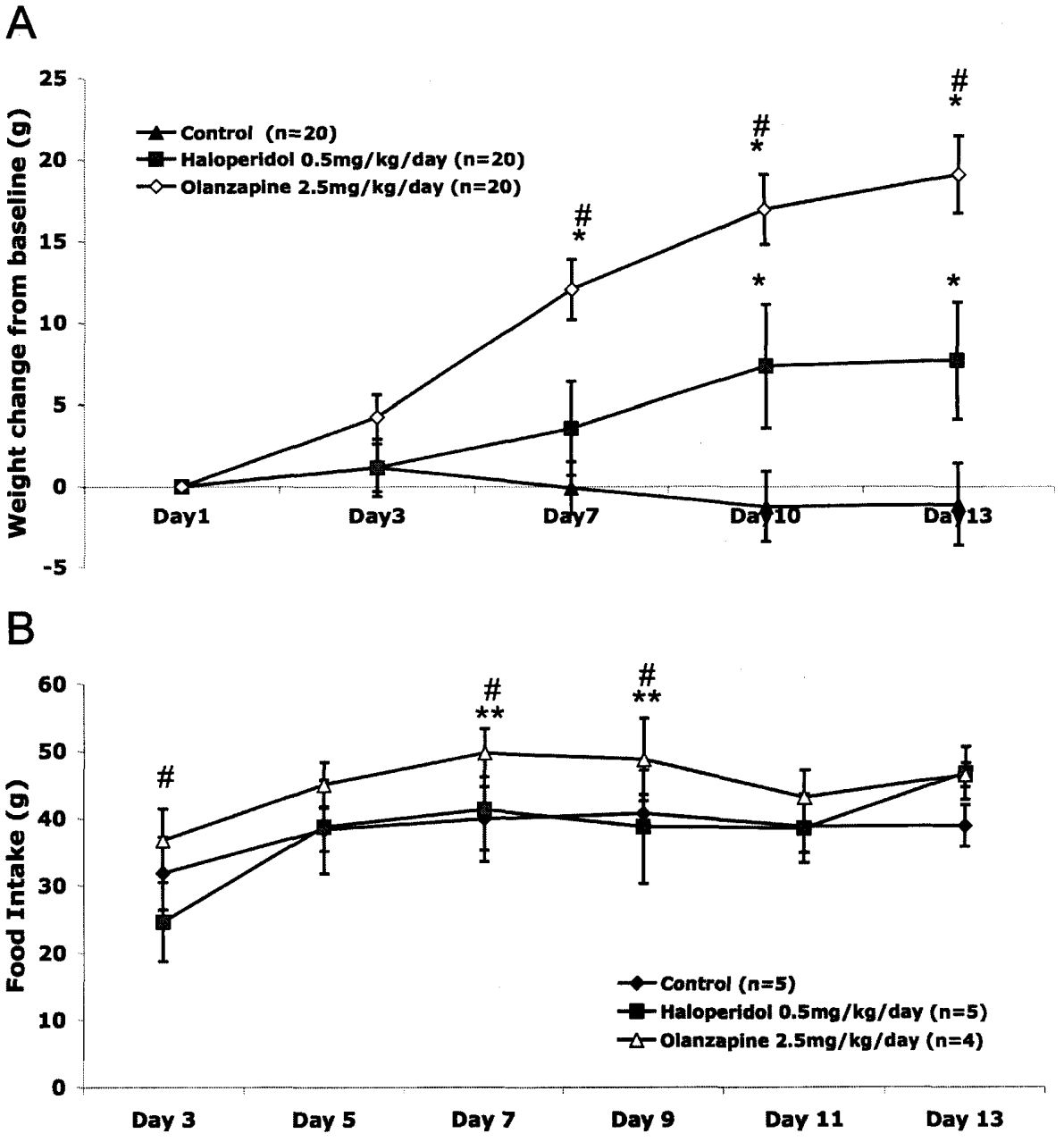

Fig. 2.2 Effect of sub-chronic olanzapine and haloperidol (0.5mg/kg/day) on mean body weight change from baseline (A) and mean daily food intake (B) over the 14 day treatment period. Data is presented as mean ± SE. ** ($p<0.01$), *($p<0.05$) designates a significant difference compared to the control group, # ($p<0.01$) designates a significant difference compared to the haloperidol group.

Effects of sub-chronic olanzapine treatment on leptin signaling in the arcuate and ventromedial nuclei of the hypothalamus.

Sub-chronic administration of olanzapine or haloperidol did not significantly alter pSTAT3 immunoreactivity throughout the ARC (Fig. 2.3). STAT3 immunolabeling in leptin and vehicle injected animals was also unaltered following sub-chronic olanzapine and haloperidol treatment (Fig 2.4). However, sub-chronic olanzapine treatment significantly reduced pSTAT3 immunoreactivity in the caudal VMH compared to haloperidol and control animals (control: 427.2 ± 32.8 SEM; p=0.009, haloperidol: 457.9 ± 32.4 SEM; p=0.002, olanzapine 277.52 ± 21.0 SEM) (Fig. 2.5). No significant difference was observed in rostral or middle divisions of the VMH. Olanzapine and haloperidol did not significantly alter the levels of STAT3 throughout the VMH in both vehicle and leptin injected animals compared to control animals (Fig. 2.6). Additionally, pSTAT3 immunoreactivity was not observed in any vehicle injected animals (Fig. 2.3).

Effects of sub-chronic olanzapine treatment on AgRP and POMC immunoreactivity in the arcuate nucleus.

Sub-chronic administration of olanzapine significantly decreased the mean number of immunoreactive POMC neurons in the caudal ARC compared to control (control: 88.2 ± 2.9 SEM; olanzapine: 79 ± 1.6 SEM; p=0.044), but not haloperidol animals (Fig. 2.7). No significant difference was found between treatment groups in the rostral or middle divisions of the arcuate nucleus. Percentage of AgRP fibre density in the PVN and ARC remained unchanged in all treatment groups (Fig. 2.8).

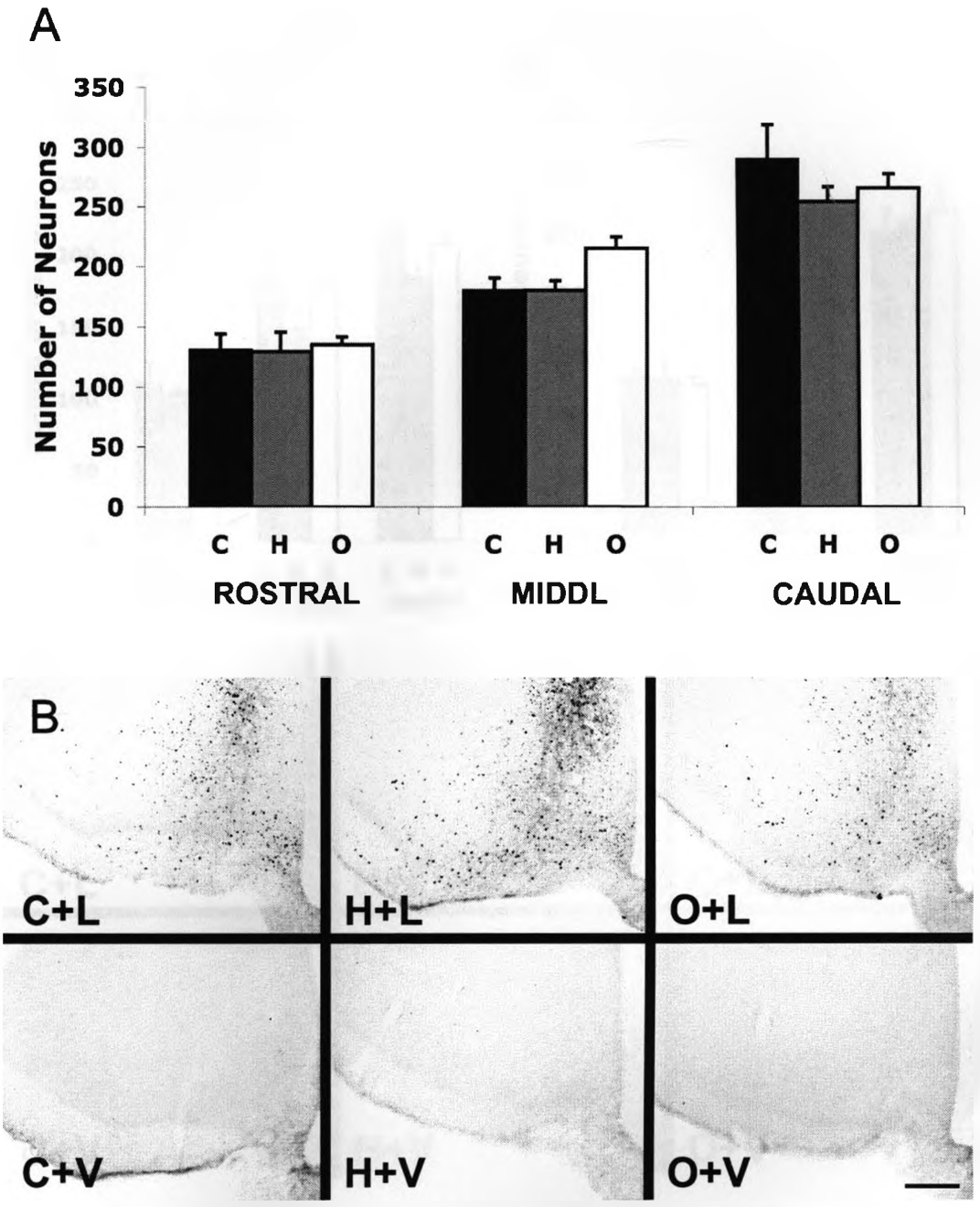

Fig 2.3 Effect of sub-chronic olanzapine and haloperidol (0.5mg/kg) on number of pSTAT3 immunoreactive neurons in the rostral, middle and caudal ARC. The graph illustrates the mean number of neurons, ± standard error, of leptin injected animals (A). Photomicrographs show representative images of pSTAT3 neurons from the rostral ARC in olanzapine (O+L, O+V), haloperidol (H+L, H+V) and control (C+L, C+V) animals injected with leptin and vehicle (B). Scale bar = 225μm.

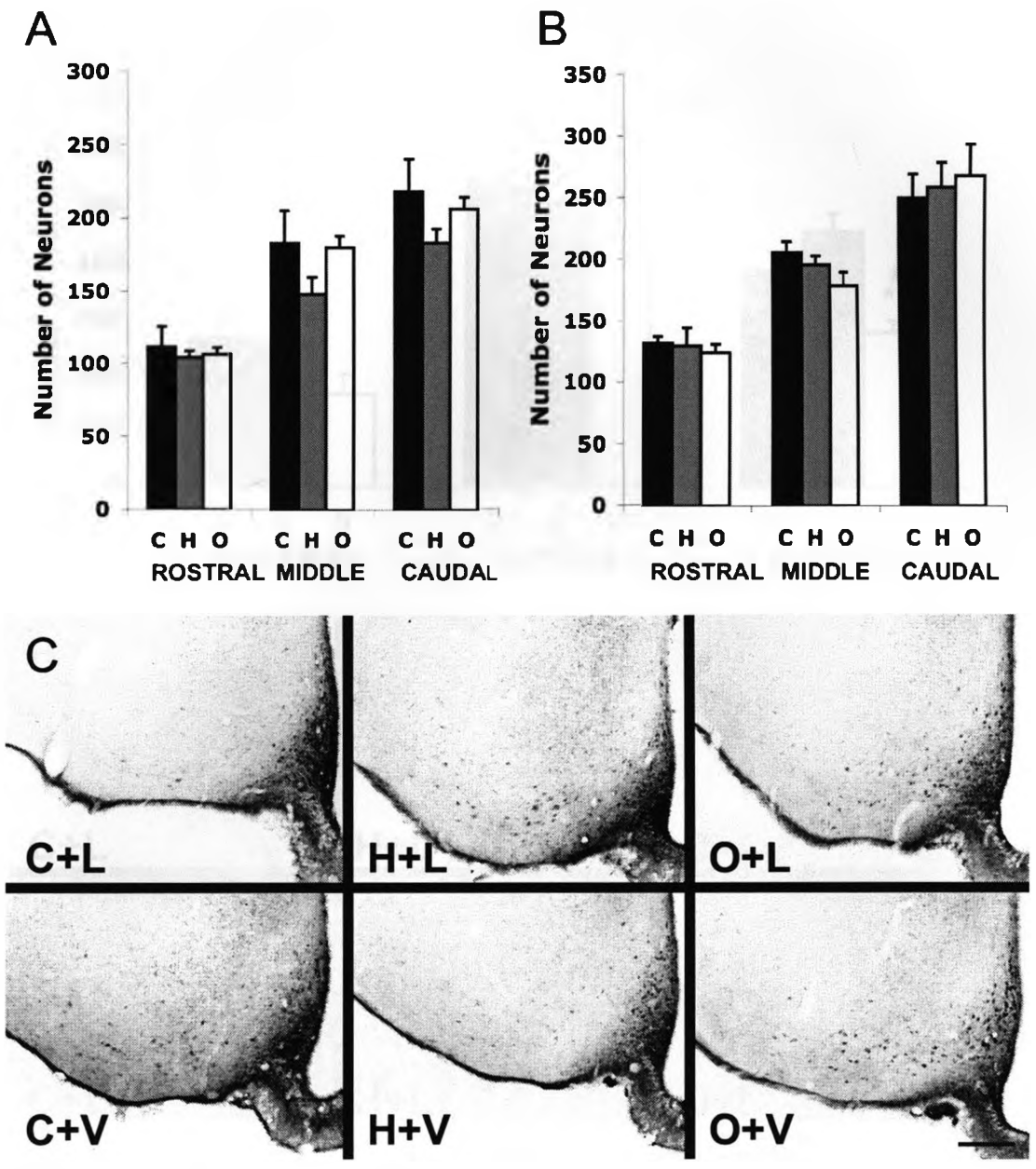

Fig 2.4 Effect of sub-chronic olanzapine and haloperidol (0.5mg/kg) on number of STAT3 immunoreactive neurons in the rostral, middle and caudal ARC. The graph illustrates the mean number of neurons, ± standard error, of leptin injected animals (A) and vehicle injected animals (B). Photomicrographs show representative images of STAT3 neurons from the rostral ARC in olanzapine (O+L, O+V), haloperidol (H+L, H+V) and control (C+L, C+V) animals injected with leptin and vehicle (C). Scale bar = 225μm.

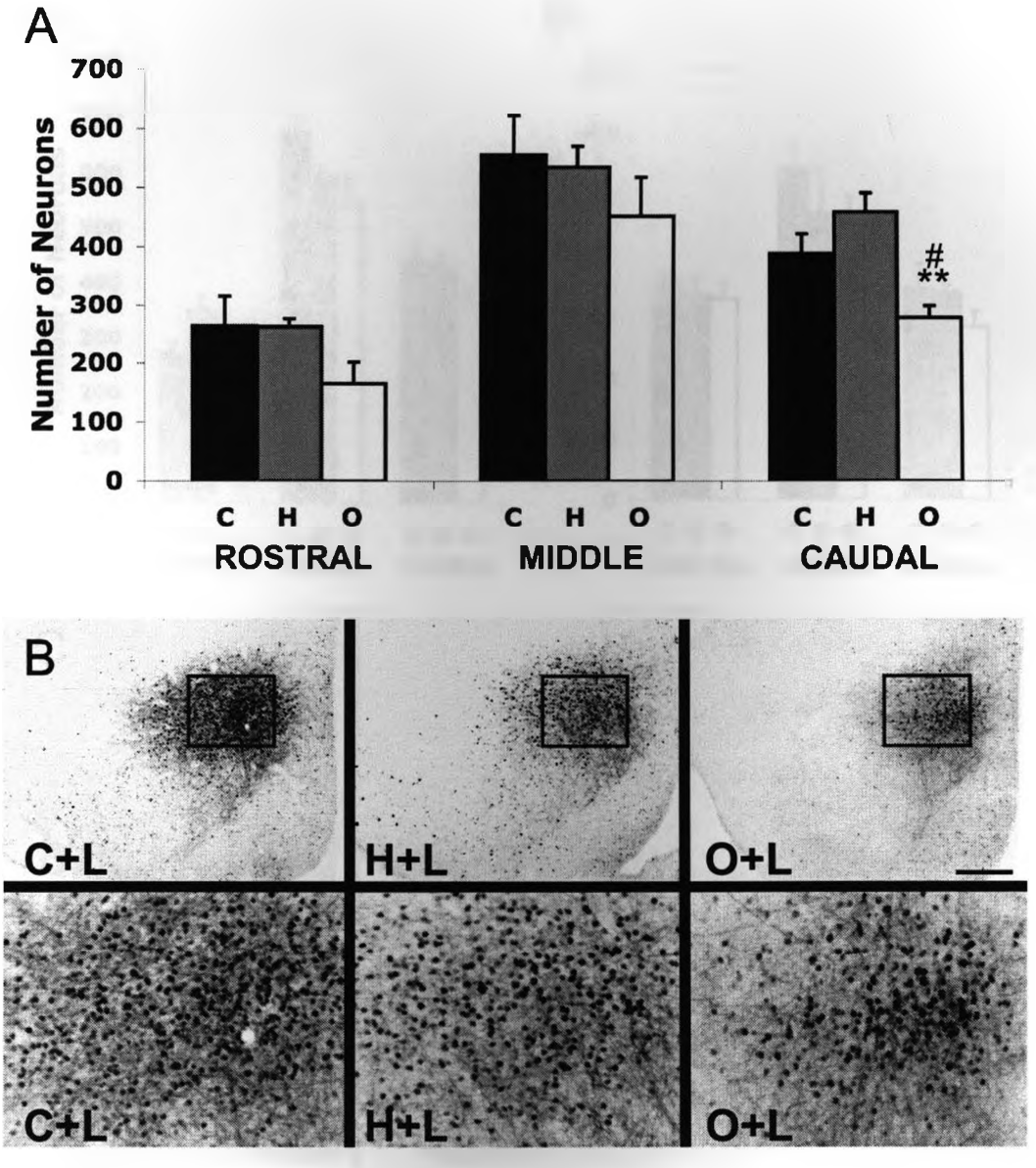

Fig 2.5 Effect of sub-chronic olanzapine and haloperidol (0.5mg/kg) on number of pSTAT3 immunoreactive neurons in the rostral, middle and caudal VMH. The graph illustrates the mean number of neurons, ± standard error, of leptin injected animals (A). Photomicrographs show representative images of pSTAT3 neurons from the caudal VMH in olanzapine (O+L), haloperidol (H+L) and control (C+L) animals injected with leptin (B). Lower panel images are higher-magnifications of each area of interest. Scale bar = 225μm.

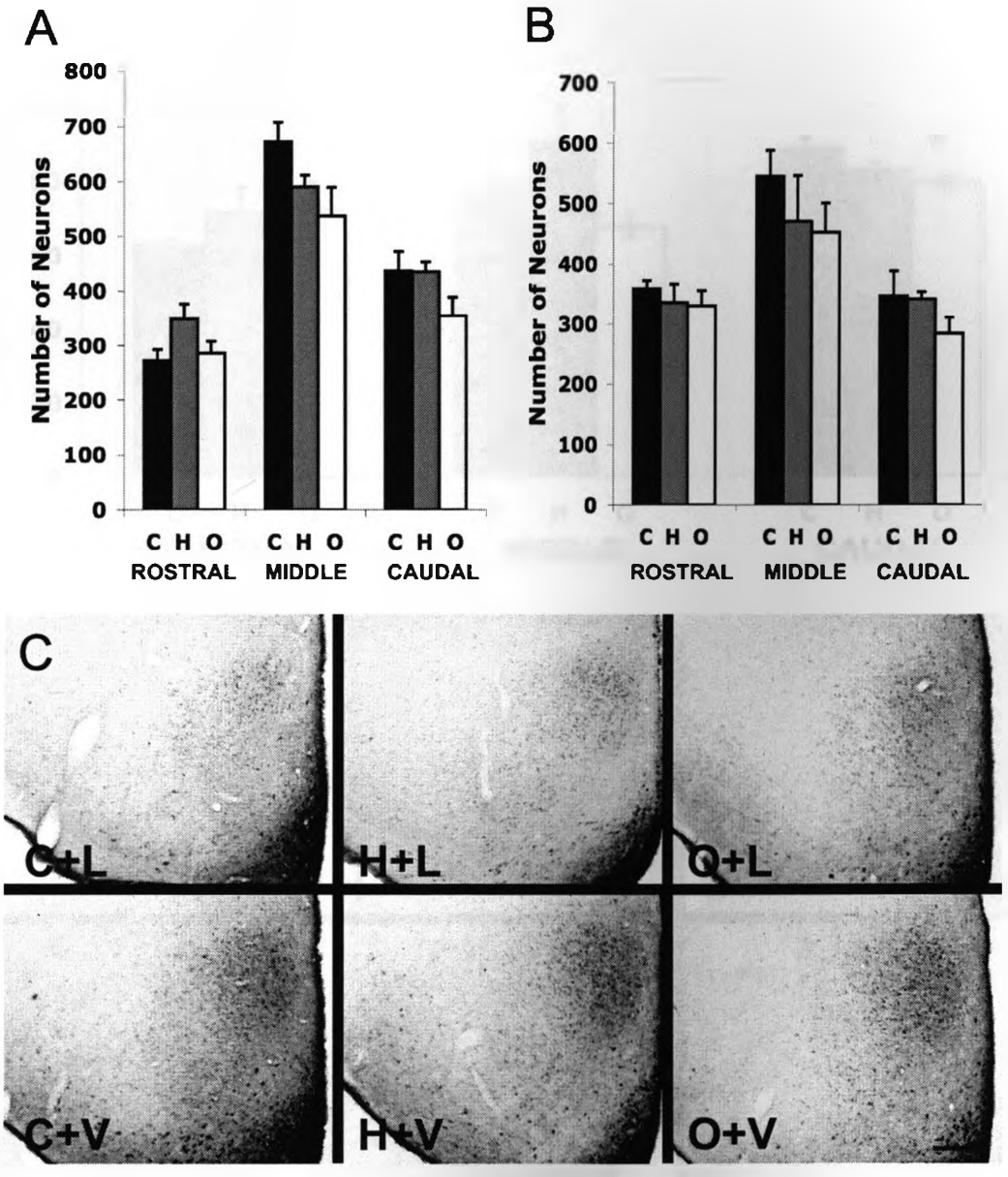

Fig 2.6 Effect of sub-chronic olanzapine and haloperidol (0.5mg/kg) on number of STAT3 immunoreactive neurons in the rostral, middle and caudal VMH. The graph illustrates the mean number of neurons, ± standard error, of leptin-injected animals (A) and vehicle injected animals (B). Photomicrographs show representative images of STAT3 neurons from the rostral VMH in olanzapine (O+L, O+V), haloperidol (H+L, H+V) and control (C+L, C+V) animals injected with leptin and vehicle (C). Scale bar = 225μm.

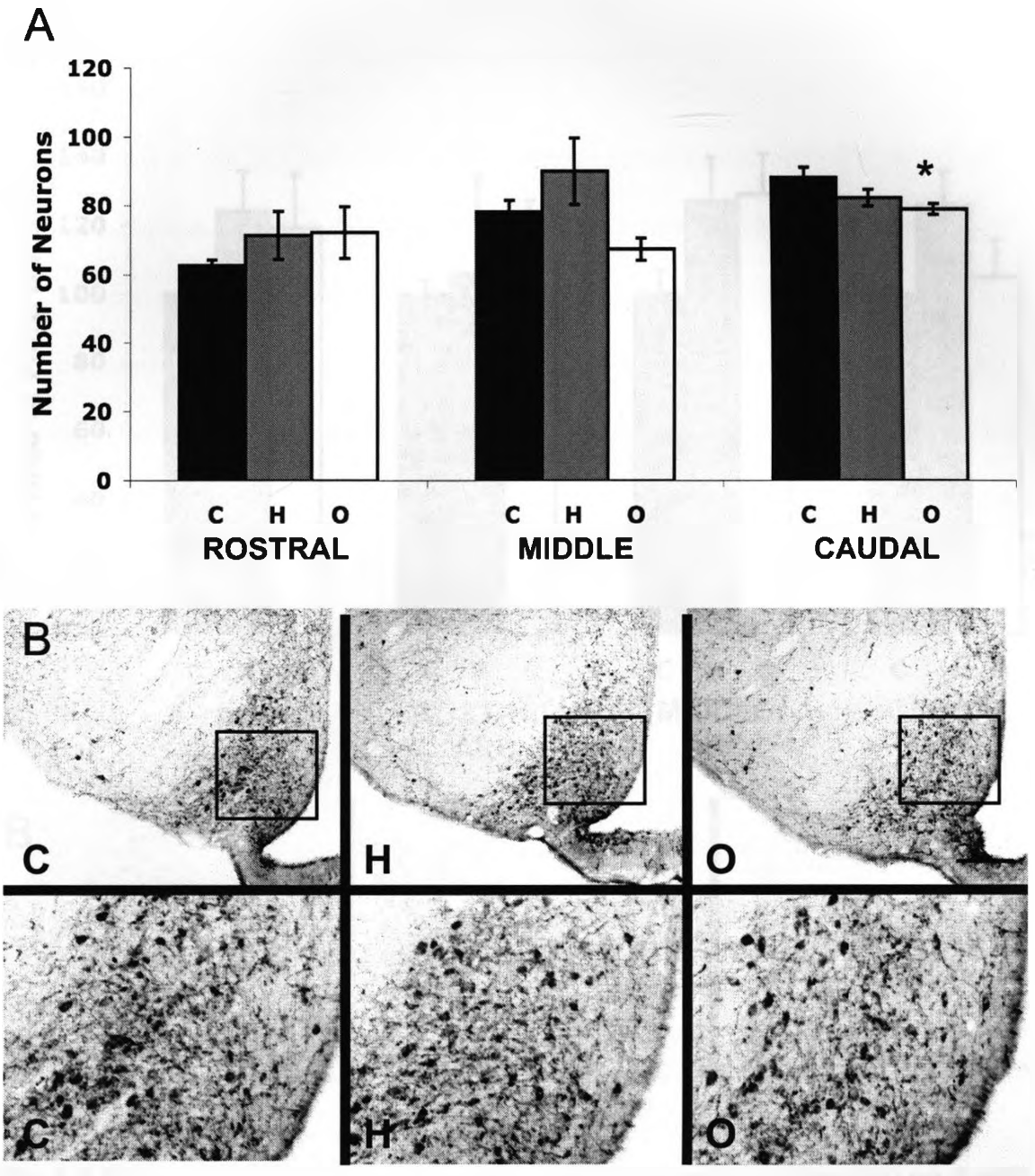

Fig 2.7 Effect of sub-chronic olanzapine and haloperidol (0.5mg/kg) on number of POMC immunoreactive neurons in the rostral, middle and caudal ARC. The graph illustrates the mean number of neurons, ± standard error (A). Photomicrographs show representative images of POMC neurons from the caudal ARC in olanzapine, haloperidol and control animals (B). Lower panel images are higher-magnifications of each area of interest. Scale bar = 225μm.

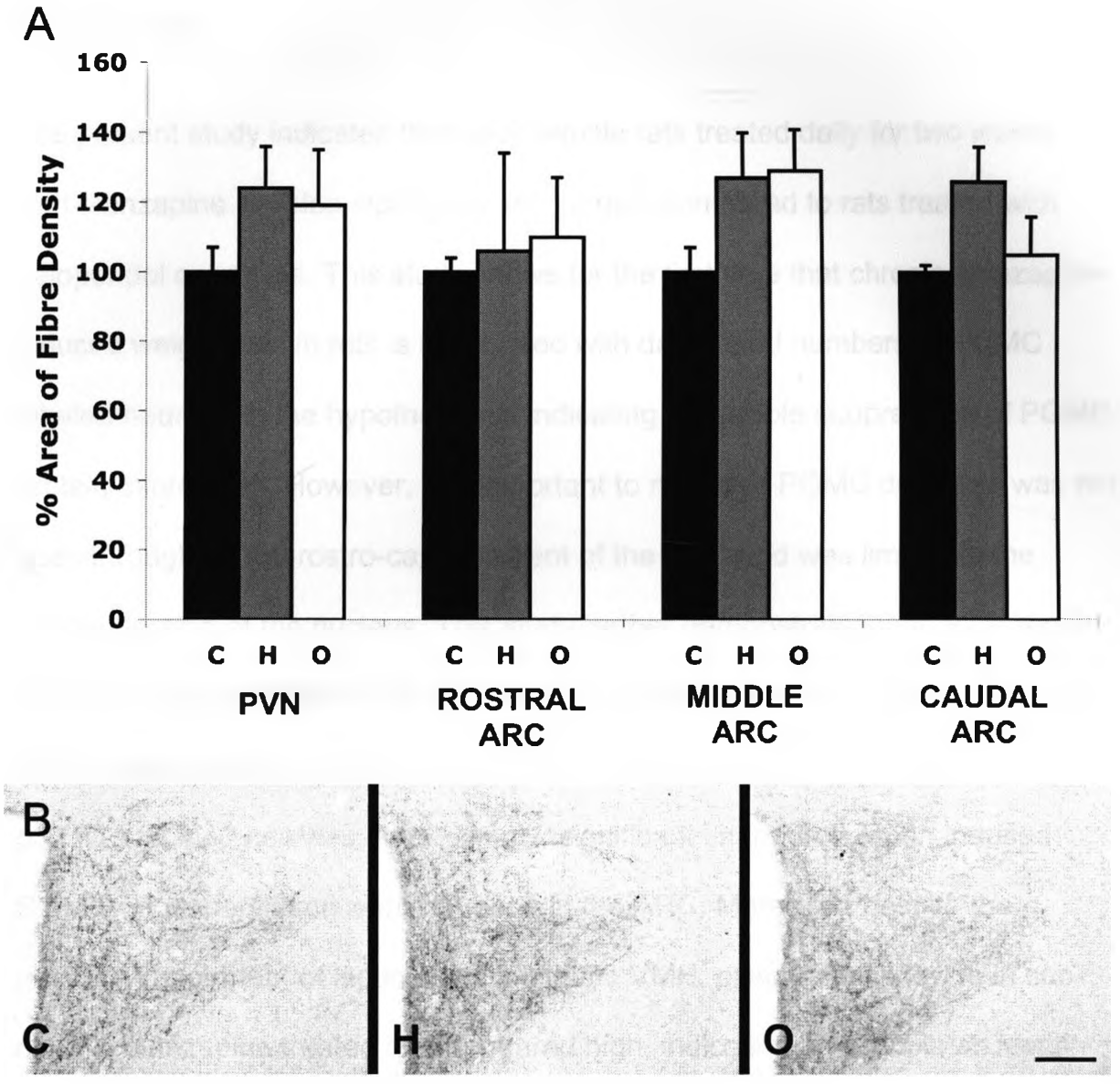

Fig 2.8 Effect of sub-chronic olanzapine and haloperidol (0.5mg/kg) on AgRP fibre density in the PVN and rostral, middle and caudal ARC. The graph demonstrates the percent area of fibre density normalized to control (A). Photomicrographs show representative images of AgRP fibres from the PVN in olanzapine, haloperidol and control animals (B). Scale bar = 125μm.

DISCUSSION

The present study indicates that adult female rats treated daily for two weeks with olanzapine develop significant weight gain compared to rats treated with haloperidol or vehicle. This study shows for the first time that chronic olanzapine-induced weight gain in rats is associated with decreased numbers of POMC labeled neurons in the hypothalamus indicating a possible suppression of POMC protein expression. However, it is important to note that POMC decrease was not seen throughout the rostro-caudal extent of the ARC and was limited to the caudal division of the nucleus. This study further demonstrates that acute leptin-induced phosphorylation of STAT3 was attenuated in the VMH compared to sub-chronic haloperidol or vehicle treated rats, indicated by the decreased number of pSTAT3 labeled neurons. Interestingly, significant changes in leptin-induced STAT3 phosphorylation were not seen in the ARC. Moreover, despite the possible impairment of leptin signaling in the VMH, plasma leptin levels in sub-chronic olanzapine treated rats appeared high, indicating an associated leptin resistance in these rats, a phenomenon described in patients as well. Daily food intake was not correlated to weight gain in these animals suggesting that a decrease in metabolism and energy expenditure might be contributing to the observed weight gain. Decreased STAT3 phosphorylation in VMH neurons in sub-chronic olanzapine treated rats may favor this notion as the VMH is often implicated in regulation of energy balance (51-55). The present results therefore support the hypothesis that altered hypothalamic function affecting the VMH and ARC may contribute to atypical antipsychotic-induced weight gain.

The first aim of the present study was to produce atypical antipsychotic-induced weight gain in the rodent. Evidence indicates that chronic olanzapine (56-59), clozapine (60, 61) or risperidone (62-64) can induce weight gain and increase food consumption (61) in rats and mice. It appears that female rodents are more prone to weight gain following chronic antipsychotic treatment compared to males (56-58, 64-68). To date, the most consistent induction of weight gain was found in adult, female rats treated chronically with olanzapine (56-59). Olanzapine at 2-4 mg/kg daily for 2-3 weeks resulted in increased body weight (an average 12-15 g), increased food intake, visceral adiposity and increased plasma levels of leptin, insulin, prolactin and adiponectin (69-74). Therefore, in the present study, olanzapine was employed as a representative atypical antipsychotic agent at a daily dose of 2.5 mg/kg. Pilot studies have indicated that daily injections, especially injections of vehicle (at pH 5.2), resulted in considerable pain and disability to animals and therefore an oral route was sought.

Additional pilot studies have indicated that young adult rats at 12 weeks continue to grow and gain weight even with vehicle treatment, thereby interfering with treatment effect. Consequently, the present study used female rats at least 20 weeks of age. With the treatment regimen used, rats rapidly gained weight until they reached close to a plateau level at approximately 14 days of treatment. A similar pattern of weight gain, though with a larger time course, has been observed in patients (51, 52). Despite their rapid metabolism of antipsychotic

drugs (>24 hrs in human vs. 2-5 hrs in rats), the present results indicate that once daily administration is sufficient to cause weight gain in rats of this age.

In the first series of experiments, daily administrations of 1.0mg/kg of haloperidol resulted in an initial decrease in food intake. Animals receiving this dose also showed a significant decrease in weight gain during this time. During the treatment period, it was observed that shortly after administration of 1.0mg/kg of haloperidol, animals became sedated. Haloperidol is known to cause sedation, especially at higher and oral doses (75). This side effect may have caused the decrease in food intake and subsequent decrease in weight. However, by the end of the treatment period, these animals were able to regain the weight that was lost, resulting in no significant changes in weight gain when compared to control animals.

The daily dose of 1.0mg/kg/day haloperidol had a significant influence on weight, but due to its sedative effects, this dose is not relevant to clinical observations in patients. In the following series of experiments, a smaller dose, 0.5mg/kg/day, was administered to minimize the sedative effect of the drug. Animals receiving the lower dose of haloperidol did not show a significant decrease in food intake or weight. Administration of 0.5mg/kg of haloperidol daily for two weeks did cause weight gain, as seen at individual time points, however overall this increase was not significantly different from baseline weight or rats that received vehicle. Therefore, haloperidol given at a dose that does not induce sedation

may have a modest but insignificant effect on weight gain in female rats. Interestingly, similar results have been observed in rats receiving oral administration of 0.08 and 0.31 mg/kg/day of haloperidol (76, 77). This is in accordance with a recent, large multi-centered study that found that after two years, patients taking haloperidol gained 7.5kg on average (78).

Davoodi and collaborators have shown that total food intake, meal size and duration of feeding was significantly increased following chronic olanzapine treatment (9). They also found that the olanzapine-induced weight gain was prevented if rats were given a restricted amount of food to the level of vehicle treated controls, suggesting increased food intake as an important factor. On the contrary, Stephanidis and collaborators have shown that chronic olanzapine-induced weight gain was associated with decreased locomotor activity, reduced brown adipose tissue temperature, and lack of increase in food intake (79). While the present results agree with the later study, a transient increase in food intake and suppressed POMC labeling in sub-chronically olanzapine treated rats suggests a potential contribution from increased feeding as a possibility.

The mechanism underlying chronic olanzapine-induced weight gain is not clear. Peripheral/endocrine disturbance, impaired central regulation of feeding and energy balance or a combination of both has been suggested as contributing factors. Only recently, studies have reported antipsychotic-induced changes in brain areas that are directly implicated in food intake. For example, decreased

density of muscarinic and cannabinoid receptors in the dorsal vagal complex (80, 81), and increased levels of AMP-kinase in hypothalamic neurons (82) were reported.

The hypothalamus plays a crucial role in controlling feeding behaviour, energy balance and in maintaining normal body weight. As previously described, leptin is produced by adipose tissue in response to food intake and fat storage. This hormone acts on leptin receptors in a variety of tissues, including the hypothalamus, to suppress food intake (83-87). In the ARC, leptin stimulates POMC-containing neurons, and inhibits a separate population of neurons containing NPY, AgRP and GABA. POMC neurons stimulate MC4 receptor-containing GABAergic neurons of the PVN to inhibit feeding behavior (88). The NPY/AgRP/GABA-containing neurons of the ARC, on the other hand, inhibit POMC neurons of the ARC and MC4 receptor-containing GABAergic neurons of the PVN via collateral projections. Consequently, POMC and MC4R containing neurons are inhibited, causing stimulation of feeding (38). The present results indicate for the first time that chronic olanzapine-induced weight gain is associated with suppressed POMC in the ARC and therefore would facilitate increasing food intake.

A few studies have measured levels of hypothalamic orexigenic peptides, such as NPY and orexin, following antipsychotic treatment however; results have been contradictory (9, 39, 40, 89). The present results show that sub-chronic

olanzapine treatment decreased the expression of POMC in the caudal ARC. Decreased POMC expression found in olanzapine treated animals was only significantly different from control animals, suggesting that this effect may not be specific to atypical antipsychotics. Nonetheless, a decrease in POMC expression may account for some of the weight gain resulting from olanzapine treatment. These results are in contrast with a recent study where POMC mRNA expression was unaltered in rats exhibiting weight gain following olanzapine treatment (9). However, the current study measured POMC protein whereas Davoodi and collaborators analyzed the mRNA levels. Furthermore, drug protocol and treatment length also differed.

Analysis of AgRP fibre density in the PVN and ARC did not identify any significant changes between treatment groups. Expression of AgRP in the hypothalamus has not been reported in relation to antipsychotic treatment. However, levels of NPY, an orexigenic peptide co-localized with AgRP in the ARC, have been investigated. A previous study, using a similar treatment protocol found that olanzapine does not alter levels of NPY mRNA in the ARC two hours following chronic treatment (89). In contrast, two studies found that chronic clozapine, a different atypical antipsychotic agent, increased NPY immunoreactivity in the hypothalamus and ARC (39, 40). Differences in drugs, dosage, frequency, treatment period, species and sex may account for the discrepancies in the results. Interestingly, elevated levels of NPY immunoreactivity have also been found following treatment with haloperidol (39).

Thus, any modifications in NPY or AgRP expression may not be specific to atypical antipychotics.

Leptin binding to its receptor causes phosphorylation of the transcription factor, STAT3 (90-92). pSTAT3 translocates to the nucleus to facilitate the transcription of POMC (93), and inhibits the transcription of AgRP (94). Therefore, as a feedback messenger, leptin acts on neurons in the hypothalamus to suppress feeding by increasing POMC levels and suppressing AgRP levels. Since the results of this study showed reduced numbers of immunoreactive POMC neurons, we expected to see impaired leptin-induced STAT3 phosphorylation in the ARC in sub-chronically olanzapine treated rats. However, this effect was not observed and may be due to the fact that pSTAT3 was not specifically examined in POMC or AgRP containing neurons.

Leptin-induced pSTAT3 expression in the VMH was significantly reduced by olanzapine. Given the involvement of the VMH and leptin signaling in the regulation of energy balance (95-99), it is reasonable to presume that reduced pSTAT3 expression in this area may be a contributing factor to olanzapine-induced weight gain. As this study is the first to identify any changes in pSTAT3 expression in the hypothalamus, the exact mechanism of decreased pSTAT3 in the VMH remains to be investigated. However, levels of unphosphorylated STAT3 were not affected by olanzapine treatment. Whether decreased pSTAT3 results from a direct interaction of olanzapine with pSTAT3 containing neurons in

the VMH is unknown. However, this evidence suggests that leptin signaling in the VMH is a consequence of treatment with atypical antipsychotic drugs.

The present results also indicate increased plasma leptin levels in olanzapine treated rats. Presence of increased plasma leptin levels with impairment of leptin signaling in the VMH suggests that chronically olanzapine treated rats that gained weight might have leptin resistance, a phenomenon frequently suggested in antipsychotic-induced weight gain in humans. Mechanisms underlying leptin resistance are currently unclear.

In conclusion, present results demonstrate that weight gain induced by atypical antipsychotic treatment can be modeled in adult, female Sprague Dawley rats. Furthermore, this effect is specific to atypical antipsychotics, as the typical, haloperidol, was unable to induce significant weight gain. Results also support the hypothesis that atypical antipsychotic drugs alter normal hypothalamic function. The reduction in leptin signaling, via pSTAT3 expression in the VMH, and decreased POMC expression in the ARC following sub-chronic olanzapine treatment, suggests a possible mechanism of atypical antipsychotic induced weight gain. In addition, this model also shows evidence of leptin resistance, a phenomenon suggested in antipsychotic-induced weight gain in patients. Consequently, the present rat model may prove useful in further studies of mechanisms of antipsychotic induced weight gain. Whether atypical

antipsychotics induce weight gain by increasing food intake and/or altering energy expenditure remains to be comprehensively investigated.

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CHAPTER 3: SUMMARY AND DISCUSSION

Atypical antipsychotic-induced weight gain presents a significant hindrance in the treatment of schizophrenia. This common complication greatly impedes patient compliance and increases mortality rate by increasing a patient's risk for cardiovascular disease. The results presented in this study provide evidence implicating altered hypothalamic function in association with atypical antipsychotic-induced weight gain and therefore facilitate future investigations. This is the first study to report an association between chronic olanzapine-induced weight gain and decreased hypothalamic anorexigenic peptide, POMC, and impaired leptin signaling.

Adult female Sprague Dawley rats at 20 weeks of age were used throughout the present study. Most studies investigating weight gain employed female rats, as the success rate seems to be greater than in males (1-7). However, antipsychotic-induced weight gain occurs equally in both male and female patients. Future studies may determine why male rats are relatively resistant to such weight gain. Interestingly, a previous study has found atypical antipsychotic induced weight gain in male rats (8), but the same group subsequently noticed that chronic olanzapine treatment in male rats resulted in increased adiposity without any change in weight (9). This evidence suggests that olanzapine-induced metabolic changes may be presented in different ways in male and female rats. Nevertheless, differences in drug treatment paradigms, such as

route and time of administration, may also account for the discrepancies between these sets of observations.

Our preliminary results from a pilot study have shown that age may also be a contributing factor to modeling antipsychotic-induced weight gain in animals. A daily dose of 2.5mg/kg of olanzapine (i.m.) was given to female Sprague Dawley rats at nine weeks of age and 20 weeks of age for two weeks. Results indicated that both the vehicle and olanzapine treated groups gained significant weight in nine-week old animals. It was concluded that the normal growth and maturation of young animals likely mask the olanzapine effects and therefore are not suitable for creating a model.

In the present study, it was hypothesized that olanzapine-induced weight gain would result from increased food intake. Only transient increases in food intake were noticed. In addition, although POMC levels were decreased in the ARC favouring increased feeding, leptin-induced pSTAT3 was impaired only in the VMH. One caveat to these observations is the timing of drug administration relative to feeding, and the profile of drug metabolism in rodents. Pilot studies have indicated that rats consumed the majority of their daily food intake during the dark/active phase. Consequently, olanzapine was administered in the present study between mid-light phase as sedation occurred for a few hours following ingestion. However, the half-life of olanzapine in rodents is approximately 2.5 hours (10). Therefore, it is possible that olanzapine was in such low levels in the

body at the time of food intake, that it did not influence meal patterns. It is important to consider that in humans, the half-life of olanzapine is over 24 hours and most consume throughout the day. In the present study, chronic olanzapine induced a considerable gain in weight than was accounted for by the daily consumption of food. Although, water intake was not monitored, it is still likely that a considerable proportion of weight gain in these animals were due to conservation of energy. Activity levels and metabolic rates of these animals during the treatment period should be monitored.

In addition to changes in leptin, clinical and rodent studies report elevated circulating insulin levels and evidence of insulin resistance. Many patients also develop type II diabetes following chronic atypical antipsychotic treatment (11-13). It is unknown whether this increase in insulin is a direct effect of atypical antipsychotic treatment or secondary to weight gain. However, it would be interesting to investigate insulin-signaling pathways in the hypothalamus. Similar to leptin, insulin secretion is influenced by body fat and is transported to the hypothalamus where it is capable of activating ARC neurons, affecting POMC and CART expression via the JAK-STAT3 pathway to reduce food intake and increase energy usage (14). In the current study, we observed a significant decrease in POMC immunoreactivity in the ARC in the absence of decreased leptin-induced pSTAT3. It is possible that olanzapine may interfere with insulin-induced activation of POMC expression. Analysis of pSTAT3 immunoreactivity in

the ARC following an acute insulin injection after 14 days of olanzapine treatment will help to test this hypothesis.

It is likely that olanzapine modulates energy balance via antagonism of the histamine H1 receptor and the serotonin 5-HT_{2C} receptor subtypes. Modulating these receptors in the hypothalamus affects feeding behavior (15, 16, 17). Specifically, activation of these receptors suppresses food intake and up-regulates metabolism (17-20). Serotonin receptors, specifically the 5-HT_{2C} receptor subtype, have been co-localized with POMC neurons in the ARC (21). Interestingly, the majority of these co-localized neurons are located in the caudal ARC (21), the area where we found a decrease in POMC immunoreactivity. Agonists of the 5-HT_{2C} receptor can depolarize and induce c-fos immunoreactivity in POMC neurons (21). This evidence may suggest that antagonists of this receptor, such as olanzapine, are able to inhibit depolarization and induction of c-fos immunoreactivity in POMC neurons. Consequently, activity of these neurons may be dampened, inhibiting their propensities to propagate satiety signals.

Unlike the ARC, the exact role of the VMH in energy homeostasis is still unclear. However, lesion and knockout studies have characterized the VMH as a satiety center (22-25). As such, our results indicating decreased leptin signaling via reduced pSTAT3 expression provide evidence that the VMH plays a more important role in atypical antipsychotic-induced weight gain than initially

hypothesized. Interestingly, a recent study has presented an association with olanzapine treatment, weight gain and changes in the VMH (26). Specifically, significant decreases in H1 receptor mRNA and receptor binding density were reported (26). We are unsure if a connection exists between reduced pSTAT3 and H1 receptor mRNA and binding density in the VMH. However, given that olanzapine is a potent antagonist to the H1 receptor, future investigations in this hypothalamic area are likely to further our understanding.

Atpyical antipsychotics may modify peptide expression in alternate areas of the hypothalamus. For example, a few studies have found increased c-fos immunoreactivity in orexin neurons located in the PFLH of rats receiving olanzapine, clozapine and risperidone treatment (27, 28). Reciprocal connections exist between the PFLH and arcuate nucleus, which allow communication between these regulatory systems. Olanzapine may act directly on the orexin-containing neurons in the PFLH to induce changes in the melanocortin system in the ARC, or vice versa.

Atypical antipsychotic-induced weight gain appears to involve complex changes in many integrated neuronal circuits, likely caused by their ability to antagonize multiple receptor types. The data presented in this thesis provides important information applicable to these mechanisms. The analysis of hypothalamic peptide expression/signaling has provided a fundamental step for future investigations into atypical antipsychotic-induced disturbances in metabolic

function. Elucidating the mechanism of action of these pharmacological agents is essential for improving treatment strategies and overall quality of life of patients with schizophrenia.

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APPENDIX 1:LEPTIN AND INSULIN IMMNOASSAY PROTOCOLS

Serum and plasma collected from blood samples were sent to the laboratory of Dr. Murray Huff at the Robarts Research Institute for analysis of serum leptin and plasma insulin levels. The leptin EIA kit (11-LEPHU-E01) and the insulin EIA kit (80-INSRT-E01) from ALPCO Diagnostic were used and are attached below.

Immunoassay Kits Beyond The Ordinary

Leptin EIA

For the quantitative determination of Leptin in human serum

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number: 11-LEPHU-E01
Size: 96 Wells
Version: February 20, 2007 6.0 – ALPCO 11/27/07

Critical Changes: Please read protocol carefully. Ex. Serum samples do not need to be diluted.

ALPCO Diagnostics

26 Keewaydin Drive Unit G • Salem, NH 03079
Phone: (800) 592-5726 • Fax: (603) 898-6854
www.alpco.com • Email: web@alpco.com

INTENDED USE
For the quantitative determination of Leptin in human serum by an enzyme immunoassay method.For *research* use only.

PRINCIPLE OF THE TEST
The principle of the following enzyme immunoassay test follows a typical two-step capture or 'sandwich' type assay. The assay makes use of two highly specific monoclonal antibodies: A monoclonal antibody specific for leptin is immobilized onto the microwell plate and another monoclonal antibody specific for a different epitope of leptin is conjugated to biotin. During the first step, leptin present in the samples and standards is bound to the immobilized antibody and to the biotinylated antibody, thus forming a sandwich complex. Excess and unbound biotinylated antibody is removed by a washing step. In the second step, streptavidin-HRP is added, which binds specifically to any bound biotinylated antibody. Again, unbound streptavidin-HRP is removed by a
washing step. Next, the enzyme substrate is added (TMB), forming a blue colored product that is directly proportional to the amount of leptin present. The enzymatic reaction is terminated by the addition of the stopping solution, converting the blue color to a yellow color. The absorbance is measured on a microtiter plate reader at 450nm. A set of standards is used to plot a standard curve from which the amount of leptin in patient samples and controls can be directly read.

BACKGROUND
Human Leptin is a 16 kDa, 146 amino acid residue, non-glycosylated polypeptide. Leptin is encoded by the OB gene. Its major source is the adipose tissue, and its circulating concentrations indirectly reflect body fat stores. Plasma or serum concentrations of leptin are increased in obese humans and strongly correlate with the degree of adiposity as expressed by percentage of body fat or body mass index. The recently discovered hormone leptin contributes to the regulation of energy balance by informing the brain of the amount of adipose tissue in the body. The brain may then make the appropriate adjustments in either energy intake or expenditure.

Many areas of leptin physiology remain to be investigated. The roles of leptin in metabolism, insulin sensitivity, as a potential therapeutic modality for weight loss as well as involvement in endocrine function are active areas of research. While the future for leptin as a therapeutic agent is not clear, its involvement in many areas of physiology undoubtedly makes this a new hormone which requires extensive study in the future to understand its physiology.

PROCEDURAL CAUTIONS AND WARNINGS
1. This kit is intended for research use only.
2. Practice the following good laboratory practices when handling kit reagents:
• Do not pipette by mouth.
• Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
• Wear protective clothing and disposable gloves when handling the specimens and kit reagents.
• Wash hands thoroughly after performing the test.
• Avoid contact with eyes; use safety glasses; in case of contact, flush with water immediately and contact a doctor.
3. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
4. Avoid microbial contamination of reagents.
5. A calibrator curve must be established for every run.
6. It is recommended to all customers to prepare their own control materials or serum pools which should be included in every run at a high and low level for assessing the reliability of results.
7. The controls (included in kit) should be included in every run and fall within established confidence limits.
8. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.

9. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
10. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the control do not reflect established ranges.
11. When reading the microplate, the presence of bubbles in the microwells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
12. The substrate solution (TMB) is sensitive to light and should remain colorless if properly stored. Instability or contamination may be indicated by the development of a blue color, in which case it should not be used.
13. When dispensing the substrate and stopping solution, do not use pipettes in which these liquids will come into contact with any metal parts.
14. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
15. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
16. Kit reagents must be regarded as hazardous waste and disposed of according to local and/or national regulations.

LIMITATIONS
1. All the reagents within the kit are calibrated for the direct determination of leptin in human serum.
The kit is not calibrated for the determination of leptin in saliva, plasma or other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Only assay buffer may be used to dilute any high serum samples. The use of any other reagent may lead to false results.

SAFETY CAUTIONS AND WARNINGS
POTENTIAL BIOHAZARDOUS MATERIAL
All serum samples should be considered as potential biohazards and handled with the appropriate precautions.

CHEMICAL HAZARDS
Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SERUM COLLECTION AND STORAGE
Approximately 0.1 ml of serum is required per duplicate determination. Collect 4-5 ml of blood into an appropriately labeled tube and allow it to clot. Centrifuge and carefully remove the serum layer.
Store at 4oC for up to 24 hours or at -10oC or lower if the analyses are to be done at a later date. Consider all human specimens as potential biohazards and take appropriate precautions when handling.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED
1. Precision pipette to deliver 20-200 ul
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Microtiter plate washer (recommended)
6. Microwell plate reader with a filter set at 450nm and an upper OD limit of 3.0 or greater

REAGENTS PROVIDED

1. Anti-Leptin Monoclonal Antibody Coated Microwell Plate-Break Apart Wells Contents:
One 96 well (12x8) monoclonal antibody-coated microwell plate in a resealable pouch with desiccant.
Storage: Refrigerate at 2-8oC
Stability: Unopened at 2-8 oC until expiration date on label.

2. Monoclonal Anti-Leptin-Biotin Conjugate
Contents: Monoclonal anti-leptin antibody conjugated to biotin in a protein-based buffer with a non-mercury preservative.
Volume: 10 ml/bottle
Storage: Refrigerate at 2-8oC
Stability: Unopened at 2-8 oC until expiration date on label.

3. Streptavidin-HRP Conjugate Concentrate
Contents: Streptavidn conjugated to horseradish peroxidase in a protein-based buffer with a non-mercury preservative.
Volume: 0.4 mL/bottle
Storage: Refrigerate at 2-8oC
Stability: Unopened at 2-8 oC until expiration date on label.

Preparation: Dilute 1:50 in assay buffer before use (eg. 40 ul of concentrate in 2 ml of assay buffer). If the whole plate is to be used dilute 240 ul of concentrate in 12 ml of assay buffer. Discard any that is left over.

4. Leptin Calibrators
Contents: Six bottles containing leptin in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of leptin. Listed below are approximate concentrations, please refer to bottle labels for exact concentrations.

Calibrator	Concentration	Volume/Bottle
Calibrator A	0 ng/mL	0.5 m:
Calibrator B	1 ng/mL	0.5 mL
Calibrator C	5 ng/mL	0.5 mL
Calibrator D	10 ng/mL	0.5 mL
Calibrator E	20 ng/mL	0.5 mL
Calibrator F	50 ng/mL	0.5 mL
Calibrator G	100 ng/mL	0.5 mL

Storage: Refrigerate at 2-8oC
Stability: Unopened at 2-8oC until expiration date on label.

5. Control
Contents: One bottle containing leptin in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of leptin. Refer to bottle label for expected value and acceptable range.
Volume: 0.5 mL/bottle
Storage: Refrigerate at 2-8oC
Stability: Unopened at 2-8oC until expiration date on label.

6. Wash Buffer Concentrate
Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 ml/bottle
Storage: Refrigerate at 2-8oC

Stability: Unopened at 2-8oC until expiration date on label.
Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of water.

7. Assay Buffer
Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
Volume: 20 ml/bottle
Storage: Refrigerate at 2-8oC
Stability: Unopened at 2-8oC until expiration date on label.

8. TMB Substrate
Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing
buffer.
Volume: 16 ml/bottle
Storage: Refrigerate at 2-8oC
Stability: Unopened at 2-8oC until expiration date on label.

9. Stopping Solution
Contents: One bottle containing 1 M sulfuric acid.
Volume: 6 ml/bottle
Storage: Refrigerate at 2-8oC
Stability: Unopened at 2-8oC until expiration date on label.

ASSAY PROCEDURE
All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.
1. Prepare working solutions of the streptavidin-HRP conjugate and wash buffer.
2. Pipette 20 ul of each calibrator, control and serum sample into correspondingly labeled wells in duplicate.
3. Pipette 80 ul of the monoclonal anti-leptin-biotin conjugate into each well.
4. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.
5. Wash the wells 3 times with of prepared wash buffer (300 ul/well for each wash) and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is highly recommended).
6. Pipette 100 ul of prepared streptavidin-HRP conjugate into each well.
7. Incubate on a plate shaker (approximately 200 rpm) for 30 minutes at room temperature.
8. Wash the wells again in the same manner as step 5.
9. Pipette 100 ul of TMB substrate into each well at timed intervals.
10. Incubate on a plate shaker for 10-15 minutes at room temperature.
11. Pipette 50 ul of stopping solution into each well at the same timed intervals as in step 9.
12. Read the plate on a microwell plate reader at 450nm within 20 minutes after addition of the stopping solution.

CALCULATIONS
1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator
concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. If a sample reads more than 100 ng/mL then dilute it with assay buffer at a dilution of no more than 1:8. The result

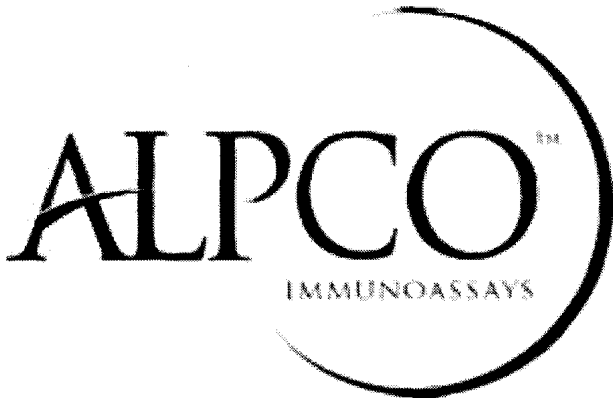
obtained should be multiplied by the dilution factor. For example, if diluting a sample (which is already 1:10
diluted) 1:8 then the obtained results must be multiplied by 8.

TYPICAL TABULATED DATA

Calibrator	OD 1	OD 2	Mean OD	Value (ng/ml)
A		0.073	0.070 0.072	0
B		0.102	0.100 0.101	1
C		0.290	0.293 0.292	5
D		0.620	0.630 0.625	10
E		1.140	1.086 1.113	20
F		1.947	1.919 1.933	50
G		2.518	2.514 2.516	100
Unknown		0.275	0.273 0.274	4.22

SENSITIVITY

The limit of detection (LoD) for Leptin is 0.50 ng/ml, as determined by use of a NCCLS protocol and with proportions of false positives (α) less than 5% and false negatives (β) less than 5%; based on 82 blank determinations; LoB=0.42 ng/ml.



Insulin (Rat) EIA

For the quantitative measurement of insulin in rat serum and plasma.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number:	80-INSRT-E01
Size:	1 x 96 Wells
Catalog Number:	80-INSRT-E10
Size:	10 x 96 Wells
Version:	v1.2: March 24, 2008

ALPCO Diagnostics

26G Keewaydin Drive • Salem, NH 03079
Phone: (800) 592-5726 • Fax: (603) 898-6854
www.alpco.com • Email: web@alpco.com

INTENDED USE

The ALPCO Insulin (Rat) EIA is designed for the quantitative determination of insulin in rat serum and plasma.

PRINCIPLE OF THE ASSAY

The ALPCO Insulin (Rat) EIA is a sandwich type immunoassay. Monoclonal antibodies specific for insulin are immoblized to the 96-well microplate as the solid phase. Standards, controls, and samples are added to the appropriate wells with a horseradish peroxidase enzyme labeled monoclonal antibody (Conjugate), resulting in insulin molecules being sandwiched between the solid phase and the Conjugate. After incubation on a microplate shaker at room temperature, the microplate wells are washed with Wash Buffer to remove unbound Conjugate. TMB Substrate is added to each well, and the microplate is again incubated on a microplate shaker at room temperature. During the second incubation, a blue color results form the TMB Substrate reacting with bound Conjugate in the wells. Stop Solution is added and this stops the reaction and changes the color from blue to yellow. The optical density (OD) is measured by microplate reader at 450nm with a reference wavelength of 620nm. The intensity of the color generated is directly proportional to the amount of insulin in the sample.

MATERIALS SUPPLIED

Single Plate Kit (80-INSRT-E01)

Components	Content	Quantity	Preparation
Insulin Microplate	1 microplate	12 x 8 well strips	Ready to use
Zero Standard (0 ng/ml)	1 vial	5 ml	Ready to use
Insulin Standards (A → E) (0.15, 0.4, 1, 3, 5.5 ng/ml)	5 vials	1 ml/vial	Ready to use
Mammalian Insulin	2 vials	0.6 ml/vial	Lyophilized
Conjugate (11X HRP Labeled anti-insulin antibody)	1 vial	0.9 ml	Concentrate
Conjugate Buffer	1 bottle	9 ml	Ready to use
Wash Buffer (21X)	1 bottle	40 ml	Concentrate
TMB Substrate	1 bottle	12 ml	Ready to use
Stop Solution	1 bottle	12 ml	Ready to use
Plate Sealers	3	-	Ready to use

MATERIALS REQUIRED BUT NOT SUPPLIED

- Precision pipettes with disposable tips capable of dispensing 10 µl, 75 µl, and 100 µl
- Repeating or multi-channel pipette capable of dispensing 75 µl and 100 µl
- Volumetric container and pipettes for reagent preparation
- Distilled (deionized) water
- Microplate washer or wash bottle
- Horizontal microplate shaker capable of 700-900 rpm
- Microplate reader with 450 and 620-650nm filter

PRECAUTIONS

1. The human blood products incorporated into this kit have been tested for the presence of HIV (Human Immunodeficiency virus), HBV (Hepatitis B virus), and HCV (Human Hepatitis C virus). Test methods for these viruses do not guarantee the absence of virus; therefore all reagents should be treated as potentially infectious. Handling and disposal should be in accordance with all appropriate national and local regulations for the handling of potentially biohazardous materials.
2. All materials derived from animal sources are BSE negative. However, all materials should be kept from ruminating animals.
3. Avoid direct contact with skin.
4. This product is not for internal use.
5. Avoid eating, drinking, or smoking when using this product.
6. Do not pipette any components by mouth.
7. Components from this kit should not be mixed with components of different lot numbers.
8. Do not use components beyond the expiration date.
9. Variations to the test procedure are not recommended and may influence the test results.

SPECIMEN HANDLING

Serum and plasma (EDTA) specimens are appropriate for use in this assay. No dilution or treatment of the sample is required. If a sample is > 5.5 ng/ml, the sample should be diluted in Zero Standard and the analysis should be repeated. Specimens can be stored at 2-8°C for 24 hours prior to analysis in this assay. For longer periods, storage at < -20°C is recommended. Avoid repeated freezing/thawing of the sample. Grossly lipemic, icteric, or hemolyzed samples do not interfere in the assay.

REAGENT PREPARATION AND STORAGE CONDITIONS

The kit should be stored at 2-8°C. The kit is stable until the expiration date on the box label. All reagents must reach room temperature prior to preparation and subsequent use in the assay. ***Conjugate (11X)*** is diluted with 10 parts Conjugate Buffer. For example, to prepare enough Working Strength Conjugate for one complete microplate, dilute 0.9 ml of Conjugate (11X) with 9 ml of Conjugate Buffer. Working Strength Conjugate is stable for 4 weeks at 2-8°C.

Mammalian Insulin Controls (Low and High) are provided in a lyophilized form. Reconstitute each control with 0.6 ml of distilled water. Close the vial with the rubber stopper and cap, then gently swirl the vial and allow it to stand for 30 minutes prior to use. The contents should be in solution with no visible particulates. The reconstituted controls are stable for 7 days stored at 2- 8°C. If desired, the controls can be aliquoted and stored at < -20°C for up to 6 months. The controls should not be repeatedly frozen and thawed. ***Wash Buffer (21X)*** is diluted with 20 parts distilled water. For example, to prepare Working Strength Wash Buffer, dilute 20 ml of Wash Buffer (21X) with 400 ml of distilled water. Working Strength Wash Buffer is stable for 4 weeks at room

temperature (18-25°C).

QUALITY CONTROL

It is recommended that the Mammalian Insulin High and Low Controls provided with the ALPCO Insulin (Rat) EIA be included in every assay. The concentration ranges of the controls are provided on the Certificate of Analysis enclosed with each kit; however, it is recommended that each laboratory establishes its own acceptable ranges.

ASSAY PROCEDURE

Bring all reagents and microplate strips to room temperature prior to use. Gently mix all reagents before use. A standard curve must be included with each assay and with each microplate if more than one is run at a time. All standards, controls, and samples should be run in duplicate.

1. Ensure that microplates are at room temperature prior to opening foil pouch. Designate enough microplate strips for the standards, controls, and desired number of samples. The remaining microplate strips should be stored at 2-8°C in the tightly sealed foil pouch containing the desiccant.
2. **Pipette 10 µl** of each standard, reconstituted control (see *Reagent Preparation)* or sample into its respective wells.
3. **Pipette 75 µl** of Working Strength Conjugate (see *Reagent Preparation)* into each well.
4. **Incubate for 2 hours,** shaking at 700-900 rpm on a horizontal microplate shaker at room temperature (18-25°C).
5. **Wash the microplate 6 times** with Working Strength Wash Buffer (see *Reagent Preparation)* with a microplate washer. Alternatively, use a wash bottle to fill the wells, and then discard the liquid, inverting and firmly tapping the microplate on absorbent paper towels between washes. After final wash with either the microplate washer or wash bottle, remove any residual Wash Buffer and bubbles from the wells by inverting and firmly tapping the microplate on absorbent paper towels (See *Microplate Locking Diagram* below).
6. **Pipette 100 µl** of TMB Substrate into each well.
7. **Incubate for 15 minutes** at room temperature (18-25°C) on a horizontal microplate shaker (700-900 rpm).
8. **Pipette 100 µl** of Stop Solution into each well. Gently shake the microplate to stop the reaction. Remove bubbles before reading with microplate reader.
9. Place the microplate in a microplate reader capable of reading the absorbance at 450nm with a reference wavelength of 620-650nm. The microplate should be analyzed within 30 minutes following the addition of Stop Solution.

CALCULATION OF RESULTS

A calibration curve is constructed from the Insulin Standards. It is preferable to use a software program to calculate the standard curve and to determine the concentration of the samples. The Zero Standard should be used as a blank with its average value subtracted from each well. The preferred calculation method is cubic spline. Plot the

standard curve using a log/log scale. **Manual Calculation:** The Zero Standard should be used as a blank with its average value subtracted from each well. The standard concentrations are plotted on the X-axis and the absorbance values are plotted on the Y-axis using log/log paper. The sample concentrations are determined by plotting the absorbance of each unknown sample against the standard curve. The corresponding value on the X-axis is the concentration of the unknown sample.

PERFORMANCE CHARACTERISTICS

Sensitivity:

The analytical sensitivity was determined by calculating the mean ±2 standard deviations for 20 replicates of the Zero Standard. The sensitivity of the assay is 0.124 ng/ml.

Precision: Within run (intra-assay) variation

The within run precision is expressed as the percentage coefficient of variation (%CV). This was determined based on the mean and standard deviation of 20 replicates of a specimen run in a single assay. The table below shows the results of 3 samples that span the range of the assay.

	Sample 1	**Sample 2**	**Sample 3**
mean	0.491 ng/ml	2.366 ng/ml	3.840 ng/ml
std. dev.	0.028 ng/ml	0.128 ng/ml	0.152 ng/ml
%CV	5.79%	5.43%	3.95%
n=	20	20	20

Precision: Between run (inter-assay) variation

The between run precision is expressed as the percentage coefficient of variation (%CV). This was determined based on the mean and standard deviation across 10 assays of duplicate measurements of a single specimen. The table below shows the results of 3 samples that span the range of the assay.

	Sample 1	**Sample 2**	**Sample 3**
mean	0.537 ng/ml	2.401 ng/ml	3.775 ng/ml
std. dev.	0.053 ng/ml	0.175 ng/ml	0.245 ng/ml
%CV	9.95%	7.29%	6.48%
n=	10	10	10

Linearity:

The linearity of the assay was determined by preparing dilutions of a sample with a high insulin concentration with the Zero Standard. The expected values were compared to the obtained values to determine a percent recovery. The range of recovery was 98-120 %.

Spike and Recovery:

The spike and recovery for the assay was determined by adding various known amounts

of insulin to a sample. This spiked sample was evaluated in the assay and the measured concentration was compared to the expected concentration (endogenous + spiked). The range of recovery was 89-113 % with an average of 100%.

Specificity:
The table below indicates the analyte and the percent cross reactivity observed in the assay.

Analyte	**% Cross Reactivity**
Human C-Peptide	<0.01
Human IGF 1	<0.01
Human IGF 2	<0.01
Human Intact Proinsulin	0.18
Lispro	100
Mouse C-Peptide 1	<0.01
Mouse IGF 1	<0.01
Mouse IGF 2	<0.01
Mouse Insulin 1β	<0.01
Mouse Insulin 1α	<0.01
Rat C-Peptide 1	<0.01
Rat C-Peptide 2	<0.01
Rat Insulin 1 Synthetic	100
Rat Insulin 1α	<0.01
Rat Insulin 1β	<0.01

Hook Effect:
No high dose hook effect was observed with insulin concentrations up to 1536 ng/ml.

APPENDIX 2: ANTIBODY CONTROLS

Non-specific binding of the primary and secondary antibodies was carried out by omitting incubation with a primary antibody (POMC, pSTAT3, STAT3 for biotinylated goat anti-rabbit secondary antibody and AgRP for biotinylated goat anti-guinea pig secondary antibody) using the immunohistochemistry protocol described in Ch 2 (Fig A1). Secondary antibodies were added to the tissues at a concentration of 1:500.

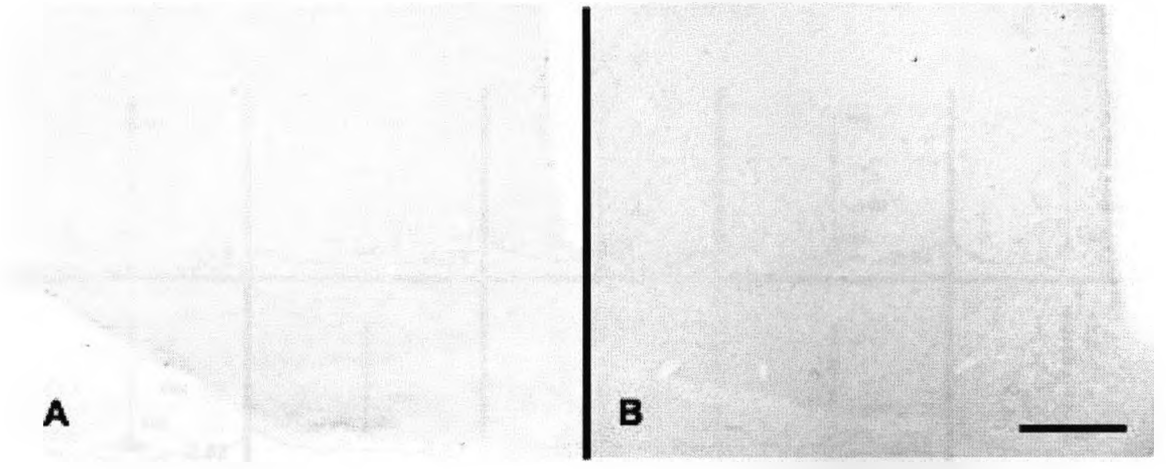


Fig A1 Controls for secondary antibodies. Incubation with biotinylated goat anti-rabbit antibody (1:500) (A) and biotinylated goat anti-guinea pig antibody (1:500) (B) in the absence of primary antibody incubation. Scale bar = 225 μm

APPENDIX 3: ROSTRAL-CAUDAL CORONAL SECTIONS

Appropriate rostro-caudal planes of the hypothalamus were identified using the Rat Brain Atlas (Paxinos and Watson; 5th edition). The following sections in relation to bregma were identified in each animal and photographed; -1.44, -1.72, -1.80, -2.04, -2.28, -2.52, -2.76, -2.92, -3.12 and -3.36. The PVN, VMH and ARC were identified using this atlas to count cells in those specific areas.

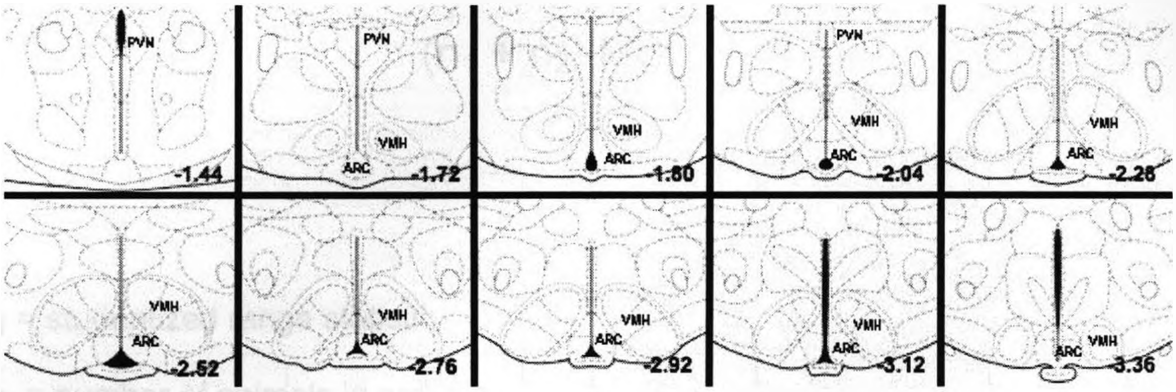


Fig A2 Rostral to caudal coronal sections of the rat hypothalamus extracted from the Rat Brain Atlas (Paxinos and Watson; 5th edition). These planes, in relation to bregma were identified in each animal and photomicrographed. Abbreviations: paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), arcuate nucleus (ARC).

APPENDIX 4: TUKEY EQUATION

Following a repeated measures ANOVA, the Tukey test was computed manually to determine significance at specific time points. Significance was determined using a critical values table for 'q'. If computed values were greater than the critical value at the corresponding degrees of freedom, then it was considered significant.

$$q = \frac{n_1 n_2 (y_1 - y_2)^2}{(n_1 + n_2)\, MS}$$

q = studentized range statistic

n_1 = number of animals in group 1

n_2 = number of animals in group 2

y_1 = group 1 mean

y_2 = group 2 mean

MS = mean square