THE INTERACTIVE AND INDIVIDUAL EFFECTS OF OREXIN A AND NEUROTENSIN IN THE BRAIN ON SPONTANEOUS PHYSICAL ACTIVITY

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Abstract

Within the brain, there are key feeding and locomotion circuits that feature the signaling of numerous peptides. Orexin A has been shown to have effects on feeding and physical activity behaviors when administered centrally or in several brain regions. This effect is site-specific and requires the presence of orexin receptors. Our lab has studied the behavioral effects of injecting orexin A into the dorsal rostral portion of the lateral hypothalamus. We have previously observed an acute increase in spontaneous physical activity as well as food intake following injection of orexin A into this area. The acute effect is also observed with bicuculline-mediated stimulation of neurons in the rostrolateral hypothalamus. It is likely that there are other peptide signaling systems which are downstream of orexin receptor activation in the rostrolateral hypothalamus that are responsible for the observed increase in spontaneous physical activity.

In chapter one, we chose to study neurotensin signaling in the ventral tegmental area as a possible downstream event of orexin receptor activation in the rostrolateral hypothalamus. Firstly we performed immunohistochemical studies which confirmed the colocalization of orexin-receptor and neurotensin-expressing neurons in the rostrolateral hypothalamus. Secondly, we coinjected orexin A in the rostrolateral hypothalamus and a neurotensin receptor antagonist in the ventral tegmental area. In this case we observed an attenuation of orexin A responsivity in the time spent ambulating in the first hour post-injection in the presence of the antagonist. Thirdly we administered a dose range of both neurotensin and the neurotensin antagonist in the ventral tegmental area. High doses of the antagonist and an intermediate dose of neurotensin both increased spontaneous physical activity within two hours post-injection. The equivalent directionality of the effects of neurotensin and the antagonist are hypothesized to occur through several potential mechanisms including partial agonist-like qualities of the antagonist and a differential activation of dopamine receptors in the nucleus accumbens. Finally after coinjecting orexin A and neurotensin in the rostrolateral hypothalamus, we found that neurotensin antagonizes the effect of orexin A on spontaneous physical activity in the rostrolateral hypothalamus. We confirm here an involvement of neurotensin signaling in the orexin A-mediated response. Further studies are needed to elucidate the dynamics of the pathways within the rostrolateral hypothalamus and the ventral tegmental area as well as to determine if other peptide signaling systems and brain sites are involved in orexin signaling.

In chapter two we sought to determine which other brain sites may respond to orexin A. We chose two sites, the ventral tegmental area and the paraventricular nucleus of the thalamus, which contain orexin receptors. To determine if an increase in spontaneous physical activity was observed with orexin A injection into these sites, we performed a dose response experiment in both sites. We found that orexin A increased the time spent moving when injected into the ventral tegmental area at a high dose, whereas orexin A had

no effect on spontaneous physical activity at any dose when injected into the paraventricular nucleus of the thalamus. We conclude that the presence of orexin receptors in a brain site does not ascertain physical activity responsiveness to orexin A, but that the effect is site-specific as we had hypothesized.

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rLH: rostrolateral hypothalamus

VTA: ventral tegmental area

PVT: paraventricular nucleus of the thalamus

SPA: spontaneous physical activity

*: asterisks symbolizes a statistically significant difference from control treatment ($p<.05$)

Chapter 1.

THE INTERACTIVE AND INDIVIDUAL EFFECTS OF OREXIN A AND NEUROTENSIN IN THE BRAIN ON SPONTANEOUS PHYSICAL ACTIVITY

I. Introduction

A. The Role of Spontaneous Physical Activity in Energy Balance

The energy balance of human and animal populations has been studied in great detail with regard to the involved behaviors: food intake and energy expenditure. Energy expenditure may be further broken down into basal metabolic rate, intentional physical activity, spontaneous physical activity, digestive thermogenesis, and thermoregulation (Popkin, Duffey & Gordon-Larsen 2005).

Spontaneous physical activity refers to motor behaviors outside of physical activity performed with the intention of weight loss and the achievement of other health or fitness-related benefits. In human populations, this spontaneous physical activity is manifested in motions such as fidgeting, walking, and standing posture (Levine et al. 2005). In adult humans spontaneous physical activity, also known as non-exercise activity thermogenesis (NEAT), has been shown to account for the variation in weight gain under conditions of excess calorie intake (Levine, Eberhardt & Jensen 1999). Individuals with a higher NEAT showed a greater resistance to weight

gain under overeating conditions than individuals with a lower NEAT (Levine, Eberhardt & Jensen 1999). Similarly in human populations spontaneous physical activity has been shown to play a role in the pathogenesis of obesity and is characterized as a familial trait (Zurlo et al. 1992, Joosen et al. 2005). In adult men spontaneous physical activity predicts lower body weight gain (Webb, Annis & Troutman 1980, Teske, Billington & Kotz 2008). Humans have been observed to burn approximately 350 kcalories daily in respiratory chambers due to spontaneous physical activity (Ravussin et al. 1986). In Amerindian Puruvian female adults, a lower SPA was associated with a higher body fat mass (Lindgärde et al. 2004). Thus spontaneous physical activity plays a large role in the development or prevention of obesity.

B. Hypothalamic Peptide Signaling and Energy Balance

The state of a positive energy balance is especially observed in human populations in the United States. An increasing availability of high-energy foods in addition to a decline in physical activity most likely contribute to this state (CASPERSEN, PEREIRA & CURRAN 2000), (Flegal et al. 1998). The feeding and physical activity control centers in the brain have shown to be important in the maintenance of energy balance through effects on spontaneous physical activity in organisms. These control centers exist in the hypothalamus and other brain regions and directly affect feeding and physical activity behaviors through the signaling of various peptides (Sahu 2003).

There are several peptides which signal in nuclei within the hypothalamus. These peptides have well-characterized receptors and down-stream cellular events as well as behavioral outcomes with regard to feeding and physical activity (Harvey, Ashford 2003). These peptides may be classified as anabolic or catabolic. Thus pathways exist in the hypothalamus which promote either an increase in anabolic characteristics such as food intake behaviors and a reduction in energy expenditure or an increase in catabolic characteristics which include a reduced appetite and an increase in energy expenditure and physical activity.

Orexin A is an important mediator of behaviors involved in energy balance within the hypothalamic pathways. Orexin functions to increase food intake behaviors (Edwards et al. 1999). Surprisingly orexin A also increases spontaneous physical activity and energy expenditure (Chen et al. 2002, Kotz 2006).

C. Characteristics of Orexin A, Orexin Receptors, and Anatomy

Orexin A is a 3.5 Kilodalton 33 amino-acid peptide derived from prepro-orexin, the 130 amino-acid precursor for orexin A and orexin B. Orexin A binds with high affinity to both the orexin receptor 1 and 2 leading to specific intracellular events (Uramura et al., 2001). Orexin receptors 1 and 2 are G protein-coupled receptors (Sakurai et al. 1998). The orexin receptor 1 and in some cases receptor 2 are associated with the q G-protein subunit and upon activation results in the opening of nonselective cation channels (Sakurai et al.

1998). Ultimately these events lead to neuronal activation or depolarization of orexin receptor-expressing neurons. The orexin receptor 2 may also be associated with either the i or o G-protein subunit and upon activation causes the opening of inward-rectifying potassium channels resulting in an inhibition or hyperpolarization of these neurons (Sakurai et al. 1998). The widespread distribution of the orexin receptor subtypes (Marcus et al. 2001) results in site-specific coupling with each of the G-protein subunits.

Orexin A has been shown to activate GABAergic and glutamatergic neurons in the hypothalamus (Li et al. 2002, Burdakov, Liss & Ashcroft 2003). The sodium-calcium exchanger has been proposed to be involved in the excitatory response observed in various brain regions (Burdakov, Liss & Ashcroft 2003). Additionally orexin neurons express glutamate vesicular transporters VGLUT 1 and 2 (Rosin et al. 2003). Orexin A also activates dopaminergic neurons in the ventral tegmental area resulting in excitation (Korotkova et al. 2003).

Orexin A is expressed in the perifornical nucleus, the lateral and posterior regions of the hypothalamus, as well as subthalamic areas: zona incerta, subincertal, and subthalamic nuclei (Sakurai et al. 1998). The orexin-expressing neurons are multipolar with no distinctive morphology (Sakurai et al. 1998). Both orexin receptors 1 and 2 are expressed throughout the hypothalamus, cortex, and hippocampus (Marcus et al. 2001). Within the hypothalamus both receptors are expressed in the paraventricular nucleus,

tuberomammilary nucleus, lateral hypothalamus, ventromedial nucleus, and dorsal raphe nucleus (Marcus et al. 2001).

Neurons which express orexin A project through the lateral hypothalamus and fibers labeled for orexin A are observed in the paraventricular nucleus of the hypothalamus, arcuate nucleus, lateral hypothalamus, perifornical area, and ventromedial nucleus (Peyron et al. 1998).

D. Behavioral Effects of Orexin A

The effect of orexin A on food intake is a profound factor with regard to the regulation of appetite (Rodgers et al. 2002). Injection of orexin A into the lateral cerebral ventricle results in an acute increase in feeding behaviors such as burrowing, searching, and face washing (Ida et al. 1999). Similarly an increase in two-hour food intake that occurs with lateral cerebral ventricular injection of orexin A is associated with an increase in c-Fos, a marker of neuronal activation, in the paraventricular and arcuate nucleus of the hypothalamus (Edwards et al. 1999). Orexin A injection into the perifornical nucleus and lateral hypothalamus results in an increase in feeding within two hours post-injection (Sweet et al. 1999). However chronic ventricular injections of orexin A do not result in an increase in body weight as would be expected by assessing the appetite-promoting effects of orexin alone (Yamanaka, Sakurai & Katsumoto 1999).

Interestingly, genetic ablation of orexin neurons results in narcolepsy, hypophagia, and obesity (Hara et al. 2001). This effect is due to the

spontaneous physical activity-increasing effect of orexin A. The physical activity-elevating effect of orexin A is likely more of a contributor to the resulting negative energy balance than the appetite-increasing effect. Ambulatory or horizontal movement as well as vertical or rearing motions are components of spontaneous physical activity in rodents. Orexin A injection into the paraventricular nucleus of the hypothalamus results in an increase in spontaneous physical activity as well as energy expenditure for two hours post-injection (Kiwaki et al. 2004). Similarly orexin A injection into the lateral hypothalamus increases spontaneous physical activity and wheel running for two hours post-injection (Kotz et al. 2002). Orexin A shows the same effect on physical activity when injected into the nucleus accumbens (Thorpe, Kotz 2005). The dopaminergic system has been shown to be involved in the spontaneous physical activity-promoting effect of orexin A. Dopamine receptor 1 and 2 antagonist given intraperitoneally abolished the increase in locomotion that is observed with Orexin A injection into the ventricle (Nakamura et al. 2000). This project focuses on the physical activity behavioral output of orexin A injection into the lateral hypothalamus.

E. The Role of Orexin A in the Rostrolateral Hypothalamus

The rostral dorsal region of the lateral hypothalamus (rLH) has been shown to feature both the food intake and spontaneous physical activity (SPA)-promoting effect of orexin A. The lateral hypothalamus features distinct neuron populations and likely activates other key feeding and locomotion pathways in

the brain (Luiten, Ter Horst & Steffens 1987). Our lab has observed well-established behavioral effects on feeding and SPA as a result of orexin A signaling in the rLH. We have shown that orexin receptors 1 and 2 are present in the rLH and likely mediate these effects. This project sought to determine which other peptides or pathways may be involved downstream of orexin receptor activation in the rLH and the consequent behavioral effects.

F. Characteristics of Neurotensin, Neurotensin Receptors, and Anatomy

Neurotensin is a 1.67 Kilodalton, 13 amino acid peptide first isolated from the bovine hypothalamus (Carraway, Leeman 1975). Neurotensin was first studied as a vasodilator that acted on cutaneous tissue in live rats (Carraway, Leeman 1975). The precursor for neurotensin is a 170 amino acid protein which gives rise to both neurotensin and neuromedin N, a related peptide expressed throughout the brain (Kislauskis et al. 1988).

Neurotensin cell bodies and fibers are found in abundance in the cortex, accumbens, caudate-putamen, septum, striatum, thalamus, hypothalamus, nucleus of the solitary tract, anterior pituitary, ventral tegmental area, dorsal raphe, locus coeruleus, and other regions (Jennes, Stumpf & Kalivas 1982). These locations feature circuits involving the autonomic-endocrine system as well as the somatosensory function of neurotensin (Jennes, Stumpf & Kalivas 1982).

Neurotensin acts on neurotensin receptors 1,2,3, and 4 found in high concentration in the Islands of Calleja, diagonal bands of Broca, magnocellular

preoptic nucleus, parasubiculum, ventral tegmental area, suprachiasmatic nucleus, anterodorsal nucleus of the thalamus, pontine nuclei, and vagus (Boudin et al. 1996). Immunohistochemical analysis shows the presence of neurotensin receptors on either or both of the dendrites and axons in combination with cell bodies of neurons throughout areas of high expression (Boudin et al. 1996). Neurotensin receptors 1 and 2 are implicated in many of the physiological and metabolic behaviors downstream of neurotensin signaling while neurotensin receptors 3 and 4 are less well characterized and are expressed within the cell (Kinkead & Nemeroff, 2006).

Neurotensin receptors 1 and 2 are G protein-coupled receptors that result in the selective activation of G protein subunits and intracellular second messengers. Activation of neurotensin receptor 1 results in the activation of phospholipase C, IP3, cyclic nucleotides and the Sodium-Potassium-ATPase (Hermans, Maloteaux & Octave 1992, Kinkead, Nemeroff 2006). Activation of neurotensin receptor 2 results in IP3 formation and the mobilization of Calcium (Turner, James-Kracke & Camden 1990). Neurotensin receptor activation differentially results in GABAergic or Glutamatergic responses in efferent neurons in the striatum, pallidum, and nigroventral thalamus (Ferraro et al. 2001, Ferraro et al. 1998). This may account for the contrasting effects of neurotensin application to various brain nuclei.

G. Behavioral Effects of Neurotensin

Initial studies on the behavioral effects of neurotensin assessed its role as an endogenous antipsychotic (Kinkead, Nemeroff 2006). Neurotensin given centrally results in antipsychotic actions (Binder et al. 2001). Other physiological roles of neurotensin include the promotion of hypothermia and analgesia. Intracisternal administration of neurotensin results in hypothermia, an intolerance to cold, hypoinsulinemia, hyperglycaemia, and hyperglucagonaemia (Bissette et al. 1976). Additionally exogenous administration of neurotensin results in the release of prolactin and luteinizing hormone (Kinkead, Nemeroff 2006, Watanobe, Takebe 1993).

Neurotensin also affects metabolism through its effects on locomotion and feeding. Neurotensin receptor antagonist studies show that neurotensin signaling is involved in the satiety response to leptin. Intracerebroventricular injection of a neurotensin antagonist blocked the inhibition of food intake evoked by central administration of leptin (Sahu, Carraway & Wang 2001). Similarly central administration of neurotensin decreases 24-hour food intake in a dose-dependent manner in rats (Luttinger et al., 1982)

Intracerebroventricular administration of neurotensin decreases locomotion at a low dose (30 ng) and increases locomotion at a higher dose (75 ng), which also resulted in dopamine efflux in the nucleus accumbens (Nouel, Costentin 1994). Injection of neurotensin into the ventral tegmental area results in an increase in locomotion as assessed by electromagnetic activity counts (Kalivas et al. 1983). However injection of neurotensin into the nucleus

accumbens decreases locomotion and hyperactivity induced by dopamine signaling in the nucleus accumbens (Kinkead, Nemeroff 2006, Kalivas, Nemeroff & Prange 1984).

The mixed effects of neurotensin on locomotion may be due to the presence of dopamine receptors 1,2, and 3 in the nucleus accumbens. For example distinct populations of neurons in the nucleus accumbens express either D2 or D3 receptors. Activation of the D2 receptor decreases mRNA expression of neurotensin while activation of D3 increases mRNA expression of neurotensin (Diaz et al. 1994b). Similarly another study found that stimulation of the D1 and D2 receptors increase and decrease the levels of neurotensin immunoreactivity respectively in the nucleus accumbens (Merchant, Gibb & Hanson 1989). Since neurotensin decreases locomotion when injected into the nucleus accumbens, we would expect that selective activation of dopamine receptors modulates locomotion behaviors elicited by neurotensin.

Broadly speaking, neurotensin has been shown to modulate the dopaminergic pathways within the brain (Nemeroff, Cain 1985). Neurotensin regulates plasticity through its mixed effects on D1 and D2 receptors in the neostriatum (Fuxe et al. 1992). Dopamine has been shown to regulate the affinity and number of neurotensin binding sites in the limbic forebrain in rats (Agnati et al. 1985). These neurotensin-dopamine interactions are the likely mechanism for the effects of neurotensin on psychosis, analgesia, hypothermia, and locomotion (Nemeroff, Cain 1985). Thus neurotensin may have pronounced effects on energy balance through its activity within the brain.

H. The Unknown Downstream events of Orexin Receptor Activation in the Rostrolateral Hypothalamus

The main question addressed in this project regards the determination of the downstream events following orexin A administration in the rLH which occur leading up to and resulting in our observed behavioral outcome: an increase in spontaneous physical activity (SPA). Our lab has shown that following orexin A injection into the rLH, an increase in c-Fos, a marker of neuronal activation, is observed in the rLH. It is assumed that these c-Fos immunoreactive neurons contain orexin receptors; the binding of orexin A to its receptors results in the depolarization of orexin receptor-expressing neurons. However it is unknown where these orexin receptor-expressing neurons project. It is likely that other brain regions which receive projections from these neurons are involved in the behavioral responses. Additionally the orexin receptor-expressing neurons in rLH may release other peptides from the axon terminals onto other neurons in these unknown involved brain regions. Thus it was of interest to determine a candidate for a peptide and signaling system that shows evidence for being involved in orexin A signaling in the rLH.

I. Evidence that Neurotensin Signaling is Downstream of Orexin A Signaling in the Rostrolateral Hypothalamus

The activity promoting effects of neurotensin in the ventral tegmental area led us to consider this peptide as a potential mediator of the effects of orexin A in the rLH on SPA. One key study by Reynolds *et al* (2006) led us to

choose neurotensin for our peptide of study in this series of experiments (Reynolds et al. 2006).

Reynolds discovered a population of neurons in the rLH which express neurotensin and also project to the ventral tegmental area (Geisler, Zahm 2005). In a study looking at the role of neurotensin signaling in locomotion elicited by these neurotensin expressing neurons, these neurons were activated by bicuculline. Bicuculline is an antagonist of inhibitory GABAergic neurons. Thus an injection of bicuculline causes a disinhibition of the surrounding neurons. Reynolds *et al* stimulated neurons in the rLH by injecting bicuculline and observed an acute increase in spontaneous physical activity. It remains unknown which cells were activated in the rLH by bicuculline. To determine the role of neurotensin signaling in the bicuculline-induced locomotion, a neurotensin antagonist, SR 142948, selective for neurotensin receptors 1 and 2, was administered to the ventral tegmental area just prior to bicuculline injection. In the presence of SR 142948, the effect of bicuculline on locomotion was abolished. This effect persisted but was less marked with intraperitoneal administration of SR 142948 prior to bicuculline injection. Thus Reynolds *et al* concluded that a pathway from neurotensin-expressing neurons in the rLH to the ventral tegmental area was responsible for the increase in locomotion.

The acute effects of both our observed orexin A injections into the rLH and the Reynolds study revealed that the neurotensin-expressing neurons in the rLH may also contain orexin receptors. Orexin receptor activation may lead to downstream events shared with the neurotensin pathway observed by

Reynolds *et al*. We hypothesized that some orexin receptor-expressing neurons in the rLH overlap with neurotensin-expressing neurons. It seemed plausible that orexin activation of orexin receptors leads to intracellular G-protein signaling cascade(s) which ultimately result in the release of neurotensin at the terminals of these neurons. Based on the Reynolds study, we proposed that these projections to the ventral tegmental area allow for neurotensin signaling within the ventral tegmental area.

Evidence supports that the locomotion-increasing effect of neurotensin in the ventral tegmental area requires the mesolimbic dopaminergic pathway from the neurotensin receptor-expressing neurons which project to the nucleus accumbens and release dopamine (Kinkead, Nemeroff 2006). Dopamine acts on excitatory neurons in the nucleus accumbens leading ultimately to motor output (Kinkead, Nemeroff 2006).

More evidence for the partnership of orexin and neurotensin signaling systems in locomotor behavior is observed in hypothalamic explants. In vitro application of orexin A to hypothalamic explants results in a release of neurotensin (Russell et al., 2000). The mechanism for this neurotensin release likely involved the activation of orexin receptors present in neurons from these explants. Intracellular second messenger pathways downstream to orexin receptor activation likely led to the release of neurotensin. Thus we found evidence that orexin receptor activation in the rLH may lead to the release of neurotensin in the ventral tegmental area.

II. Hypothesis

We hypothesized that activation of orexin receptors will result in the release of neurotensin from neuron terminals in the ventral tegmental area. Neurotensin will activate neurotensin receptors in the ventral tegmental area leading to the observed increase in spontaneous physical activity (Figure 1-1)

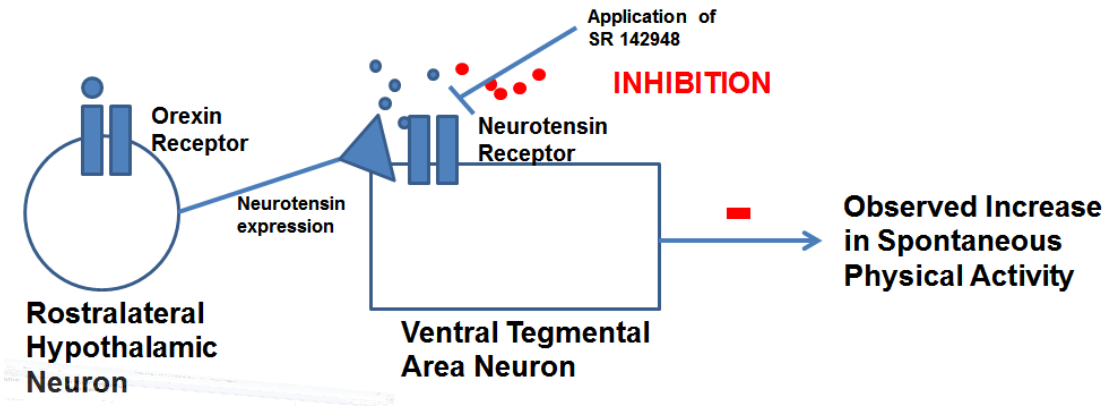

Figure 1-1: Our hypothesis is that neurotensin signaling in the VTA is downstream of orexin A signaling in the rLH.

Our approach to test our hypothesis included a series of immunohistochemical and behavioral studies outlined below:

1. If orexin A activates orexin receptors present in the rLH and these same neurons release neurotensin upon orexin receptor activation and downstream intracellular events, we would expect neurons to be present in the rLH which express both orexin receptors and neurotensin. To determine if this is the case,

we performed immuohistochemistry on brain sections to determine the presence of overlap of cells expressing orexin receptors and neurotensin.

2. If neurotensin activation of neurotensin receptors in the VTA is downstream to the events which take place in the rLH following the administration of orexin A, we would expect that blocking neurotensin signaling in the VTA will reduce or abolish the observed spontaneous physical activity-promoting response to orexin.

3. Based on the outcome of the second point, we used a series of behavioral studies to further assess the relationship between orexin A and neurotensin signaling in the brain and the effects on activity. These studies included:

- Dose response of the neurotensin antagonist in the VTA
- Dose response of neurotensin in the VTA
- Dose response of neurotensin in the rLH
- Coadministration of neurotensin and orexin A in the rLH

III. Methods

A. Immunohistochemistry

i. Tissue

Male Sprague Dawley rats were sacrificed by an overdose of Sodium Pentobarbital (100 mg/kg given intraperitoneally). Transcardial perfusion was performed using 500 ml of .9% sodium chloride and 500 ml of 4% paraformaldehyde. Brains were dissected and stored for 4-6 hours in 4%

paraformaldehyde for post-fixation. Brains were transferred to a 20% sucrose solution for 48-72 hrs and stored in cryoprotectant until sectioning. The brains were sectioned using a sliding microtome into 40um slices and stored in cryoprotectant until immunohistochemical processing.

ii. Orexin Receptor Immunohistochemistry

Sections were rinsed 10 minutes X 6 in a 1 M phosphate buffer solution (PBS). All rinsing steps and incubation steps were performed with gentle agitation and rotation respectively. Unless specified, all steps were performed at room temperature. A blocking incubation step included using 5% normal horse serum, .3% Triton-X PBS. Sections were rinsed in PBS for 10 minutes and incubated for 48 hrs in a Millipore rabbit anti-orexin receptor 1 or 2 (1:200), 3% normal horse serum, .3% Triton-X PBS at 4 degrees Celsius. Sections were rinsed for 10 minutes X 3 in PBS and incubated with horse anti-rabbit biotinylated secondary antibody (Vector Labs, Burlingame, CA) 3% normal horse serum .3% Triton-X PBS for 1-2 hours. Sections were rinsed with PBS for 10 minutes X 3. Sections were incubated with an avidin-biotin complex (Vector Labs, Burlingame, CA) .3% Triton-X PBS and rinsed thereafter for 10 minutes X 3. Sections underwent the 3'3'-diaminobenzidine (DAB) reaction with Nickel (2.5%) enhancement for development of a dark purple-black stain.

iii. Neurotensin Receptor Immunohistochemistry

Tissue processed with the orexin receptor 1 or 2 antibody was rinsed for 10 minutes X 6 in PBS. A blocking incubation step included using 5% normal donkey serum and .3% Triton-X PBS. Sections were rinsed in PBS for 10 minutes and incubated for 48 hrs in a Santa Cruz Biotechnology (Santa Cruz, CA) goat anti-neurotensin (1:250), 3% normal donkey serum, .3% Triton-X PBS at 4 degrees Celsius. Sections were rinsed for 10 minutes X 3 in PBS and incubated with donkey anti-goat biotinylated secondary antibody (Vector Labs, Burlingame, CA) 3% normal donkey serum .3% Triton-X PBS for 1-2 hours. Sections were rinsed with PBS for 10 minutes X 3. Sections were incubated with an avidin-biotin complex (Vector Labs, Burlingame, CA) .3% Triton-X PBS and rinsed thereafter for 10 minutes X 3. Sections underwent the 3'3'-diaminobenzidine (DAB) reaction enhancement for development of a dark brown stain.

iv. Tissue Mounting

Sections processed for double-labeling of orexin receptors and neurotensin were transferred to a .1 M phosphate buffer solution and mounted onto gelatin-coated slides using paintbrushes. After slices were allowed to dry overnight, slides were dehydrated in a series of ethanol and xylene washes. Finally the slides were coverslipped using permount and left to dry.

v. Visualization

Immunohistochemical observation and photoshooting were performed with a light fluorescent microscope with a camera and computer attached.

vi. Controls

Negative control sections were developed with the double-staining procedure lacking either or both of the primary antibodies (Figure 1-2). Additionally a region of the brain known to be devoid of orexin receptors and neurotensin, the cingulum, was assessed (Figure 1-3). No inappropriate staining was observed under either condition.

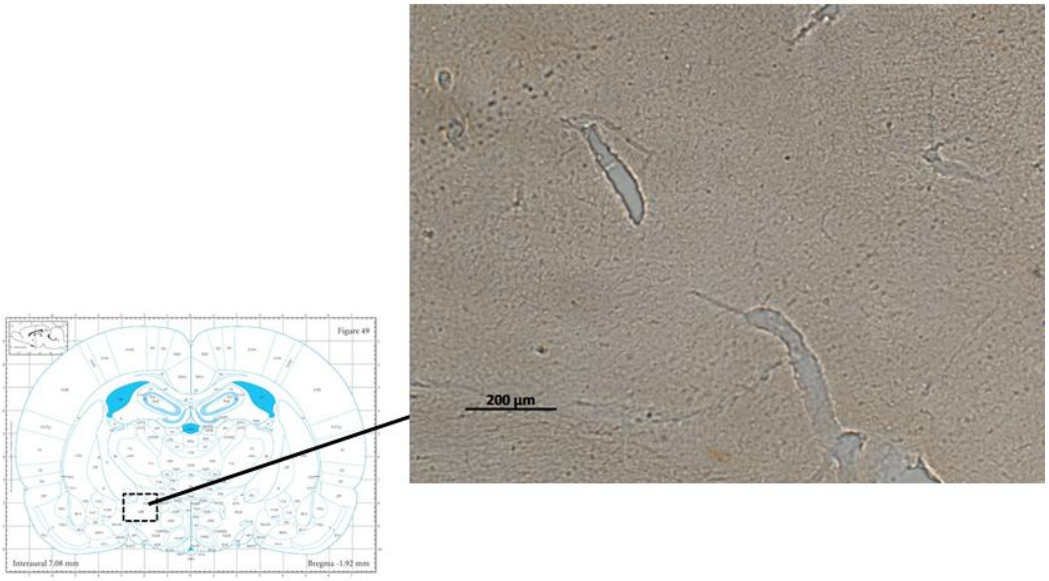

Figure 1-2: Double-staining procedure lacking primary antibodies. No staining is observed in the rLH.

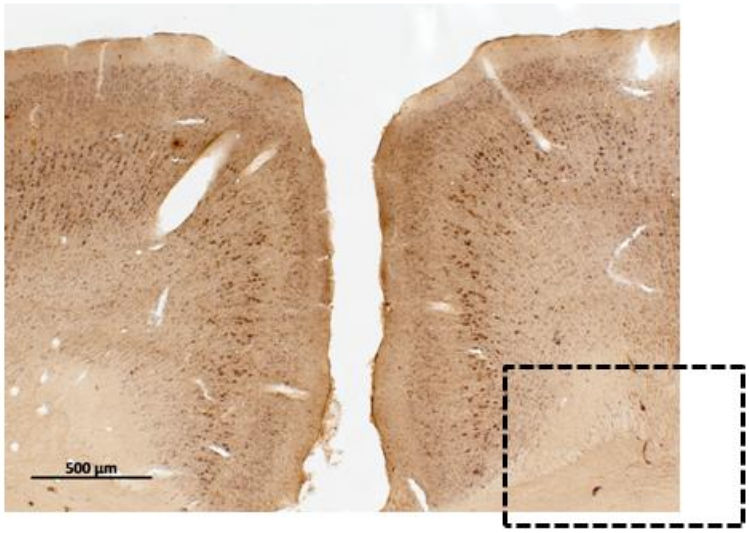

Figure 1-3: Orexin receptor 1 and neurotensin double staining in the cortex. Staining is absent in the cingulum (contained in the dotted black box).

B. Behavioral Studies

i. Cannulation Surgery

19 male Sprague Dawley rats (250-300 g) were allowed to acclimate for one week on a standard laboratory chow diet and housed in wire-hanging cages. The rats were anesthetized using sodium pentobarbital (60 mg/kg given intraperitoneally). The heads of the rats were shaved and placed into a stereotaxic apparatus and held in place with ear and nose bars. The scalp was rubbed with Povidone-Iodine as an antiseptic and the eyes were protected with eye ointment drops. A midline incision on the scalp was made and tissue was held back with hemostats. The scalp was dried with ethanol and swabs. The following coordinates relative to Bregma were used for the rostrolateral hypothalamus: -2.2 mm in the posterior direction, -1.9 mm lateral, and -7.2

ventral and for the ventral tegmental area: -5 mm in the posterior direction, .8 mm lateral, and -7.5 mm ventral. These coordinates were chosen based on the "The Rat Brain in Stereotaxic Coordinates" Paxinos atlas (Paxinos, Watson 2007). Holes were drilled over the rLH and VTA and in two additional locations for screws. Contralateral unilateral cannulae (8mm) were placed into both brain sites and glued in place with dental cement. Two sutures were made at the top and bottom of the midline incision and rats were left to recover for one week.

ii. Mock Injections and Acclimation

The rats were given mock injections of aCSF prior to experimental testing sessions to remove any effects of the novel injection procedure. The rats were acclimated to the spontaneous physical activity (SPA) chambers for two hours on two separate days. The SPA chambers contained food and water. SPA chambers detect motion in three dimensions.

iii. Drugs

Our dose of orexin A (250, 500 pmol, American Peptide Company, Sunnyvale, CA) was chosen based on our lab's prior work which shows the effectiveness of this dose range in increasing SPA. Our dose of SR 142948 (5, 10, 15 ng, Tocris Biosciences, Ellisville, MO) was based on the study by Reynolds *et al* which found this dose effective for antagonism of rLH neuron activation-elicited locomotion (Reynolds et al. 2006). Our dose of neurotensin (1.25-5 µg, Tocris Biosciences, Ellisville, MO). was based the Kalivas *et al* study which showed that this dose range given in the VTA was effective in

increasing locomotion (Kalivas et al. 1983). Drugs were solubilized in aCSF. SR 142948 was first dissolved in a very small quantity of water and brought to concentration with aCSF.

iv. Injection Treatments

Drugs were administered using a microsyringe with a 9mm injector needle which extended 1 mm beyond the cannula over 30 seconds with a 10 second waiting period for drug diffusion. The majority of treatments were separated by approximately 48 hrs and given in a Latin square design orientation.

v. Spontaneous Physical Activity Measurements

The rats were placed in chambers 43.2 cm by 43.2 cm (ENV-515 Test Environment, Med Associates, St. Albans, VT) equipped with three 16 beam infrared arrays to detect movement in x, y and z coordinates. The “Activity Monitor” (Med Associates, St. Albans, VT) software was used for collection and processing of locomotion parameters. The final output sent to the computers connected to the SPA chambers are: distance traveled, time spent ambulating, time spent in vertical movement, and time spent moving in stereotypic behavior. The first 20 minutes of data are eliminated due to injection and handling effects. Thus hour one refers to 20-80 minutes, hour two refers to 80-140 minutes, and hours 0-2 refers to 20-140 minutes. These times were chosen based on the standard acute effects of orexin A on SPA observed with injections into hypothalamic areas.

vi. Statistics

The data from all of the behavioral experiments were analyzed with a one-way analysis of variance with a repeated-measure design. A Tukey post-hoc group comparison test was used to detect differences between treatments. P values less than 0.05 were deemed significant.

vii. Placement Analysis

Following the experimental testing period, the rats were sacrificed by carbon dioxide euthanasia and decapitation. Prior to brain removal a green dye was injected into cannulae for clear identification of cannulae tracks. The brains were removed and transferred to a 20% sucrose solution for 5 hrs. The brains were sliced into 50 um sections using a cryostat and immediately mounted onto gelatin-coated slides. The slides were coverslipped and visualized with a light microscope to ascertain that cannulae track endings were contained in the rLH or VTA (Figures 1-4 – 1-6). Track endings were estimated at 1 mm ventral to the cannulae ending due to the 1 mm extension of the injector from the cannula. Data from 4 rats with misplaced cannulae were discarded from analysis.

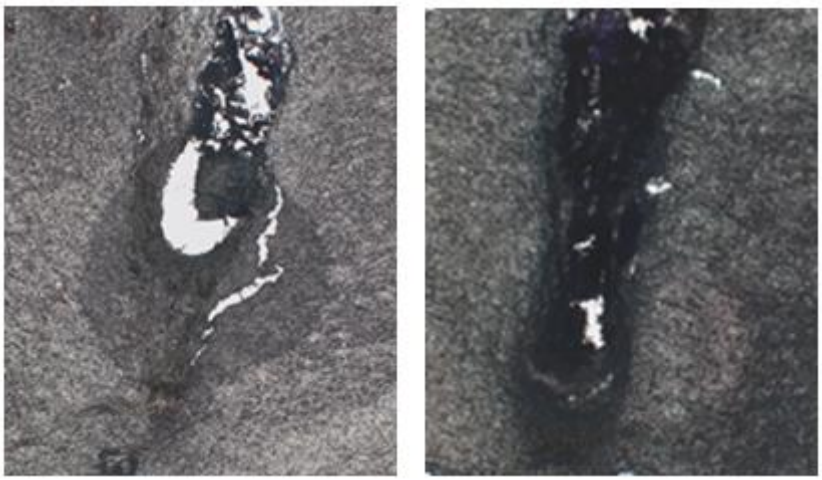

Figure 1-4: Tips of cannula tracks in the rLH (left) and the VTA (right). A green dye was injected into the cannulae just prior to brain removal for identification of cannulae tracks.

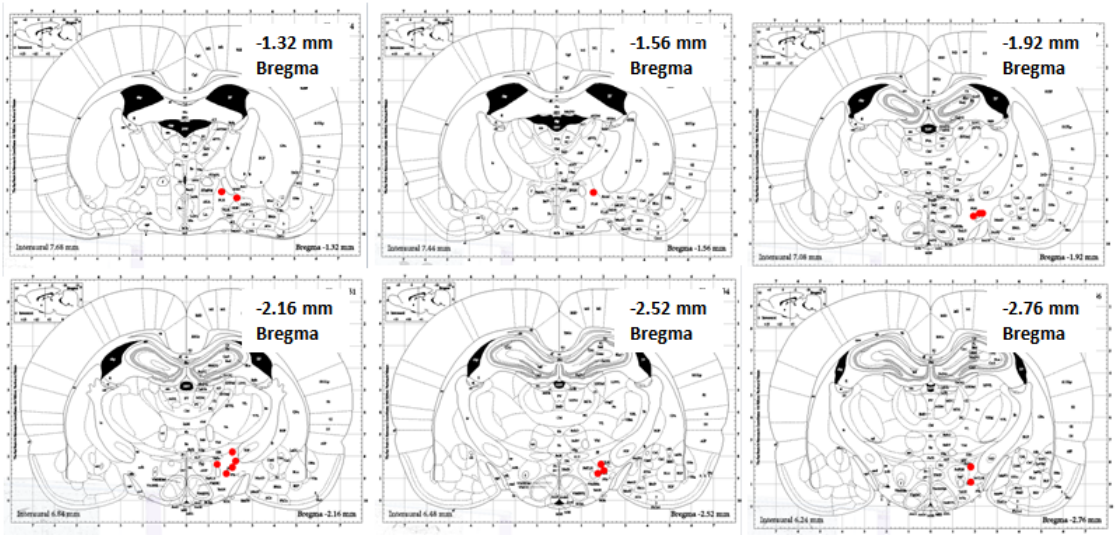

Figure 1-5: Cannulae placements in the rLH. The red dots indicate cannulae placements for individual rats. Placements included in data analysis were located between 1.32 and 2.76 mm posterior to bregma.

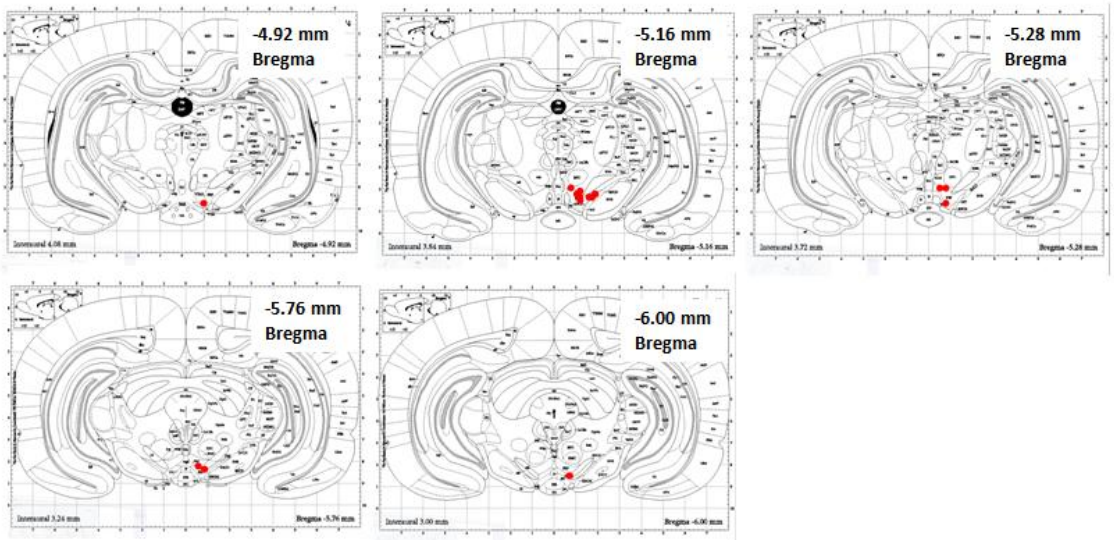

Figure 1-6: Cannulae placements in the rLH. The red dots indicate cannulae placements for individual rats. Placements included in data analysis were located between 4.92 and 6.00 mm posterior to bregma.

C. Experiment 1-1: Coadministration of the Neurotensin Antagonist and Orexin A

Rationale: To determine if neurotensin signaling in the VTA is downstream of orexin A activation in the rLH and the observed increase in spontaneous physical activity, we administered a neurotensin receptor antagonist in the VTA just prior to orexin A administration in the rLH.

Treatment	**VTA**	**rLH**
1	aCSF	aCSF
2	15 ng SR 142948	aCSF
3	15 ng SR 142948	250 pmol Orexin A
4	aCSF	250 pmol Orexin A

Table 1-1: Treatments for Experiment 1-1.

D. Experiment 1-2: Dose Response of the Neurotensin Antagonist in the VTA

Rationale: To confirm the activity-increasing effect of SR 142948 in the VTA from the results of experiment one, we conducted a dose response of SR 142948 in the VTA. The results of the dose response gave us a means to further assess the activity of SR 142948.

Treatment	**VTA**
1	aCSF
2	5 ng SR 142948
3	10 ng SR 142948
4	15 ng SR 142948

Table 1-2: Treatments for Experiment 1-2.

E. Experiment 1-3: Dose Response of Neurotensin in the VTA

Rationale: To determine what effect neurotensin injection into the VTA has on spontaneous physical activity, we performed a dose response.

Treatment	VTA
1	aCSF
2	1.25 µg Neurotensin
3	2.5 µg Neurotensin
4	5 µg Neurotensin

Table 1-3: Treatments for Experiment 1-3.

F. Experiment 1-4: Dose Response of Neurotensin in the rLH

Rationale: To determine what effect neurotensin injection into the rLH has on spontaneous physical activity, we performed a dose response of neurotensin.

Treatment	rLH
1	aCSF
2	1.25 µg Neurotensin
3	2.5 µg Neurotensin
4	5 µg Neurotensin

Table 1-4: Treatments for Experiment 1-4.

G. Experiment 1-5: Coinjection of Neurotensin and Orexin A in the rLH

Rationale: To determine if neurotensin and orexin A signaling in the rLH have opposing or synergistic roles in spontaneous physical activity, we injected neurotensin just prior to orexin A in the rLH. We chose a dose of orexin A (500

pmol) which has been shown to robustly increase activity. Our dose of neurotensin (5 µg) was found to have no significant effect on activity by itself.

Treatment	Neurotensin	Orexin A
1	aCSF	aCSF
2	5 µg	aCSF
3	aCSF	500 ρmol
4	5 µg	500 ρmol

Table 1-5: Treatments for Experiment 1-5.

IV. Results

A. Double-staining of Neurotensin and Orexin Receptors

Immunohistochemical observation of neurotensin and orexin receptor 1 or 2 revealed profuse staining for these antibodies throughout the brain. Both neurotensin and orexin receptor staining was observed in the cortex and the hypothalamus in high concentrations. The region of highest staining was the paraventricular nucleus of the hypothalamus. Staining was present in moderate concentrations in the rostrolateral hypothalamus. Overlap between nickel-enhanced DAB-stained orexin receptor 1 or 2 neurons and DAB stained neurotensin neurons was evident in many neurons in the rLH (Figure 1-7).

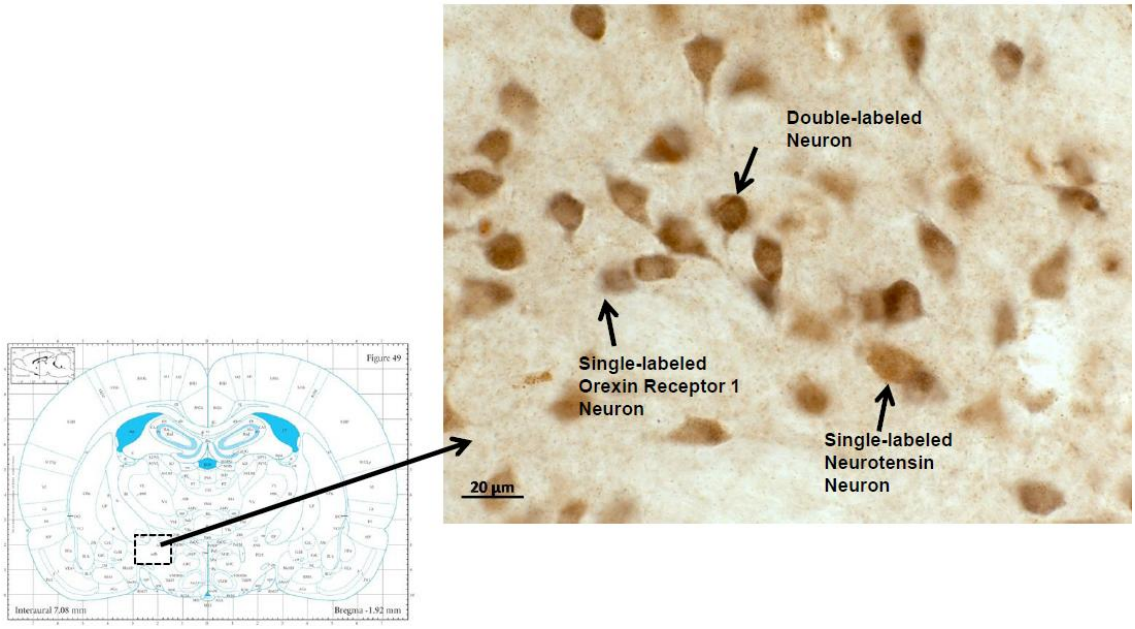

Figure 1-7: Double-labeling of Orexin Receptor 1 and Neurotensin-expressing neurons in the rLH. These cells are in the rLH: within the dotted box on the atlas page (Paxinos). Orexin receptor 1-expressing cells are stained with Nickel-enhanced DAB (black) and neurotensin-expressing cells are stained with DAB (brown). Single and double-labeled neurons are identified with black arrows. Similar double-staining patterns in the rLH were observed with the orexin receptor 2 antibody.

B. Coinjection of Orexin A in the rLH and Neurotensin Antagonist in the VTA

Coinjection of orexin A in the rLH and SR 142948 in the VTA resulted in differences in orexin A responsivity. Orexin A responsivity refers to the difference in time spent moving in the presence and absence of orexin A injection into the rLH. In the absence of SR 142948, orexin A increased the time spent ambulating in the first hour. In the presence of SR 142948, there was no increase in time spent ambulating in the first hour relative to the antagonist

alone (no orexin A) (Figure 1-8). Baseline values for time spent ambulating in the first hour between both control groups (aCSF + aCSF and 15 ng SR 142948 + aCSF) showed no significant difference.

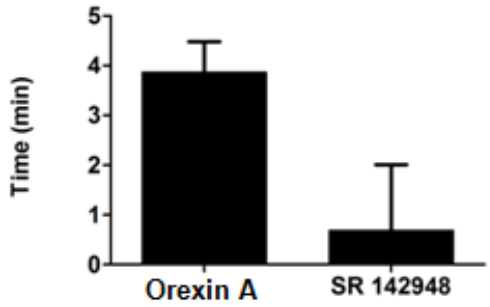

Figure 1-8: Time Spent Ambulating in the First Hour Post Injection In the presence of SR 142948, orexin A responsivity (change in time spent ambulating in the first hour post-injection) is abolished (p = 0.03; N = 15)

The same effect on orexin A responsivity was observed in the total time spent moving in ambulatory and vertical directions in the two-hour period post-injection. In the presence of SR 142948, orexin A responsivity was abolished (Figure 1-9, left). However, there was a significant increase in time spent moving in two hours post-injection in SR 142948 + aCSF-treated rats relative to aCSF + aCSF controls (Figure 1-9, right). For this reason we chose to perform a dose response of SR 142948 in the VTA to further assess dose dependent effects of the antagonist administered alone.

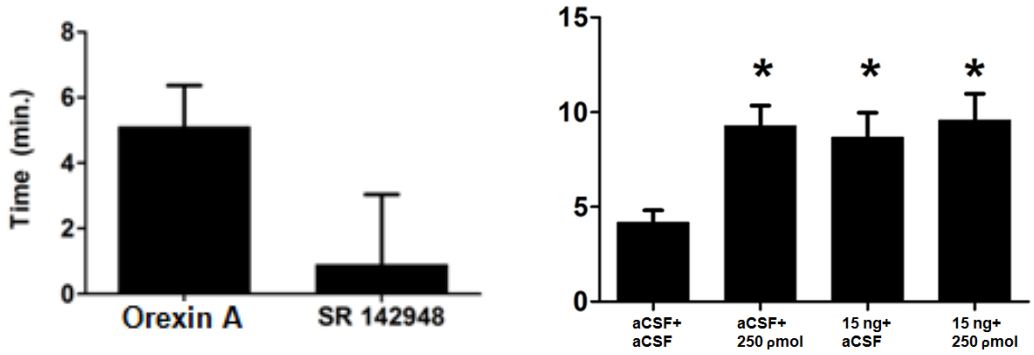

Figure 1-9: Total time spent moving in two hours post injection in the presence of SR 142948. Orexin A responsivity (change in total time spent moving in the two hours post-injection) is abolished (p = 0.045; left). Artificial CSF + SR 142948 increases the total time spent moving in two hours post injection (right: dose in the VTA + dose in the rLH). The asterisks symbolize a significant difference (p < 0.05) between treatment and control. N = 15

C. Dose Response of SR 142948 in the VTA

SR 142948 increased activity at high doses. At 10 ng and 15 ng, SR 142948 given to the VTA increased the distance traveled in the first hour and in the two hours post-injection relative to aCSF controls. SR 142948 also increased time spent ambulating in the first hour, total time spent moving in the first hour, time spent ambulating in two hours post-injection, and total time spent

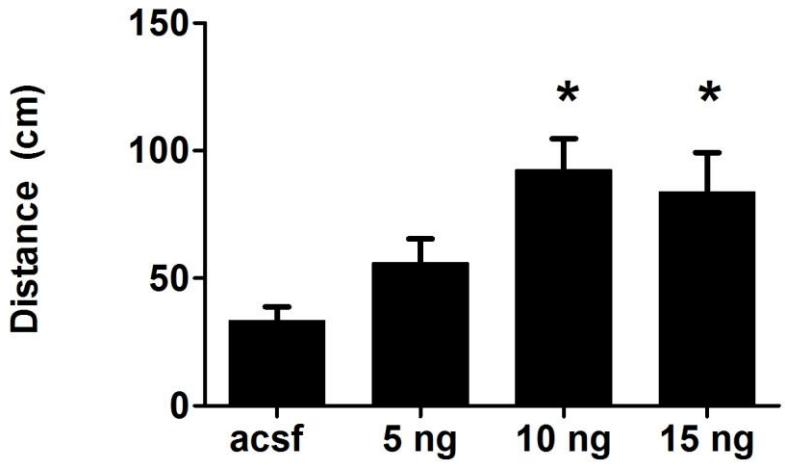

Figure 1-10: : Distance Traveled in Two Hours Post Injection of SR 142948 in the VTA Asterisks symbolize a significant different ($p < 0.05$) between treatment and control. N = 11.

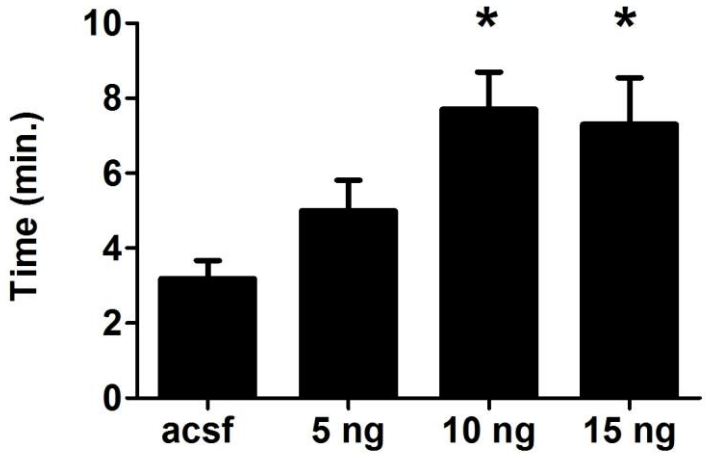

Figure 1-11: Time spent ambulating in two hours post injection of SR 142948 in the VTA. Asterisks symbolize a significant difference ($p < 0.05$) between treatment and the aCSF control. N = 12.

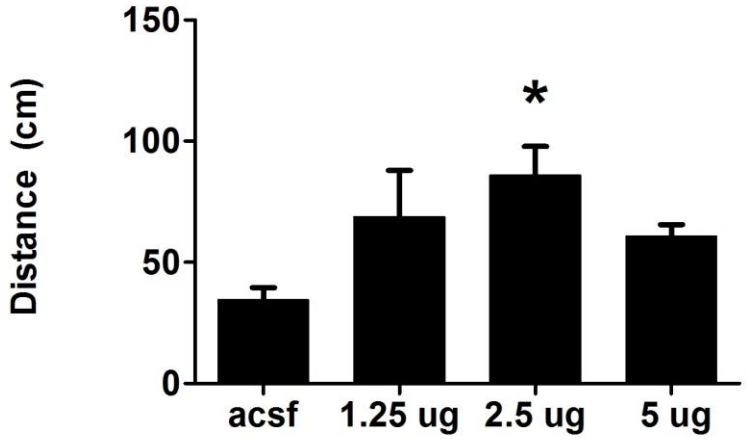

Figure 1-12: Distance traveled in two hours post injection of neurotensin in the VTA. Asterisk symbolizes a significant difference ($p < 0.05$) between treatment and the aCSF control. N = 12.

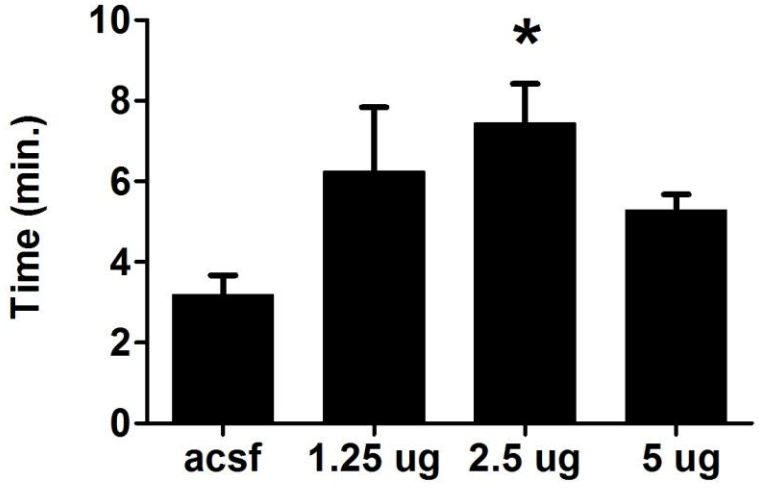

Figure 1-13: Time spent ambulating in two hours post injection of neurotensin in the VTA. Asterisk symbolizes a significant difference ($p < 0.05$) between treatment and the aCSF control. N = 12.

moving in the two hours post-injection relative to aCSF control (Figures 1-10, 1-11).

D. Dose Response of Neurotensin in the VTA

Neurotensin increased activity at the intermediate dose of 2.5 µg. A significant increase in distance traveled in the first hour, time spent ambulating in the first hour, distance traveled in two hours post-injection, and time spent ambulating in two hours post-injection relative to the aCSF control was observed (Figures 1-12, 1-13). There were no effects of neurotensin on total time spent moving.

E. Dose Response of Neurotensin in the rLH

Neurotensin decreased activity at the intermediate dose of 2.5 µg. There was a significant decrease in distance traveled in both one hour and the first two hours post-injection with 1.25 µg, 2.5 µg, and 5 µg relative to the aCSF control (Figure 1-14). At 2.5 µg there was a significant decrease in the time spent ambulating in the first hour, and in two hour post-injection relative to aCSF controls (Figure 1-15). There were no effects of neurotensin on total time spent moving.

F. Coinjection of Orexin A and Neurotensin in the rLH

Coinjection of orexin A (500 pmol) and neurotensin (5 µg) resulted in no change in either the distance traveled or time spent ambulating within the first two hours post-injection (Figures 1-16, 1-17). This dose of neurotensin given

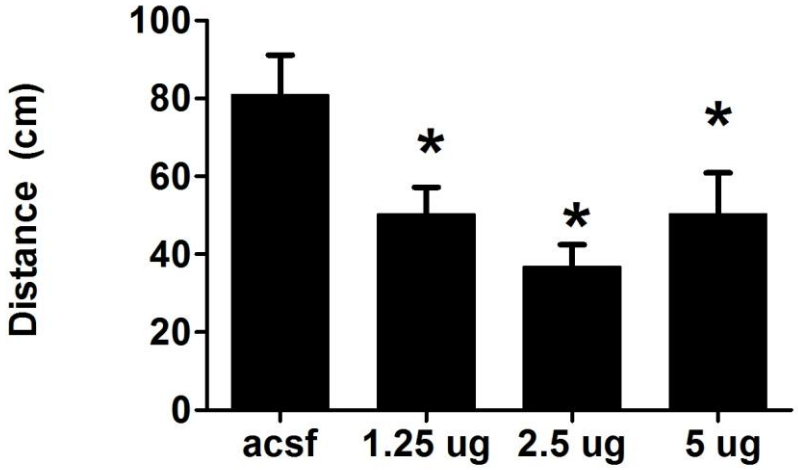

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Figure 1-14: Distance Traveled in Two Hours Post Injection of Neurotensin in the rLH. Asterisk symbolizes a significant difference ($p < 0.05$) between treatment and the aCSF control. N = 12.

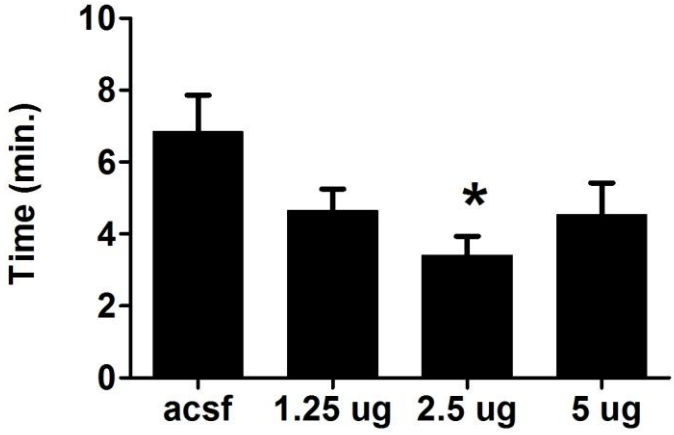

Figure 1-15: Time (min) spent ambulating in two hours post injection of neurotensin in the rLH. Asterisk symbolizes a significant difference ($p < 0.05$) between treatment and the aCSF control. N = 12.

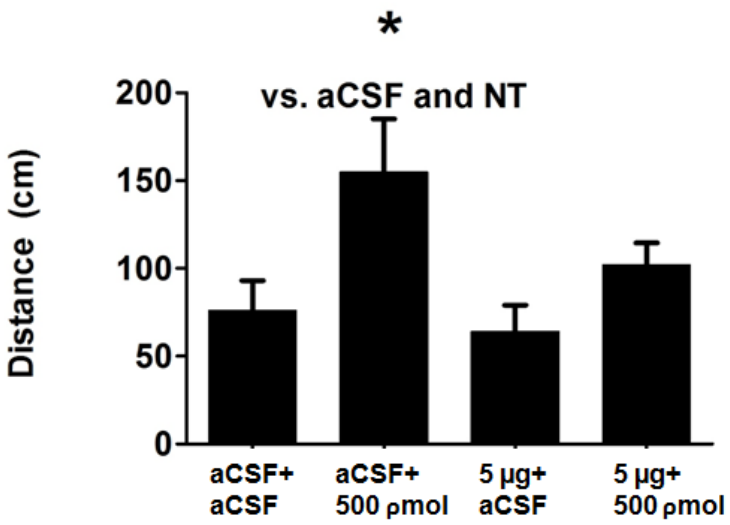

Figure 1-16: Coinjection of neurotensin and orexin A results in no change in the distance traveled in the two hours post-injection. aCSF + Orexin A (500 pmol) resulted in a significant increase in the distance traveled relative to aCSF + aCSF and neurotensin (5 µg) + aCSF treatments.

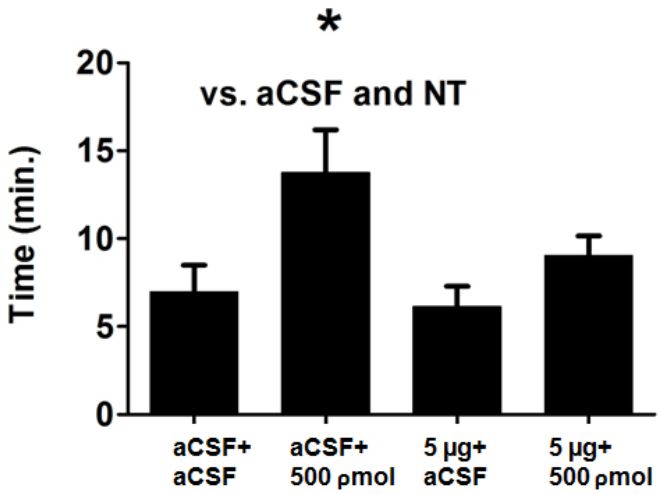

Figure 1-17: Coinjection of neurotensin and orexin A (CI) results in no change in the time spent ambulating (TSA) in the two hours post-injection. Orexin A (500 pmol) + aCSF resulted in a significant increase in the time spent ambulating relative to aCSF + aCSF and neurotensin (5 µg) + aCSF treatments.

with aCSF resulted in no significant change in physical activity relative to aCSF + aCSF controls (Figures 1-16, 1-17). The aCSF + Orexin A treatment resulted in a significant increase in the distance traveled as well as the time spent ambulating in the first hour and in two hours post-injection as expected based on prior data (Figures 1-16, 1-17).

V. Discussion

A. An Unclear Role of Neurotensin signaling in the VTA in the SPA-increasing Effect of Orexin A in the rLH

The coinjection of orexin A in the rLH and SR 142948 in the VTA initially revealed an abolishment of orexin responsivity in the presence of the antagonist. This effect was seen in time spent ambulating in the first hour with no differences between the aCSF + aCSF control and SR 142948 + aCSF control. However the same effect in total time spent moving in two hours post-injection was accompanied by a physical activity-increasing effect of SR 142948 alone (SR 142948 + aCSF). Thus it is possible that the activity-promoting effect of SR 142948 by itself is masking the true role of neurotensin signaling in the SPA-increasing effect of orexin A. The dose of 15 ng of SR 142948 was not found to have any activity-promoting effects in the first 30 minutes post-injection (these 30 minutes include the actual time post-injection and no 20 minute segment was eliminated) into the VTA in the study by Reynolds *et al* which measured distance traveled as the locomotion parameter (Reynolds et al. 2006). We were uncertain if this increase in activity was evidence of agonist-like properties of this drug, an artifact of the experimental setting, or was due to

some other unknown mechanism. To confirm the activity-increasing nature of the 15 ng dose of SR 142948 we proceeded to perform a dose response of this drug in the VTA.

B. SR 142948 and Neurotensin Injection into the VTA Increases Activity

Our dose response of SR 142948 confirmed that at higher doses of 10 ng and 15 ng, there was is a significant increase in activity within the first hour and in two hours post-injection as assessed by distance traveled, time spent ambulating, and total distance traveled. This data was clearly unusual considering that previous studies by Kalivas et al showed that neurotensin injection into the VTA leads to a similar increase in activity measured by electromagnetic activity counts. Our dose response data with neurotensin injection into the VTA showed a similar activity-promoting effect. Our dose treatments of neurotensin treatments in the VTA increased both distance traveled and time spent ambulating in the first hour and two hours post-injection. This data supports Kalivas *et al*'s data as well as the underlying hypothesis of this study.

However, the question that is left unanswered regards how SR 142948 also increases activity (Figure 1-18). There are a few potential mechanisms for this observed effect. It is commonly known that at certain doses, antagonists may have agonist-like properties. Our dose response data seems to support that at the two higher doses, SR 142948 may have been acting as a [partial] agonist (Zhu 2005). Another mechanism could involve the diffusion of SR

142948 into nearby brain regions which facilitate activity-reducing effects of neurotensin. We recall studies which showed that neurotensin injected into the nucleus accumbens and given in intracerebroventricularly decreases activity (Kinkead, Nemeroff 2006). Thus antagonism of neurotensin signaling in regions of the brain which feature this decrease in activity could result in an increase in activity. The regions surrounding the VTA could exhibit such activity-lowering properties of neurotensin.

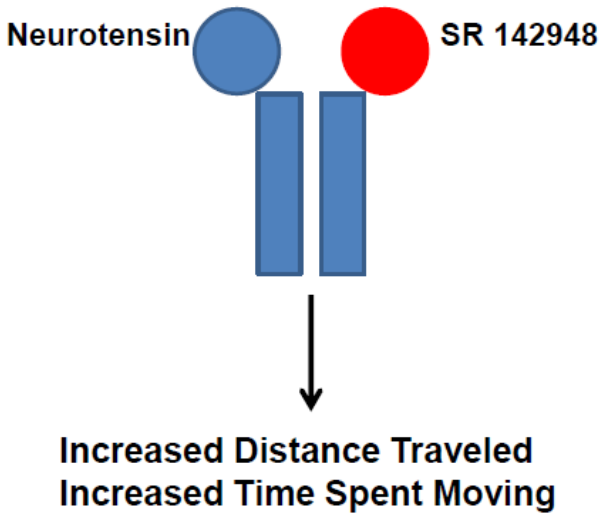

Figure 1-18: Both neurotensin and SR 142948 increase physical activity when injected into the VTA

Finally, the mechanism for the activity-increasing effect of SR 142948 may involve differential dopamine signaling in the VTA's projection site: the nucleus accumbens. There are two populations of dopamine receptor-expressing neurons present in the nucleus accumbens which affect neurotensin in opposing manners. Dopamine 3 receptors, which are expressed on neurons located in the ventromedial portion of the shell in the nucleus accumbens

positively affect the expression of neurotensin. Conversely dopamine 2 receptors, expressed mainly on neurons in the core of the nucleus accumbens, negatively affect neurontensin expression (Diaz et al. 1994a). Upon the blocking of neurotensin receptors by SR 142948, it is possible that neurons which contain these receptors and project to the nucleus accumbens differentially affect dopamine-receptor expressing neurons in the nucleus accumbens. For example, evidence suggests that certain neurons in the nucleus accumbens make projections and synaptic contact with neurons in the VTA (Carr, Sesack 2000). It may be that neurons which express dopamine 3 receptors project to the VTA and release neurotensin as a negative feedback signal to the SR 142948-mediated reduction in dopamine release to VTA neurons (Figure 1-19). Neurotensin release in the VTA would then increase physical activity. Alternatively, dopamine 2 receptor-expressing cells in the nucleus accumbens may project to the neighboring neurons and exhibit a decrease in the amount of neurotensin released. Since neurotensin application to the nucleus accumbens decreases activity, it seems reasonable to expect that such a decrease in neurotensin release within the accumbens may increase physical activity (Kalivas, Nemeroff & Prange 1984).

C. Neurotensin Injection into the rLH Decreases Activity at Low Doses and Antagonizes the Orexin A-mediated Increase in SPA at a Higher Dose

The fourth experiment injecting neurotensin into the rLH was based on an in situ hybridization and receptor autoradiography study which showed that neurontensin receptors are present throughout the lateral hypothalamus

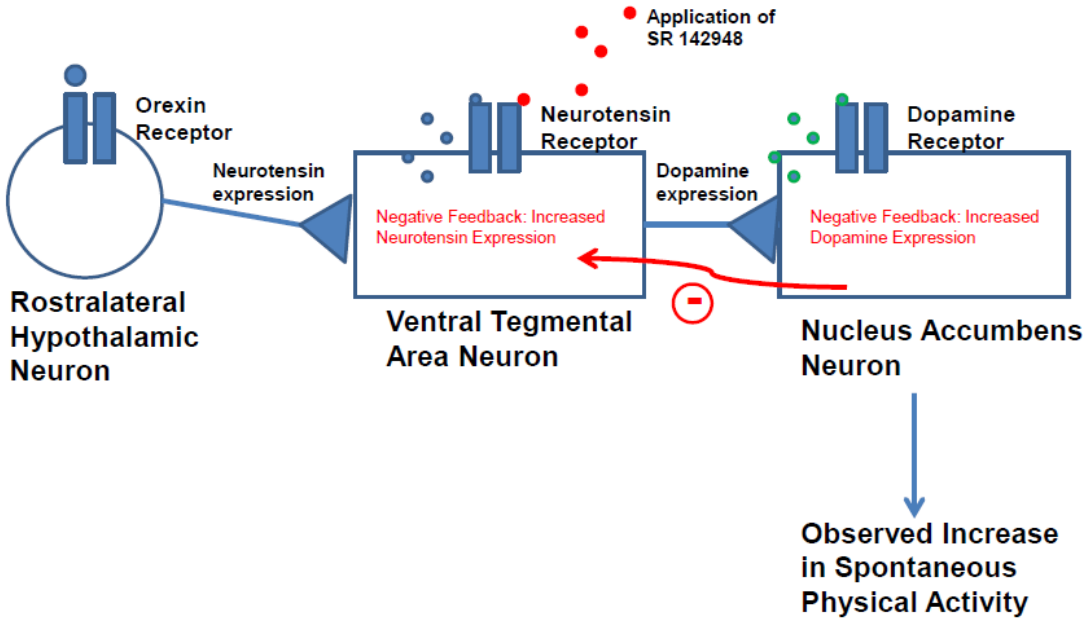

Figure 1-19: Negative feedback mechanism for the SPA-increasing effect of SR 142948 injection into the VTA. Dopamine 3 receptor-expressing neurons may project back to the VTA and release neurotensin in response to decreased neurotensin signaling due to SR 142948.

including the rLH as determined by images of receptor staining (Nicot, Rostene & Berod 1994). Taking into account the differential effects of neurotensin on physical activity when injected into particular brain regions (e.g. activity is increased in the VTA, but decreased in the nucleus accumbens upon injection of neurotensin), we predicted that there may be an effect of neurotensin signaling within the rLH on physical activity in either direction. Our observation that neurotensin injection into the rLH at the lower dose of 2.5 µg decreases physical activity suggests internal cellular mechanisms for the observed decrease in physical activity that are dependent on the concentration of neurotensin present.

For the coinjection on orexin A and neurotensin in the rLH we observed that at the high dose of neurotensin, 5 µg, there was no effect of neurotensin alone (5 µg + aCSF) on activity. This dose was found to have little effect on activity in our dose response done previously as well. However when this high dose of neurotensin was administered along with orexin A, there was no increase in physical activity. Our data here show that orexin A given to the rLH increases SPA relative to aCSF controls and in some cases the 5 µg neurotensin + aCSF-treatment. This effect was abolished when neurotensin was coinjected with orexin A. Thus, in addition or perhaps in contrast to our underlying hypothesis, neurotensin and orexin may work in opposing pathways *within* the rLH.

In the rLH neurotensin activity may perform several functions to inhibit the activity of orexin A (Figure 1-20). For example neurotensin receptor-

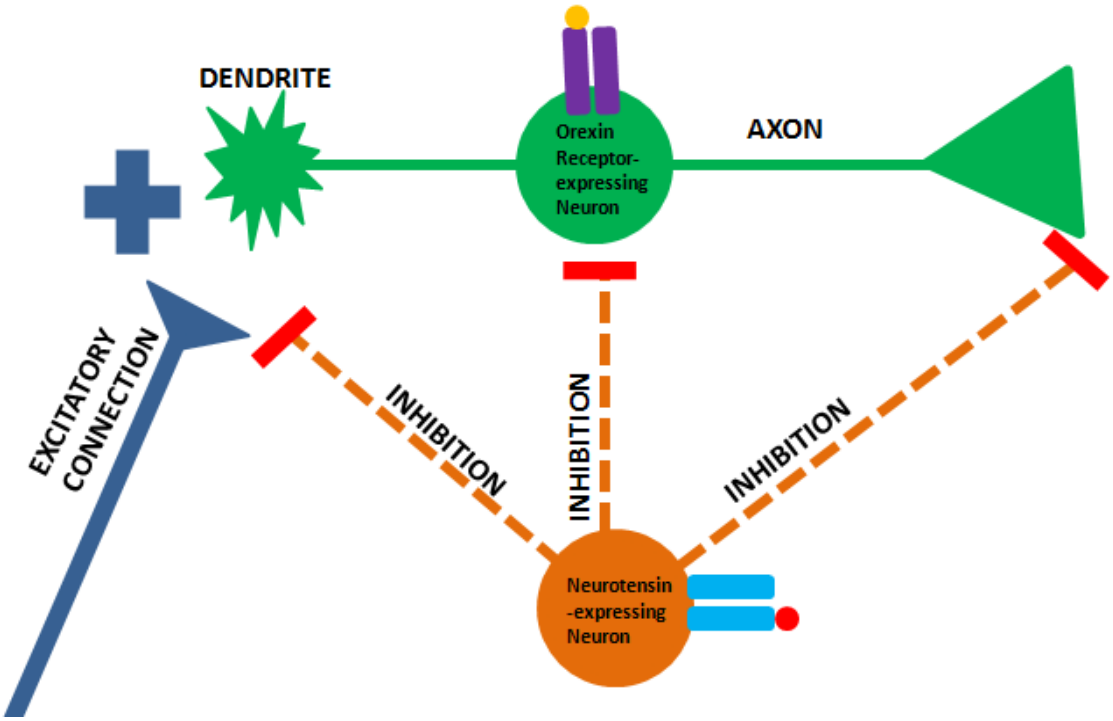

Figure 1-20: Neurotensin neurons may inhibit orexin-receptor expressing neurons in the rLH. Upon activation of neurotensin-expressing neurons, orexin receptor-expressing neurons may be inhibited by three potential mechanisms (dotted inhibitory lines): 1) Inhibition of excitatory synaptic contact from other neurons onto the dendrites 2) inhibition of somatic activity (chemical or electrical) 3) inhibition of synaptic activity at nerve terminals.

expressing neurons may inhibit orexin receptor-expressing neurons upon activation of neurotensin receptors. These inhibitory connections may be made on the cell soma or on the dendrites of orexin receptor-expressing neurons. Another possibility is an inhibitory synapse onto the dendrites of other neurons which synapse onto orexin receptor-expressing neurons and contribute to the basal activity of these neurons. In this case, neurotensin activity may reduce the excitability of orexin receptor-expressing neurons.

D. Future Research

Further studies looking at anatomical distributions of neurons within the rLH would be useful in assessing the mentioned possibilities. Anterograde and retrograde tracers may be used to identify other brain sites that involve rLH signaling. For example anterograde tracers would reveal brain sites which rLH neurons project to. Retrograde tracers would reveal which brain sites project to the rLH. Additionally immunohistochemistry for other peptides in combination with neuronal tracers may elucidate other candidates for signaling systems that are responsible for the observed behavioral effects of orexin A signaling in the rLH. A combination of these techniques would reveal the cellular phenotypes of cells in other brain region that form synaptic and dendritic connections with the rLH.

Once other peptide candidates and brain sites of interest are revealed, similar behavioral studies as those performed here may be performed. Antagonism of the relevant peptide signaling in the appropriate brain site just

prior to injection of orexin A in the rLH with SPA or a related behavioral measure as an output would clarify the axonal tracer and immunohistochemistry studies. It is possible and likely that multiple pathways and peptides are involved in orexin A responses in regions of the hypothalamus which contain orexin receptors and respond to orexin A.

VI. Conclusion

Our study reveals evidence that neurotensin signaling in the VTA may be downstream to orexin A signaling in the rLH. This evidence includes the absence of orexin A responsivity in time spent ambulating in the first hour post-injection in the presence of the neurotensin antagonist as well as the observed physical activity-increasing effect of neurotensin in the VTA. To further assess this theory, effective antagonism of neurotensin receptors in the VTA will prove necessary. The dynamics of SR 142948 activity on neurotensin in the VTA may involve multiple mechanisms and requires further elucidation. Finally neurotensin signaling in the rLH may play an opposing role to the effect of orexin A signaling in the rLH on physical activity.

Chapter 2.

THE EFFECT OF OREXIN A ON SPONTANEOUS PHYSICAL ACTIVITY IN THE VENTRAL TEGEMENTAL AREA AND THE PARAVENTRICULAR NUCLEUS OF THE THALAMUS

I. Introduction

The spontaneous physical activity-increasing effects of orexin A in the lateral hypothalamus, paraventricular nucleus of the hypothalamus, nucleus accumbens, and with the central administration of orexin A have been well established (Kiwaki et al. 2004, Kotz et al. 2002, Thorpe, Kotz 2005). However, other regions of the brain which contain orexin receptors are less well-studied. To determine whether orexin A also increases physical activity when administered to other brain regions, we choose to study the ventral tegmental area (VTA) and the paraventricular nucleus of the thalamus (PVT). Both orexin receptors 1 and 2 are present in these regions in high concentrations (Marcus et al. 2001).

A. The Role of Orexin A Signaling in the VTA

The VTA functions in the reward system circuitry to modulate motivational behaviors such as feeding and addiction (Laviolette, Van Der Kooy 2001). Orexin A signaling has been observed in the VTA with regard to several

behaviors. Orexin A signaling in the VTA has been shown to be required for the behavioral sensitization and synaptic changes that occur with cocaine addiction (Borgland et al. 2006). Stimulation of opioid signaling in the nucleus accumbens has been shown to increase the appetite for a high-fat diet. Orexin A signaling in the VTA is also required for this increase in appetite (Zheng, Patterson & Berthoud 2007). Orexin neurons present in the lateral hypothalamus make projections to the VTA and most likely release orexin A (Fadel, Deutch 2002). Orexin A activation of orexin receptors present in the VTA is responsible for these observed behavioral effects. Neurons present in the VTA project to the nucleus accumbens and release dopamine onto dopamine-receptor expressing neurons thereby activating the mesolimbic dopaminergic pathway (Kalivas et al. 1983). Activation of the mesolimbic pathway has been shown to be involved with responses in locomotion (Kalivas et al. 1983). Thus we predicted that orexin administration to the VTA could result in an increase in spontaneous physical activity (Figure 2-1).

B. The Role of Orexin A Signaling in the PVT

The PVT is a nucleus present along the midline of the thalamus that regulates autonomic and visceral functions (Kirouac, Parsons & Li 2005). Glutamatergic neurons in the PVT which project to the prefrontal cortex are activated by orexin A (Huang, Ghosh & van den Pol 2006). This circuit may be involved in a feedforward system that enhances cognitive arousal (Huang, Ghosh & van den Pol 2006). Similarly orexin-releasing projections are made in

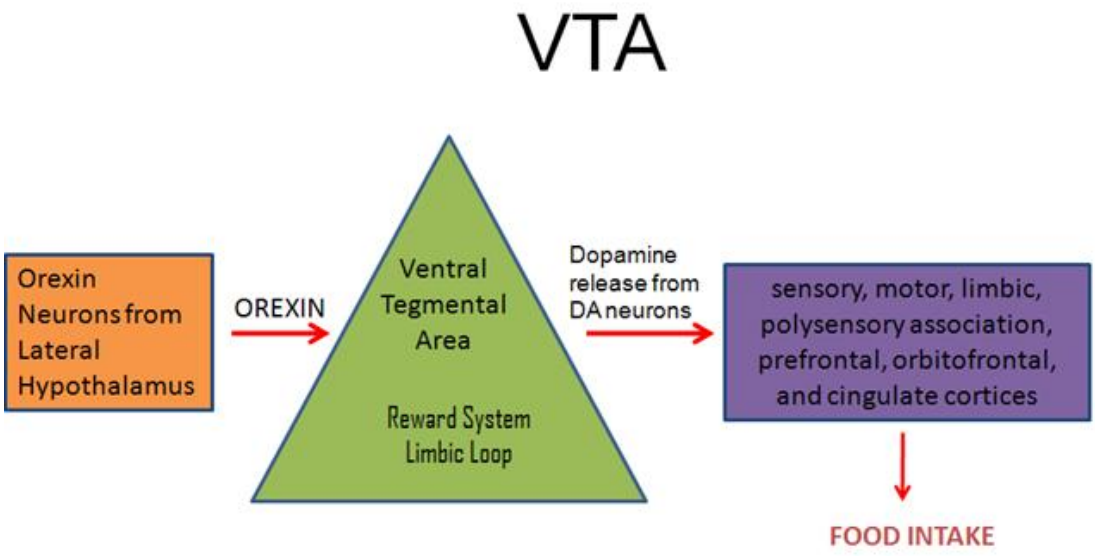

Figure 2-1: Possible role of orexin in VTA signaling. The VTA receives input from orexin neurons present in the lateral hypothalamus. Activation of DA neurons leads to the release of dopamine to forebrain areas responsible for motivational behaviors such as food intake.

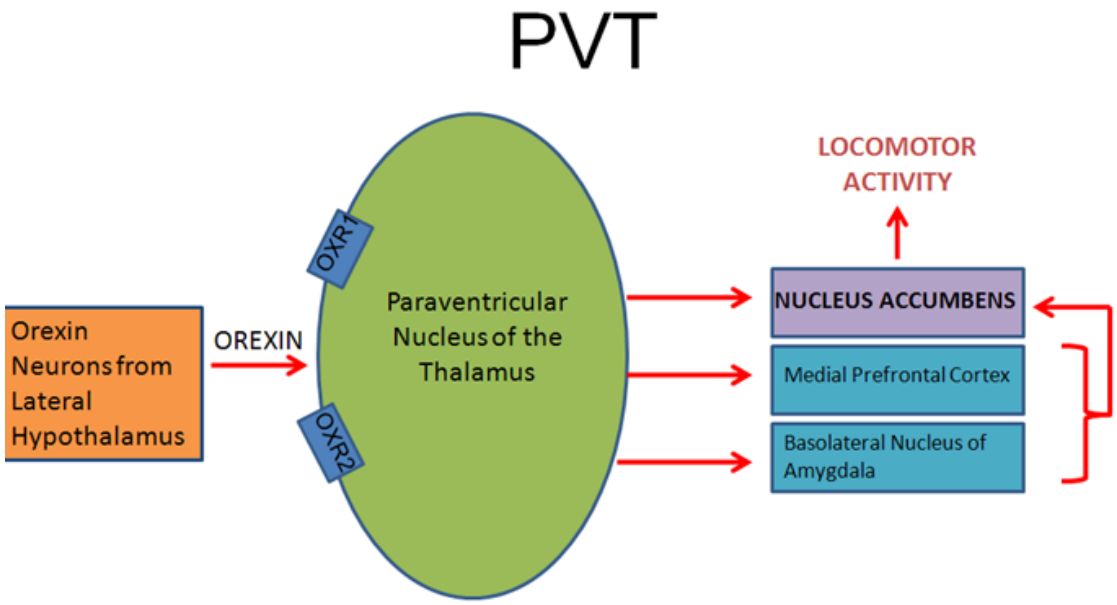

Figure 2-2: Possible role of orexin in PVT signaling. The PVT orexin receptors (OX1R, OX2R) receive input from orexin neurons present in the lateral hypothalamus. The PVT projects to the nucleus accumbens either directly or indirectly through the medial prefrontal cortex or the basolateral nucleus of the amygdala. The nucleus accumbens is a key player in the reward circuit invovled in motivational behaviors such as locomotor activity.

high concentrations within the PVT (Kirouac, Parsons & Li 2005). These neurons in the PVT project to the nucleus accumbens and amygdala and thus also may be involved in motivational or physical activity-related pathways through the dopamine system (Kirouac, Parsons & Li 2005). We predicted that orexin A administration to the PVT will increase spontaneous physical activity (Figure 2-2).

II. Hypothesis

Our hypothesis was that orexin A, administered at previously used doses, will increase spontaneous physical activity when injected into either the VTA or PVT.

III. Methods

A. Cannulation Surgery

16 male Sprague Dawley rats (250-300 g) were allowed to acclimate for one week on a standard laboratory chow diet housed in wire-hanging cages. The rats were anesthetized using sodium pentobarbital (60 mg/kg given intraperitoneally). The heads of the rats were shaved and placed into a stereotaxic apparatus and held in place with ear and nose bars. The scalp was rubbed with Povido-Iodine as a local anesthetic and the eyes were protected with eye ointment drops. A midline incision on the scalp was made and tissue was held back with hemostats. The scalp was dried with ethanol and swabs. The following coordinates relative to Bregma were used for the ventral tegmental area: -7.5 mm in the posterior direction, -1 mm lateral, and -5.2

ventral and for the paraventricular nucleus of the thalamus: -1 mm in the posterior direction, 0 mm lateral, and -4.2 mm ventral. These coordinates were chosen based on the “The Rat Brain in Stereotaxic Coordinates” Paxinos atlas (Paxinos & Watson, 2007). Holes were drilled over the VTA and PVT and in two additional locations for screws. Cannulae (8 mm) were placed into both brain sites and glued together with dental cement. Two sutures were made at the top and bottom of the midline incision and rats were left to recover for one week.

B. Mock Injections and Acclimation

The rats were given mock injections of aCSF prior to experimental testing sessions to remove any effects of the novel injection procedure. The rats were acclimated to the spontaneous physical activity (SPA) chambers for two hours on two separate days. The SPA chambers contained food and water. SPA chambers detect motion in three dimensions.

C. Drugs

Our dose range of orexin A (50, 100, 250 pmol, American Peptide Company, Sunnyvale, CA) was chosen based on our lab’s prior work which shows the effectiveness of this dose range in increasing SPA in the rostrolateral hypothalamus. Drugs were solubilized in aCSF.

D. Injection Treatments

Drugs were administered with a microsyringe with a 9mm injector needle which extended 1 mm beyond the cannulae over 30 seconds with a 10 second

waiting period for drug diffusion. The majority of treatments were separated by approximately 48 hrs and given in a Latin square design orientation.

E. Spontaneous Physical Activity Measurements

The rats were placed in chambers 43.2cm by 43.2 cm (ENV-515 Test Environment, Med Associates, St. Albans, VT) equipped with three 16 beam infrared arrays to detect movement in x, y and z coordinates. The "Activity Monitor" (Med Associates, St. Albans, VT) software is used for collection and processing of locomotion parameters. The final output sent to the computers connected to the SPA chambers are: distance traveled, time spent ambulating, time spent in vertical movement, and time spent moving in stereotypic behavior. The first 20 minutes of data are eliminated due to injection and handling effects. Thus hour one refers to 20-80 minutes, hour two refers to 80-140 minutes, and hours 0-2 refers to 20-140 minutes. These times were chosen based on the standard acute effects of orexin A on SPA observed with injections into other hypothalamic areas.

IV. Results

Orexin A injection into the VTA increased the time spent ambulating in the first hour post injection at the highest dose (250 pmol; Figure 2-3). Orexin A injection into the PVT had no effect on spontaneous physical activity with any dose used (Figure 2-4).

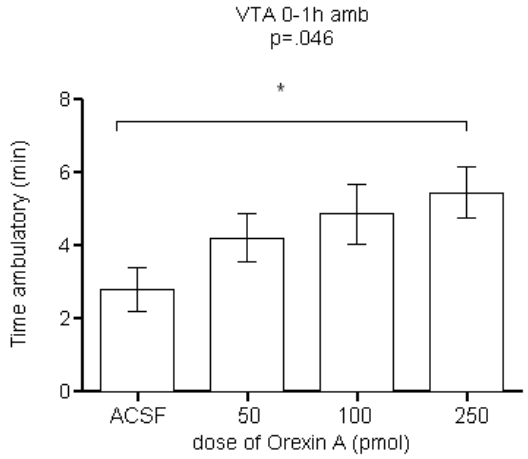

Figure 2-3: Orexin A increases SPA in the first hour post-injection into VTA. At the highest dose used (250 pmol) Orexin A causes a significant increase in the time spent ambulating in the first hour post injection.

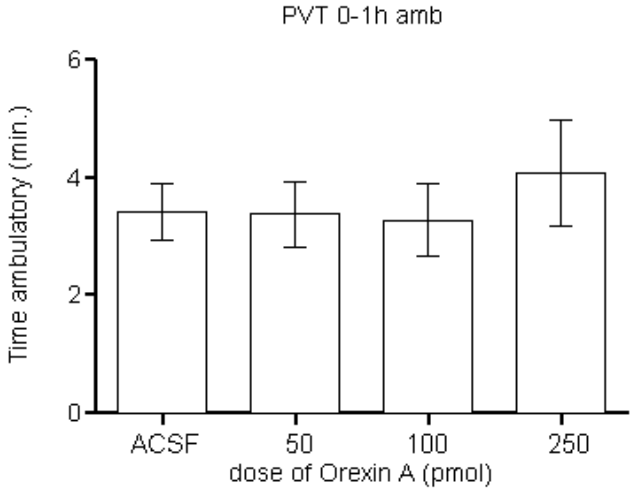

Figure 2-4: There was no effect of orexin A in the PVT on SPA during the first hour post-injection.

V. Discussion and Conclusion

Injection of orexin A in the VTA causes a significant increase in the time spent ambulating in the first hour post-injection. Ambulatory motion may be a result of increased search behavior suggesting that Orexin A in the VTA may be affecting the limbic reward circuitry. Activation of dopaminergic neurons by Orexin A may be causing increased motivation for locomotor activity (Beninger 1983).

Orexin A in the PVT had no effect on ambulatory activity, suggesting that the PVT does not play a role in orexin A-induced physical activity. It is possible that increased doses and/or other regions of the PVT could be sensitive to the activity-producing effects of orexin A.

As other brain areas (e.g. paraventricular nucleus of the hypothalamus and rostrolateral hypothalamus) are sensitive to the activity –promoting effects of orexin A, there is likely an interaction between orexin activity in various brain locations, and other neurotransmitters involved. Orexin signaling in one location may precede activation of neighboring neurons which send and receive projections to other brain sites. Measurement of c-Fos activity in the PVT, VTA, and other brain sites following orexin A injection would help uncover interactions present in orexin brain circuitry.

CONCLUDING REMARKS

We have characterized several effects of orexin A and neurotensin peptide signaling within the brain. We have shown that neurotensin modulates spontaneous physical activity in a site-specific and dose-dependent manner. Namely, neurotensin increases the time spent moving and the distance traveled when injected into the ventral tegmental area. Whereas, neurotensin reduces these behaviors when injected into the rostrolateral hypothalamus. There are likely variations in intracellular as well as pathway projection systems which allow for the differential effect of neurotensin on spontaneous physical activity. Alternative approaches are required to determine the effect of antagonizing neurotensin receptor signaling within the ventral tegmental area as related to the role of this activity in the orexin-mediated response in the rostrolateral hypothalamus. Agonist-antagonist binding assay assessment in combination with the measurement of other neurotensin-evoked behaviors such as analgesia and hypothermia in the presence of this antagonist would ascertain the physiological dose-dependent effectiveness of this antagonist.

We have also shown that orexin A increases spontaneous physical activity when injected into the ventral tegmental area but not the paraventricular nucleus of the thalamus, which suggests, in combination with prior data, that orexin A has a locomotion-promoting effect that is dependent on the site of administration. It is likely that variation in G-protein subunit coupling of orexin receptor subtypes within each site as well as neuronal partners in synapse

formation with orexin receptor-expressing neurons dictate the effectiveness of the orexin response.

Finally we have determined that there are likely interactions between the neurotensin and orexin signaling systems and the respective spontaneous physical activity responses. In particular, our studies show evidence that within the rostrolateral hypothalamus, neurons which respond to neurotensin may inhibit the response to orexin administration to this area. This effect may be due to inhibitory synapses from neurotensin receptor-expressing neurons onto orexin receptor-expressing neurons within the rostrolateral hypothalamus. In a broader sense, it maybe concluded that energy balance is maintained through multiple peptides and signaling centers within the brain in addition to intra-hypothalamic anabolic and catabolic pathways.

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