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Examination of the Effects on Orexin A on Rodentive Appetitive and Consumatory Behaviors Using a Progressive Ratio Schedule of Reinforcement

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Examination of the effects of orexin A on rodent appetitive and consumatory behaviors using a progressive ratio schedule of reinforcement

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Examination of the effects of orexin A on rodent appetitive and consumatory behaviors using a progressive ratio schedule of reinforcement

ABSTRACT

Orexin has important functions in feeding, arousal, and motivation. However, the mechanism by which orexin influences these processes is not well understood. The goals of the present study were 1) demonstrate that orexin A enhances appetitive and consumptive behaviors (lever presses, licks, rewarded licks, and breakpoint) using a progressive ratio schedule of reinforcement for sucrose reward and 2) Determine if gamma-aminobutyric acid receptor blockade could attenuate those behaviors. However, preliminary analysis did not reveal an effect of orexin A (30 nM) when infused into the lateral ventricle of rats. Of the measured operant behaviors, the orexin A and saline group did not differ. Therefore the second phase was not conducted. Multiple explanations for the lack of orexin's effect are possible are possible: aged rats, endogenous orexin levels, incorrect dose, misplaced cannula, and use of a different schedule of reinforcement than that previously used with orexin. The unique function of orexin in these behaviors prompts further analysis into the reason behind the outcomes of this study.

Energy balance involves the integration of metabolism, food intake, reward value, experience, and locomotion. The lateral hypothalamus (LH) is the central regulator of energy homeostasis. It has efferents leading to the prefrontal cortex, amygdala, ventral striatum, paraventricular nucleus, and suprachiasmatic nucleus, and as a result has the ability to control both the want for food and the locomotion towards it. Located within the ventral striatum, the nucleus accumbens (NAc) may also have a role in the central regulation of these behaviors. More specifically, current research demonstrates that the molecular basis of this integration in the NAc may involve the neuropeptide orexin, although the complete mechanism has not been clearly revealed. The goals of this project were to investigate orexin's function in motivation for food intake and activation of enhanced motor responses, as well as the role of the neurotransmitter GABA and the NAc in orexin A induced food intake. However, inconclusive results halted the execution of the second phase of the experiment.

Discovery of the orexin neuropeptide

In 1998 two independent laboratories using two separate methods isolated the orexin neuropeptides. In one laboratory, the search was for an endogenous ligand that would activate orphan g- protein- coupled receptors (GPCRs) (Sakurai et al 1998). Using a method described as "reverse pharmacology", this laboratory produced over 50 transfected cell lines, each expressing a distinct orphan GPCR cDNA. High-resolution HPLC fractions of extracts from rat and bovine brains were subsequently assessed for ligand binding on these recombinantly expressed orphan GPCRs. Two neuropeptides were found to have agonist activity: orexin A and B. Early investigations into the peptide

found that central administration increased feeding. In recognition of these studies documenting of the peptides' ability to induce feeding, the peptides were given the name orexin, which is derived from the Greek work "orexis" meaning "appetite".

Conversely, de Lecea et al (1998) isolated the same precursor peptide using a cDNA library derived from the hypothalamus. The precursor peptides were named hypocretin 1 and hypocretin 2. This name is derived from its sequence similarity to the pancreatic peptides, incretins, and the origination of the peptide in the hypothalamus. The nucleotide sequence between Orexin A and B to hypocretin 1 and 2 are identical but de Lecea's sequence also predicts amino acids in the mature peptide not found in natural orexin. Compared to native orexin, the additional amino acids had the effect of creating weaker agonists on cells expressing human orexin receptors (Smart et al 2000). Despite this difference the name orexin and hypocretin are used interchangeably in the literature.

Orexin A and B are derived from a single gene, pre- pro- orexin (Sakurai et al 1998). This gene is composed of two exons and an intervening intron. In humans and rodents the pre- pro- orexin is 131, and 130 residues long, respectively. The pre- pro- orexin gene is found on chromosome 17q9.

Mammalian orexin A is a 33 amino acid peptide of 3562 Da with an N- terminal pyroglutamyl residue, C terminal amidation, and two sets of intrachain disulfide bonds. Remarkably, the primary structure of orexin A is completely conserved among human, rat, cow, pig, and mouse genera (Sakurai et al 1998, Sakurai et al 1999). Orexin A is more stable than orexin B in vivo due the disulfide bonds. Mammalian orexin B is a 28 amino- acid, C terminally amidated peptide of 2937 Da. The conservation of sequence observed in orexin A is not seen in orexin B between generae; human orexin B has two

amino acid substitutions compared to rat and mouse amino acid sequences, which are identical. Compared to orexin A, orexin B has a simpler tertiary structure without disulfide bridges. Orexin B has a 46% amino acid identity to the orexin A sequence. The orexin A peptide has yet to be visualized; however, x- ray crystallography of orexin B reveals two alpha helices at a 60-80 degree angle to each other (Lee et al 1999).

Orexin is broadly distributed

Now that we have knowledge of the background and structure of orexin, the distribution of the peptide will be highlighted. Neurons containing orexin are shown to project widely through the brain, corroborating the theory that orexin influences a wide variety of behaviors. Peyron et al (1998) used immunohistological techniques to investigate the distribution of orexin immunoreactive fibers and neurons. Orexin is generated mainly in the lateral hypothalamus with fewer orexigenic neurons in the posterior hypothalamus. Although orexin producing cells are few in number and confined to a few areas, orexin neurons project widely throughout the brain. Significant projections lead to the cerebral cortex, olfactory bulb, hippocampus, amygdala, septum, diagonal band of Broca, thalamus, anterior and posterior hypothalamus, midbrain, brainstem, and spinal cord (Peyron et al, (1998). Of these, the densest staining of immunoreactive nerve endings were in the paraventricular nucleus of the hypothalamus, arcuate nucleus of the hypothalamus, locus coeruleus, and the tuberomamillary nucleus.

Outside of the nervous system orexin immunoreactivity is found in cells of the gastrointesintal tract such as the enteric nervous system, stomach and intestinal endocrine cells, and the pancreas. Orexin mRNA has been found in the testes (Kirchgessner and Liu

1999, Sakurai et al 1998, Voisin et al 2003).

Orexin receptors are G protein linked

Naturally, orexin mRNA localization is highly correlated with the presence of the orexin receptor. When Sakurai et al (1998) identified the orexin neuropeptides through reverse pharmacology, two receptor subtypes were consequently isolated. Two orexin receptor subtypes have been identified in mammals, termed orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R). These receptors are found throughout mammals with high conservation of amino acid structure between each other and across mammalian species. They have 64% homology with each other in contrast to the 25-35% amino acid identity with other GPCRs (Sakurai et al 2003). In addition human OX1R has 94% amino acid identity with the rat sequence, and 95% for OX2R. OX1R has a tenfold greater affinity for orexin A over B, whereas OX2R has similar binding affinities for orexin A and B (Sakurai, 1998).

The OX1R is 425 amino acids long and its gene is encoded on chromosome 1. OX1R is coupled to the G_q subclass of GPCR. G_q's are linked to nonselective cation channels leading to influx of positive charge (most likely extracellular calcium) in neurons and resultant depolarization and excitation of the postsynaptic neuron (Sakurai et al, 1998, Date et al, 1999, Uramura et al, 2001). In addition, ligand binding also activates the phospholipase C signaling pathway resulting in cleavage of phosphatidylinositol into inositol triphosphate and diacylglycerol. These second messengers then modulate the activity of other proteins including calcium channels of the smooth endoplasmic reticulum, calmodulins, and protein kinase C.

The OX2R gene is found on chromosome 6 and encodes for a peptide sequence 444 amino acids long. OX2R is coupled to both G_q and $G_{i/o}$ heteromeric G proteins. Thus OX2R is capable of modifying intracellular calcium levels as well as intracellular potassium levels through G- protein gated inwardly rectifier potassium channels (Sakurai et al 1998, Date et al 1999). These $G_{i/o}$ receptors are inhibitory whereas G_q receptors are excitatory.

Orexin receptors are localized throughout the brain

Following this discussion of the structure and mechanism of the orexin receptor let us move forward to the distribution of the receptors. Trivedi et al (1998) and Marcus et al (2000) used *in situ* hybridization of mRNA in the rat brain to demonstrate that orexin receptors (OX1R and OX2R) are expressed in widely different areas. For example OX1R mRNA is predominantly expressed in the hypothalamus, tenia tecta, hippocampus, dorsal raphe nucleus, locus coeruleus, arcuate nucleus, and ventromedial hypothamalmus. However, OX2R mRNA is mainly expressed in the cerebral cortex, NAc, subthalamus, paraventricular hypothalamic nucleus, anterior pretectal nucleus, and septal nuclei. Both orexin 1 and 2 mRNA receptors have been located in the adrenal medulla (Sakurai, 1998).

Orexin has diverse physiological functions related to energy balance

Studies of orexin revolve around three areas: sleeping, food intake, and physical activity. It is easy to see the relationship between these three behaviors, when they are

integrated together in energy homeostasis. The following section will highlight some of the findings in regard to these three areas.

Orexin and regulation of the sleep- wake cycle

Multiple studies across mammals have shown the importance of orexin in the maintenance of sleeping. Animal studies support orexin as a regulator of the sleep-waking cycle and circadian rhythms without the severity of narcolepsy. Orexin A (icv) increased wake time and also decreased REM and non- REM time in rats. An indicator of neuronal activation, *fos* labeling of orexin neurons increased in the dark cycle, the waking time for nocturnal rats, and decreased during REM and non- REM sleep (Estabrooke et al 2002).

Studies of disrupted of orexin signaling has illuminated the sleeping disorder narcolepsy. Narcolepsy is characterized by excessive daytime sleepiness, cataplexy, and fragmented rapid eye movement sleep. For example, dachshund, Doberman pinscher, and poodle pedigrees carry a higher prevalence for narcolepsy than other breeds. This was found to be due to a defective OX2R gene. Using a narcoleptic dog model, Lin et al (1999) found the canine narcolepsy gene, *canarc-1*, has deletions in the intron region. This mutation creates defects in mRNA splicing and the subsequent production of non-functional OX2R.

Research in the laboratory of Chemelli et al involving defects in mice OX1R and OX2R genes support the canine studies. OX2R knockout mice displayed characteristics of narcolepsy (Chemelli et al 1999, 2003). OX1R knockout mice displayed fragmented sleep- wakefulness cycles however the phenotype is only partially consistent with

narcolepsy. Therefore, both receptors may be required to manifest narcolepsy because loss of signaling through both displayed the most severe narcoleptic symptoms. Orexin is important to sleep wake cycles and arousal. Abolishment of the orexin neuropeptide through orexin knockout mice creates a phenotype highly similar to human narcolepsy (Chemelli et al 1999). These mice show disrupted of sleep wake cycling in the dark phase as well as narcoleptic and cataplexic attacks in the dark phase.

Based on animal studies it is reasonable to hypothesize that human narcolepsy involves dysfunctional orexin signaling. However, human narcolepsy does not display familial inheritance and is theorized to involve environmental factors (Mignot, 1998). The loss of orexin producing neurons through an immune response is a possible mechanism for narcolepsy, due to the strong association between human leukocyte antigen class II haplotypes DR2 and DQB1 * 0602 with sporadic human narcolepsy. Clinical studies of orexin support its function in narcolepsy. Correlation studies also indicate that orexin and narcolepsy are related. Peyron et al (2000) found that orexin mRNA and peptides were undetectable in narcoleptics but were present in controls. Nishino (2000) found similar results when investigating orexin A in narcoleptic and controls. Orexin A was deficient in 7 of 9 subjects with narcolepsy and present in all of the normal controls.

One could imagine that the opposite of narcolepsy is insomnia, the inability to fall asleep. Two separate pharmaceutical companies Actelion and Glaxo- Kline- Smith are currently developing an orexin receptor antagonist purpose to decrease insomnia (Brisbare-Roch et al, 2007; Glaxo- Kline- Smith, 2007). ACT-078573 is the first oral orexin receptor antagonist that penetrates the blood-brain barrier and is capable of

inducing a transient and reversible blockade of the two receptors, OX1 and OX2. Glaxo Smith Kline is currently working on GW649868, a similar drug to Acetelion's. Both of these orexin receptor antagonists offer promise in promoting deep sleep and increased REM over the current standard for insomnia treatment, Ambien. This evidence supports the orexin's function producing arousal and maintenance of the sleep wake cycle.

Orexin and induction of food intake

Orexin is one of several neuropeptides produced in the hypothalamus that serve as the molecular basis of ingestive behaviors. The distinctive production of orexin solely in the LH suggests that it has a major role in feeding and homeostatic metabolism. Early studies of the LH revealed that lesions of the area created a failure to eat and drink in rats (Anand and Brobeck, 1951). In addition, Bernandis (1971) found that ablation of neurons of the LH caused a decrease in weight and "set point", the weight at which the subject persists at despite changes in caloric intake and physical activity). Nonetheless, the LH is not the end all with regard to feeding as animals still ate after the lesions were performed, simply not as much.

Sakurai et al (1998) found that, indeed, an acute injection of orexin A into the lateral ventricle, early in the light phase, increased feeding. Orexin B under the same protocol had a similar effect; although less robust and for a shorter duration than orexin A. Further studies have shown similar increases in feeding (during the light phase) with central injections to the LH, PVN, NAc, and the locus coeruleus (Kotz et al 2002, Kiwaki et al 2003, Thorpe and Kotz, 2005, Hagan et al 1999).

In general, the hypothalamus, the location of orexin expression, functions to

maintain homeostasis in the body through diverse projections throughout the brain and lesser projections to the pituitary gland. Injection of orexin A (icv) during the light phase increased oxygen consumption and carbohydrate metabolism (Lubkin and Stricker-Kongrad, 1998). Kiwaki et al (2003) also used indirect calorimetry to measure oxygen consumption following orexin administration into the PVN. Similar increases in oxygen consumption were recorded.

Orexin antagonists can be used to disrupt natural feeding. SB-334867-A, a selective OX1R antagonist, injected intraperitoneally disrupted feeding stimulated by food restriction or icv injection of orexin A as compared to a saline control (Arch, 2000). In addition, intracisternal administration of an anti- orexin polyclonal antibody dose dependently reduced feeding (Yamada et al, 2000).

In comparison to other neuropeptides mediating food intake, orexin does not stimulate feeding as robustly. Ida et al (1999) found that orexin A increased appetite although the effect was far less than that of neuropeptide Y (NPY), another orexigenic peptide produced in the hypothalamus. NPY synthesis is upregulated during models of negative energy balance or increased metabolic demand including starvation, insulin dependent diabetes mellitus, lactation, and physical exercise (Inui et al 2000). NPY is also considered the most potent neuropeptide involved with increasing feeding.

Orexin increases physical activity and arousal

In addition to feeding, energy homeostasis also involves arousal and physical activity. To that end, investigators have linked increased orexin concentration to not only appetite but also increased physical activity and arousal. Valenstein et al (1970) noted

that in addition to increased feeding and drinking, electrical stimulation of the hypothalamus could elicit changes in aggression, sex, hoarding, and other motivated behaviors. Lesions of the LH also destroyed a portion of the nigrostriatal dopamine pathway, one of four major dopamine pathways and particularly important to the generation of movement (Ungerstedt, 1970). Thus reward seeking behaviors were abolished, other than food, eliciting catalepsy, sensory neglect, a failure of sensorimotor integration, and a resultant inability to respond to acute stimuli (Marshall et al, 1979).

More recent studies have placed orexin, a neuropeptide expressed in the LH, in a role to control motor behaviors. Kotz et al (2006) demonstrated that administration of orexin A to regions where orexin neurons terminate, such as the rostral LH, paraventricular nucleus, and substantia nigra pars compacta, significantly increases physical activity. In addition, orexinergic neurons project to the motor columns of the spinal cord (Peyron et al 1998; van den Pol, 1999). Electrical stimulation of feline LH resulted in a complex sequence of depolarizing and hyperpolarizing potentials (Yamuy et al 2004). Also application of SB- 334867, an OX1R antagonist, decreased the amplitude of depolarizing potentials. Lastly, extracellular administration of orexin A to the motor neurons resulted in depolarization.

Orexin A also regulates arousal. Previously it has been mentioned that Fos expression of orexin neurons is increased during the dark (active) period in rats and is lower during the light (inactive) period (Yoshida et al 2001). Sakurai (2005) hypothesizes that the activity of orexin neurons is linked not only to feeding but also to arousal and rest behavioral states. Intracerebroventricular administration of orexin-A provoked a dose-

related increase in physiological responses to stress including mean arterial pressure and heart rate in conscious rats (Shirasaki et al 1999).

Returning to the anatomical framework created before, the locus coeruleus receives dense orexin innervation. This region is important for the physiological response to panic and stress (Ashton- Jones et al 1981). Application of orexin A increases firing of noradrenergic neurons of the locus coeruleus. In addition measures of arousal, including grooming, increased body temperature, and locomotor activity.

Some research suggests that orexin A is better correlated to motor activity and less to caloric intake and metabolism. Orexin A immunoreactivity in rats following intense negative energy balance including 48 hour food deprivation, laboratory induced diabetes, or a combination of the two were not significantly different as compared to the controls (Swart et al 2001). Fujiki et al (2001) examined orexin A levels across a 24 hour period and during periods of food restraint. Similar to previous studies orexin levels fluctuated with the circadian cycle: during the light (inactive) phase, orexin A levels dropped to 40% to that of the dark (active) phase. However, following 72 hours of food deprivation during the light phase, the orexin A concentration was increased to levels comparable to that of the dark phase. In addition, orexin A levels in canine csf did not change following 48 h of food deprivation but was correlated with physical activity during sleep deprivation (Wu et al, 2002).

Conclusion: orexin connects multiple components of energy balance

The specific role of orexin in energy homeostasis is certainly complex. However, it is a requisite to coordinating feeding responses and activity levels. Feeding behavior

results from coordinated stimulation and inhibition arising between numerous modulators. Although we have looked at feeding, sleep- wake cycles, and physical activity separately, in living organisms it is the interactions of these behaviors that determine energy balance. Evolutionarily joining these behaviors has vast significance in goal directed behaviors and enhanced survival. For example, when an animal incurs a negative energy balance due to reduced food availability or physical exertion, they become more awake and alert as they engage in food seeking behaviors. One could venture that increased arousal and movement would help them find food and remain vigilant of predators.

Based on the evidence supporting orexin's role in feeding behavior and motor facilitation we designed an experiment to investigate orexin A's effect on motivation for a sucrose solution reward and motor behaviors. Although further work was designed to tease apart this mechanism and determine the role of the NAc in these behaviors, it could not be conducted due to a lack of affect in the first phase.

EXPERIMENTAL PROCEDURES

MATERIALS AND METHODS

Subjects.

Male Sprague-Dawley rats weighing between 250 and 400gm at study onset served as Ss for experiment. Ss were between 6 and 12 months old at the beginning of operant training. Ss were housed independently in Plexiglas cages in a humidity- and temperature-controlled vivarium maintained on a 12hr light/dark cycle (lights on from 0730 to 1930 h and dark from 1930 to 0730 h). Food and water were available ad libitum.

Surgery.

All Ss were implanted with a single 27ga. guide cannula directed at the right lateral ventricle (stereotaxic coordinates: -0.3 mm AP; +0.9 mm LM; -2.4 mm DV from bregma). In addition a second set of guide cannulae that were not used in this experiment were directed bilaterally into the nucleus accumbens (stereotaxic coordinates: +1.4 mm AP; +-1.8 mm LM; -5.5 mm DV from bregma). The rats undergoing surgery were anesthetized with isoflurane vapor in 100% O_2 and mounted in a standard stereotaxic frame with the tooth bar initially positioned 5 mm dorsal to the interaural line. Ss were tested using a tail pinch method for maintenance of plane of anesthesia every 15 min during surgery. The cranium was exposed, and two small burr holes were drilled into the bones underlying the NAc. Following placement of the cannula into the NAc, the tooth bar was lowered to 0 mm and the guide cannula was placed unilaterally into the left ventricle in a similar fashion as before. Cannula were held in place with dental acrylic

affixed to three anchor screws placed in the nasal and parietal bones. A stylet was inserted into the cannula to maintain patency. Topical lidocaine (4%) was administered to the area surrounding the skin surrounding the dental cement. Ss were then placed under a heat lamp following surgery. Ss were given a 10-day interval following surgery before the onset of behavioral procedures.

Apparatus.

Ss were trained in eight standard operant chambers (Med-Associates, St. Albans, VT) measuring 30.5 cm x 24.1cm x 21.0 cm and housed within sound attenuating cubicles equipped with high-output ventilation pole fan blowers. A lever was situated on the center pane of one wall 10 cm above the grid floor. Directly above the lever was a house light situated two cm from the ceiling. Twenty percent (wt/vol) sucrose solution was available through a lick spout located to the left of the lever at the same height. A stimulus light was situated two cm above the lick spout. To the right of the lever was an inactive food hopper and head poke sensor. All paradigmatic events were controlled by a microcomputer running Med PC IV software (Med-Associates). Data sorting and analysis was accomplished through Neuroexplorer and SPSS. Except for the cumulative activity records, which were created in Neuroexplorer, all figures were created in Excel.

PROCEDURES

Operant behavior

Licks were defined as the times when the subject completes a circuit by touching its tongue to the sucrose solution fountain and simultaneously touching at least one of the

four grate bars closest to the fountain. Rewards are the drops of sucrose solution delivered from the lick fountain. Fluid was delivered over a 10 s interval following the required response of the reinforcement schedule. During periods of access to reward, the lever press retracted, the house light turned off, and the stimulus light was activated.

Prior to surgery, Ss underwent daily training sessions. Rats were water deprived for the first training session. Initial training began on a fixed ratio (FR1) where access to the sucrose solution reward was available for 10 s following one lever press. The sessions ran 12 h. Following completion of the session, Ss received free access to water in the home cage. After successful completion of the FR1 task (ie access to the reward to 100 times) the required response ratio was increased to five lever presses (FR5) and access to the sucrose reward was contingent upon five lever presses. The FR5 training sessions lasted 6 h. Upon successful completion of the FR5 schedule of reward (defined as access to the reward 20 times), Ss were trained on a variable response schedule of reinforcement (VR) where required responses were randomly chosen from an integral value 1-10 but cumulatively averaged to 5 (VR5). Session length for VR5 training was 3 h.

Following successful completion of VR5 (ie 100 lever presses) Ss were switched to a progressive ratio schedule of reinforcement (PR). This schedule requires an increasing number of lever presses before the onset of access to reward. Ss were administered PR5 sessions (1.5 h long) where required in session response sequences increased by 5 (ie the sequence of required responses was 5, 10, 15, and so on).

Next Ss received training for a geometric progressive ratio schedule of reinforcement (PRG). The number of required responses is given by the equation " $5e^{ik}$-5", where i is the reward number and k is a constant (Richardson and Roberts, 1996;

Nicola and Deadwyler, 2000). For this experiment *i* was set to 0.22, creating the following sequence of required lever presses prior to reward access: 1, 3, 7, 12, 19, 29, 44, 65, 95, 137, 198, 284, 406, and 581. For example, to access the first 10 s period of reward the Ss must perform one lever press, the second period of reward requires an additional three lever presses, the third period of access requires an additional seven, etc. The breakpoint is defined as the last number of lever presses completed of the PRG sequence before the 1.5 h cut off time for the PRG sessions.

Following surgery, Ss received 10 reacquisition sessions of the PRG schedule using sessions 1.5 h in duration. Before beginning the restraint sessions, stability in measured operant behavior and sucrose solution intake was assessed from day to day.

Drug infusion.

Prior to the first drug infusion, Ss received three restraint sessions and one sham session. During the restraint sessions, Ss were gently hand restrained for 3 min immediately before being placed into the operant chamber. During sham infusion sessions, Ss were being hand restrained and the obturators were removed. Next a 30-gauge injection cannula, extending 2.0 mm beyond the ventral tip of the guide was inserted into the guide. The injector needle was held in place for 3 min. The obturators were then replaced and the rats placed in the operant chambers.

In subsequent drug sessions, the 30- gauge injection cannula was inserted and held in place 1.5 min prior to drug administration. Drug was infused at a rate of 1.67μL per minute over a 3 min period. Following drug infusion, the injector was held in place for an additional 1 min to ensure complete flow through.

16 Ss were assigned to one of two drug treatments: saline or 30 nM orexin A (in 5μl saline vehicle) injected into the lateral ventricle in a counterbalanced Latin Square design 3-5 h into the light phase. With this design each Ss received both treatments with at least 4 days between injections. Ss were separated into groups of 4; however, the experimental procedures were identical excluding the drug dose. Following the drug infusion, Ss were placed immediately into the operant chamber and the PRG session was initiated. Session length was 1.5 h regardless of responding.

Histology.

Cannula placement will be determined by postmortem histological verification. Following the completion of all testing, each subject will be administered a lethal dose of sodium pentobarbital (100mg/kg). Ss will be perfused transcardially with normal saline and brains will be fixed, sectioned, and stained according to standard procedures. Cannula tracks will be mapped with the aid of a light microscope. Histological examination of the cannula tracks has not yet occurred. Following termination of testing in this experiment, Ss were used in another study and therefore histological testing has not been carried out.

RESULTS

Following recovery from surgery, all Ss received reacquisition training prior to drug infusions. Ss were monitored for two successive sessions for behavioral stability before initiation of the restraint sessions. Pearson R correlation analysis show that day-to-day performance of drops rewarded ($R= 0.598$, $p=0.018$, $n=15$), licks ($R= 0.896$, $p=$

0.000 n=15), and lever presses (R= 0.0626, p=0.012, n=15) did not differ significantly. Figure 1 compares task performance on the two sessions prior to the first restraint session.

<INSERT FIGURE 1 HERE>

One subject had a clogged cannula due to a broken injector and was not used for subsequent behavioral testing and was excluded from the following analysis. Recorded drops delivered by the lick fountain were assessed as a measure of sucrose solution intake. Following stable responding of performance under the PR schedule, infusions of either orexin A (30nM) or saline were executed. Paired t- tests did not demonstrate that administration of orexin A altered consumption of sucrose solution reward (208.077± 75.93; mean ±SEM) compared to the saline control (240.46± 85.54) ($t_{.05, 12}$= -.670 p= .797). In addition licks at the fountain, rewarded or not, were analyzed (saline: 369.92±108.58; orexin A 30 nM: 350.15± 121.47). Paired t- tests failed to find a significance of effect due to orexin ($t_{.05, 12}$= -1.364 p= .198).

<INSERT FIGURE 2 HERE>

Breakpoints and lever presses were analyzed as a function of appetitive behaviors. As shown in figure 3, orexin A did not affect breakpoint of the number of lever presses (saline: 41.38± 22.99; orexin A 30 nM: 38.85± 22.12). Paired t- tests failed to establish a significantly different effect of orexin A on breakpoint ($t_{.05, 12}$= -.733 p= .477). lever

presses are an appetitive behavior and also a measure of motivation since performance of this operant is required to gain access of reward. Similar to breakpoint, paired t- tests failed to reveal a statistical difference between orexin A (77.92± 43.22) and saline control (90.00± 54.08) on lever pressing ($t_{.05, 12}$= -1.514 p= .156).

<INSERT FIGURE 3 HERE>

In order to determine whether orexin A infusion altered the pattern of operant responding, data from a subset of 10 Ss for which behavioral timestamps were obtained for both drug sessions were assessed. The Ss from the smaller subset had similar levels of operant responding as compared to the larger sample. The number of drops (saline: 203.2± 25.56; 30 nM orexin A: 239.1± 23.35), licks (saline: 352.9± 31.79; 30 nM orexin A: 339.2± 42.71) and lever presses (saline: 89.3± 17.97 ; 30 nM orexin A: 76.9± 15.69) were comparable to the full group data measures. Examination of cumulative records reveals that the profile of behavior following orexin infusion was similar to that observed for saline control. Figure 4 contains representative records of two Ss; one where operant responding was higher following orexin A infusion and a second Ss, where operant responding was reduced in comparison to orexin infusion. Note that the majority of activity occurred within the first 15 minutes of the session with only short intervals of activity later in the session.

<INSERT FIGURE 4 HERE>

Although the total values of measured operant behavior and sucrose solution did not differ, it is possible that orexin A was affecting the finer patterns of responses. Figure 5 displays the period between the last required lever press in a PRG sequence and the first lick at the fountain. This interval is defined as the lick latency. Lick latency is a measure of motor response and could demonstrate motor facilitation as suggested by the literature or a possible motor disruption. Analysis of lick latency using paired t-tests failed to observe a statistical difference between orexin A (0.83± 0.28) and saline (0.98± 0.56) administration ($t_{.05, 9}$= -.895 p= .394).

<INSERT FIGURE 5 HERE>

Another measurement of motor activation is lick frequency. This is derived from the taking the reciprocal of the interspike interval of licks at the fountain. Next the reciprocal is obtained, giving the frequency of licks in the units licks per second or hertz. The mode lick frequency was determined as the frequency at which the most licks frequencies occur. As shown in figure 1 these did not differ due to orexin infusion (saline: 7.28± 2.09; orexin A 30 nM: 7.045± 0.47). Paired t-tests do not reveal an effect of drug on lick frequency ($t_{.05, 9}$= 1.308 p= .736).

<INSERT FIGURE 6 HERE>

DISCUSSION

In the present experiment, we examined the ability of orexin A to increase appetitive and consumatory behaviors under a PR schedule of reinforcement. Administration of orexin A did not alter sucrose solution intake as compared to the saline controls. Although orexin A failed to increase consumption of reward, it is possible that it affected motivational responses. However, neither the breakpoint nor the number of lever presses were changed. In addition to sucrose solution intake and motivation, administration of orexin A could be facilitating motor responses. Similar to other findings, orexin A infusion did not increase lick frequency or decrease lick latency.

The findings that orexin A administered icv failed to alter sucrose solution consumption, motivational, and motor responses is highly unexpected. Past literature heavily supports the role of orexin A in all of these functions.

However, there is a precedence found in the literature detailing circumstances when orexin A fails to increase feeding and drinking. The first possible reason is that the Ss were too old. Takano et al, (2005) demonstrated that orexin A infusion icv does not induce food intake in rats older than 24 months. Similarly Kotz et al (2005) found that orexin A infused into the LH failed to have a effect on measures of feeding in old rats (24 months) but also the power of orexin A diminished in effect so that only the highest dose increased feeding in rats 12 months old. The Ss in this study varied in age from 6 months to 12 months at the onset of the study. However by the time of drug infusions Ss were aged 12- 14 months. It could be the case that the study included rats that were entering middle age during the phase of orexin A administration and had decreased sensitivity to orexin A.

The second reason for an attenuated effect occurs when orexin levels are already high due to fluctuations in orexin A levels in concordance with photocircadian cycles (Yoshida et al 2001). In this study, the concentration of orexin A gradually increased with the onset of the dark periods (active phase) until just before the beginning of the light period (rest phase) in rats. From then orexin A levels continue to decrease until shortly before the dark phase. Although the concentration of orexin A was not measured, the experiment was designed to take into account circadian cycling of orexin. Orexin infusions occurred during the period of 11 h and 13 h. These are intervals of reported low concentrations of orexin; therefore, it is not likely that this is the cause of the lack of effect. Nonetheless, Thorpe et al (2005) demonstrate that infusion of orexin A at 14 h can increase food intake.

Food and water deprivation can also cause increased orexin A levels. Pre-pro orexin levels are shown to increase in response to food deprivation for 48 h (Sakurai et la 1998). Cai et al (1999) also observed the increase in pre-pro-orexin mRNA following 48 h food restriction, but in contrast found that chronic fasting for six days resulted in unchanged levels of the mRNA. Water deprivation does not alter the number of orexin A immunoreactive neurons in the LH of rats (Yao et al 2005). Nevertheless, similar to food deprivation, pre- pro- mRNA levels upregulated in water deprived rats. This experiment did not use either food or water deprivation, but an aqueous sucrose solution, thus combining results of the results from caloric and water intake. The conflicting observation of increased orexin mRNA without similar increases in orexin is hypothesized to be due to increased release of the neuropeptide with swift degradation without reuptake. However, regardless of pre- pro orexin precursor peptide levels or

peptide levels, administration of orexin A has been shown to increase appetite and physical activity when the rats have not been food or water deprived and it was expected to do the same in this experiment as the Ss were not placed on any fasting diet.

A third possibility for the negative results lies in the range of orexin A doses cited in the literature that increase water and/ or food intake. In the original paper detailing the discovery of the orexin neuropeptide, Sakurai et al (1998) stimulated food intake in rats with both a 3 nM and a 30 nM dose of orexin A. The lowest concentration of orexin A infused into the ventricle and still increase drinking occurred at 3 nM. However, Takano et al (2004) used the same dose of orexin A, 3 nM, and failed to see an effect on drinking in young rats (note that the same dose and a higher dose, 30 nM failed to alter water intake in old rats as well). However, Takano et al demonstrated that food intake increased in young rats using the lower dose, 3 nM. Two differences in the research paradigms are the most likely cause of this inconsistency: 1) Takano et al used the lowest dose given by Kunii et al and 2) Kunii only allowed access to water during this period and Takano allowed free access to food and water during the sessions. The second hypothesis is supported by Siegel (2005), who argues that orexin non- selectively activates motor responses, and that rats will increase whichever physical behavior is available to them.

In addition to reasons found in the literature, laboratory procedures can possibly account for the lack of effect of orexin administration. The first is incorrect cannula placement. As of the writing of this manuscript, histological examination of the cannula tracks had not been performed. It was deemed ethically and morally sound to allow use these Ss in a different set of experiments for educational purposes prior to sacrificing these Ss. The use of those Ss in a second experiment prevented unnecessary surgery and

sacrifice of rats. In addition, those experiments would not in any way affect the results of this experiment since data collections terminated before their use.

A second account of the difference between our results and previous studies includes the use of PRG schedule of reinforcement. This PRG schedule has not been used in research examining the effects of orexin. Most studies of the PRG schedule use it in conjunction with drug studies of motivation. In comparison to another study investigating orexin and motivation Thorpe et al (2005) used a PR5 ratio, ie after the third reward, the PR5 schedule of reinforcement allowed more access to reward than the PRG used in this study. Thus the PRG schedule may be less sensitive to neuropharmacological manipulation in comparison to PR schedules where the increase in required responses is fixed. In addition access to reward is restricted to 10 s. following the last lever press in a requirement sequence. Observations that the Ss continued to lick following this forced cut off to reward suggest that Ss would have continued drinking at the fountain for a longer period. Thus the duration of rewarded licks (lick bouts), another function of motivation, were not recorded.

Orexin A is a multifaceted peptide and of high interest to this laboratory. It is important for future work that the reasons behind the failure of orexin A to increase consumption, motivation, and physical activity be accounted for. It is important to solve the cause behind the problem.

Future studies

This study was designed to act as the first step in a series to elucidate the mechanism by which orexin acts on motor and reward systems to govern sucrose intake

and physical activity. It is hypothesized that orexin acts on the NAc through an inhibitory effect and that blocking other means of inhibition through a $GABA_A$ antagonist will result in a decrease in feeding and drinking. To test this hypothesis, those behaviors were to be monitored in non- food or drink deprived rats following co- administration of orexin A into the lateral ventricle and the $GABA_A$ antagonist bicuculline into the NAc.

Although the LH has long been recognized as the center of feeding, the mechanism has been elucidated only in recent years. Studies have demonstrated that multiple orexinergic and anorexinergic efferents exit the LH. However, the role of external input into the LH remains a mystery. Neurotransmitters applied to the NAc, including AMPA and GABA, are shown to modulate feeding through the LH (Maldonado- Irizzary et al 1995; Stratford et al 1998; Stratford and Kelley 1999). Also, both core and shell sub regions of the NAc are networked to control taste, perception, energy balance, and movement control (Ricardo and Koh, 1978; Saper 1982, and McDonald and Jackson, 1987) Further studies in this lab will hopefully capitalize on knowledge and data gained from this experiment and utilize knowledge of the NAc connections with the LH.

Nucleus Accumbens and its role in feeding

The NAc is composed of two distinct regions, the core and the shell, which are distinct with regard to anatomy and output behavior (Maldonado- Irizzary and Kelley, 1994). The core is embryonically derived from the striatal complex and serves in conditioning tasks. The NAc core (NAcc) region connects extensively to basal ganglia output structures including the ventral palladium, subthalamic nucleus, and the substantia

niagra (Groenewegen and Ruschen, 1984, Groenewegen 1980). During embryonic development, the NAc shell (NAcSh) is derived from the amygdala. As the stimulus reward center, the shell serves a different role from the core. It projects to subcortical limbic regions including the LH, ventral tegmental area (creating the mesolimbic pathway), and the locus coeruleus. Each of these has an important function with regard to feeding and reward. The LH functions in homeostasis. The ventral tegmental area contributes to the processing of reinforced. The locus coeruleus is a key modulator of arousal and locomotor activity in response to sensations of panic and stress. Hence, due to the NAc's position within the mesolimbic pathway and innervation from the hypothalamic pituitary axis, it is placed in a prominent position to translate hunger into reward seeking through homeostatic mechanisms established in the hypothalamus.

GABA mediation of the NAcSh and motivation

The majority of projections from the NAcSh are medium spiny neurons (MSNs) (Meredith et al 1993). GABA receptors are located presynaptically and postsynatpcially in MSNs. Thus they are modulated by GABAergic input and are GABAergic meaning they have an inhibitory affect on postsynaptic terminals.

MSN projections onto the LH are demonstrated by injection of the $GABA_A$ agonist muscimol into the NAc and the subsequent increase in the number of neurons in the LH expressing Fos. NAcSh MSN projections function in ingestive behaviors. Inhibition of neural activity by MSN in the NAcSh by stimulation of $GABA_A$ or $GABA_B$ receptors induces robust effect of eating in laboratory chow (Basso and Kelley, 1999).

Also, blockade of AMPA receptors reduces excitation of MSNs, and also elicits a feeding response (Maldonado-Irizarry, Swanson, and Kelley, 1995).

Zheng et al (2002) and Kelley et al () showed through c-fos and orexin double labeling, injection of the $GABA_A$ agonist muscimol into the NAcSh increased Fos labeling in the hypothalamus as compared to saline control rats. In addition, in the arcuate nucleus, Fos activation was significantly lower in neurons co expressing cocaine and amphetamine related transcript (CART) and proopiomelanocortin (POMC). Zheng et al al suggests that the NAcSh regulates food intake through the coordinated stimulation of hypothalamic neurons expressing orexinergic peptides and suppression of anorexinergic peptides.

Food intake is induced by infusion of muscimol into the NAcSh (Stratford and Kelley 1999). Inhibition of GABAergic MSNs would result in suppressed release of GABA onto neurons. As a result neurons of the LH would be disinhibited and release more orexin neuropeptide, leading to an increase in feeding. However, as a corollary to the $GABA_A$ agonist theory on the modulation of feeding, it would be expected that $GABA_A$ antagonism would have the opposite effect and decrease feeding. Znamesky et al (2001), have demonstrated that neither $GABA_A$ nor $GABA_B$ antagonists alter feeding on their own. Yet, Kandov et al (2006) showed that following food deprivation infusion of bicuculline or saclofen decreases deprivation induced feeding. Although, immunohistochemistry was not included in the study, the food deprivation has been shown to increase orexin levels.

In an experiment similar to the one proposed here, Kokare et al (2006) infused rats with orexin A icv and then delivered bicuculline intraperitoneally. As a result feeding

was attenuated. However, the design of this experiment only demonstrates that orexin and GABA interact, it does not specify where and in what context.

However, investigation into the role of GABA and the NAcSh could not be carried out due to a lack of effect of orexin A in this current study. Icv administration of orexin A failed to alter of appetitive and consumatory behaviors. For this reason and future laboratory goals it is important to determine the cause of the negative results in our experiment. Besides orexin A's motivational and motor behaviors studied in this experiment orexin is hypothesized to have function in other goal directed behaviors including addiction.

In conclusion, this study failed to show that orexin A icv altered measures of appetitive and consumatory behaviors in rodents. I believe that the most likely reason is that the rats were too old. However, other possible reasons include incorrect cannula placement and endogenous orexin A levels. Future studies will use younger rats.

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Figure Captions.

Figure 1. Performance stability. Line of best fit shows the stability of responding between two days prior to restraint sessions. The x and y axis represents performance on day 1 and day 2 respectively. Each point represents one subject. A) Drops delivered; B) licks; and C) lever presses

Figure 2. Effect of orexin A on drops delivered and licks. A) shows the effect of orexin A on drops earned. Saline and orexin A have a comparable effect on the drop rewards. B) displays the licks performed by Ss. Once again saline and orexin A have comparable effects.

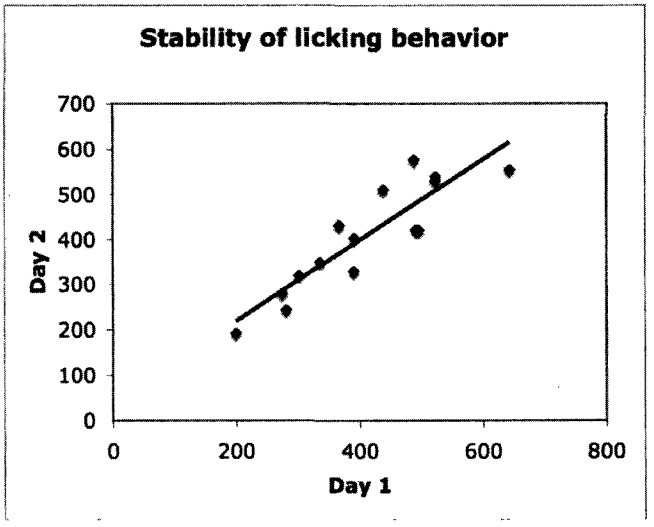
Figure 3. Representative cumulative activity records of progressive ratio performance following icv infusions of saline (grey) or 30 nM orexin A (black). A) Drops delivered; B) licks; and C) lever presses.

Figure 4. Effect of orexin A on breakpoint and lever presses. A) shows the mean breakpoint. Breakpoint was similar between the saline and orexin A treatments. B) displays the mean lever presses performed by Ss. Orexin A treatment did not differ from the saline control treatment.

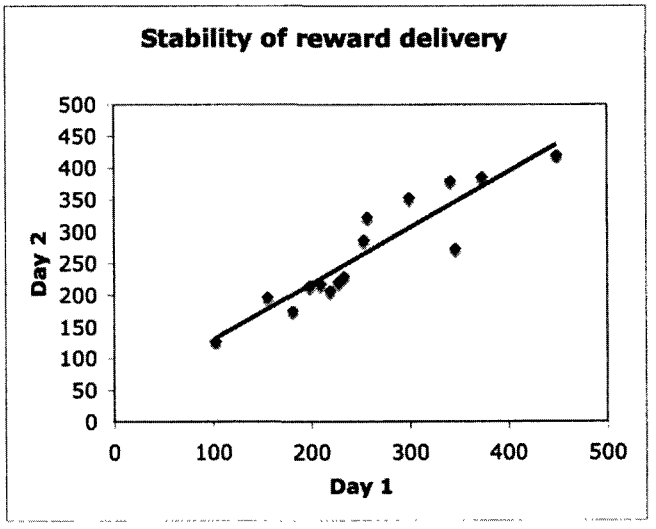
Figure 5. Effect of orexin A on lick latency. The interval of time between the last lever press and rewarded lick. Lick latency of the orexin A dose was similar to the saline control.

Figure 6. Effect of orexin A on lick frequency. Orexin A infusion did not alter the mode lick frequency as compared to the saline dose.

A)


Stability of licking behavior
Day 2
Day 1
0
100
200
300
400
500
600
700
0
200
400
600
800

B)


Stability of reward delivery
Day 2
Day 1
0
50
100
150
200
250
300
350
400
450
500
0
100
200
300
400
500

C)

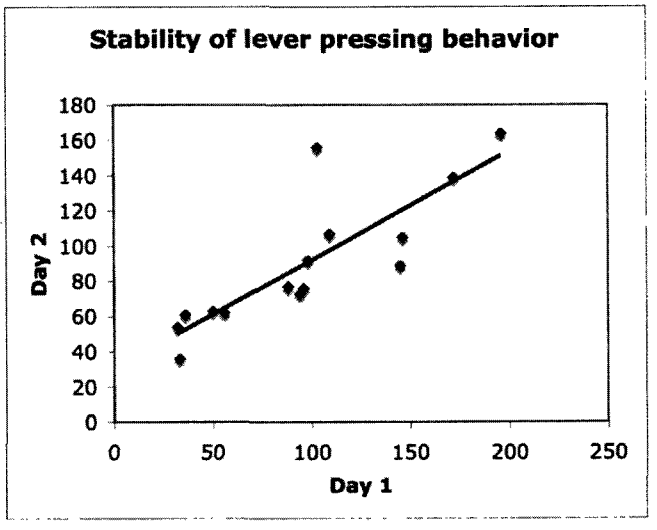
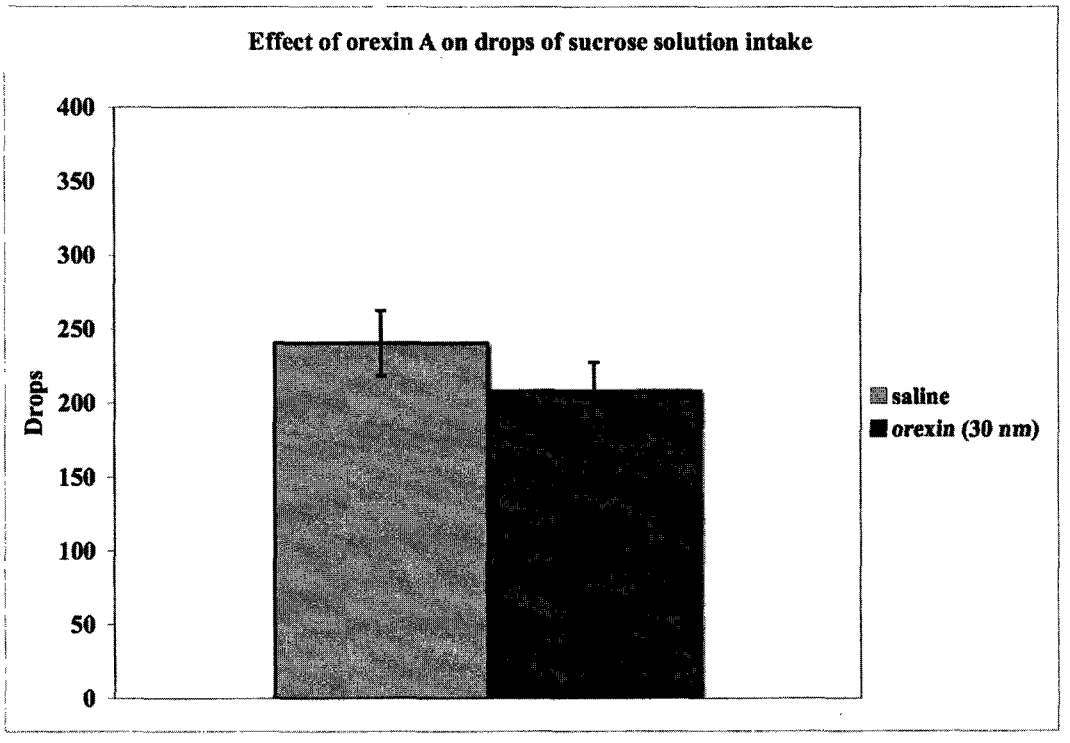

Stability of lever pressing behavior
Day 2
Day 1
0
20
40
60
80
100
120
140
160
180
0
50
100
150
200
250

Figure 1.

A)


Effect of orexin A on drops of sucrose solution intake
Drops
400
350
300
250
200
150
100
50
0
saline
orexin (30 nm)

B)

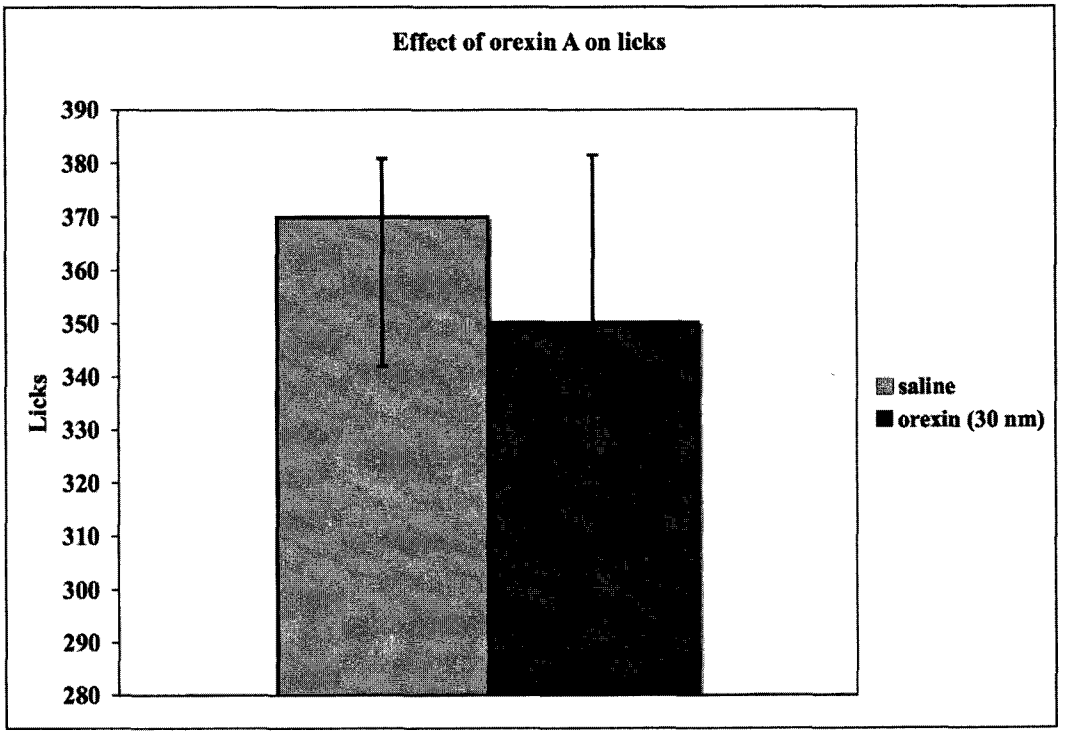
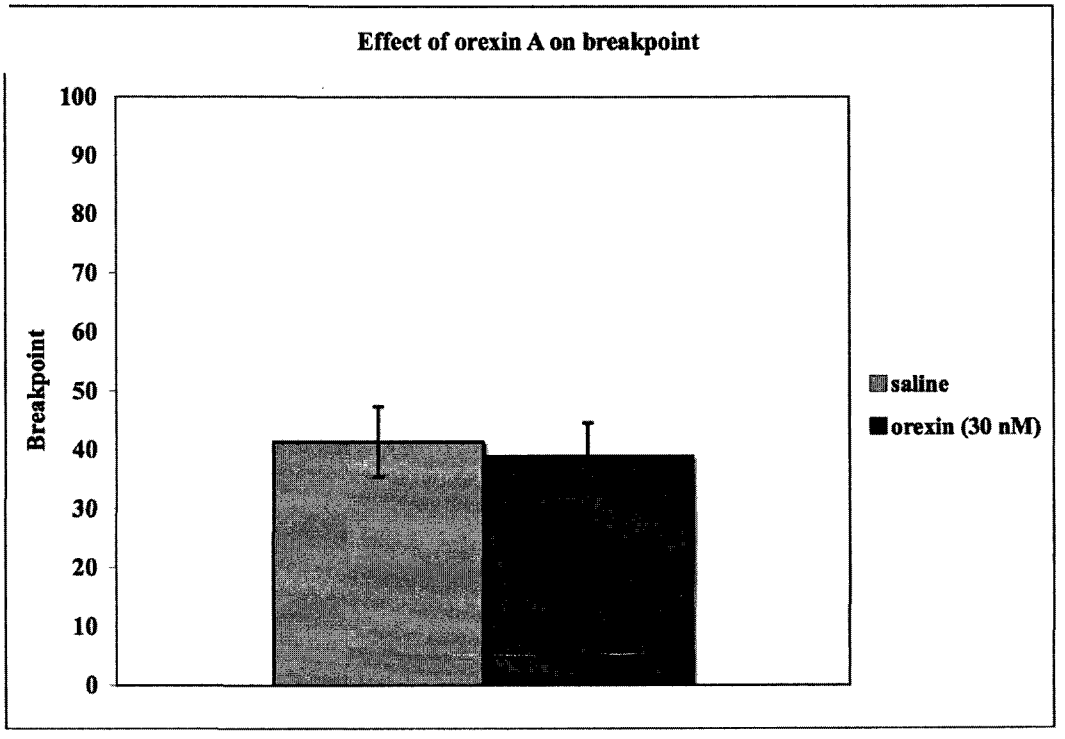

Effect of orexin A on licks
Licks
390
380
370
360
350
340
330
320
310
300
290
280
saline
orexin (30 nm)

Figure 2.

A)


Effect of orexin A on breakpoint
Breakpoint
100
90
80
70
60
50
40
30
20
10
0
saline
orexin (30 nM)

B)

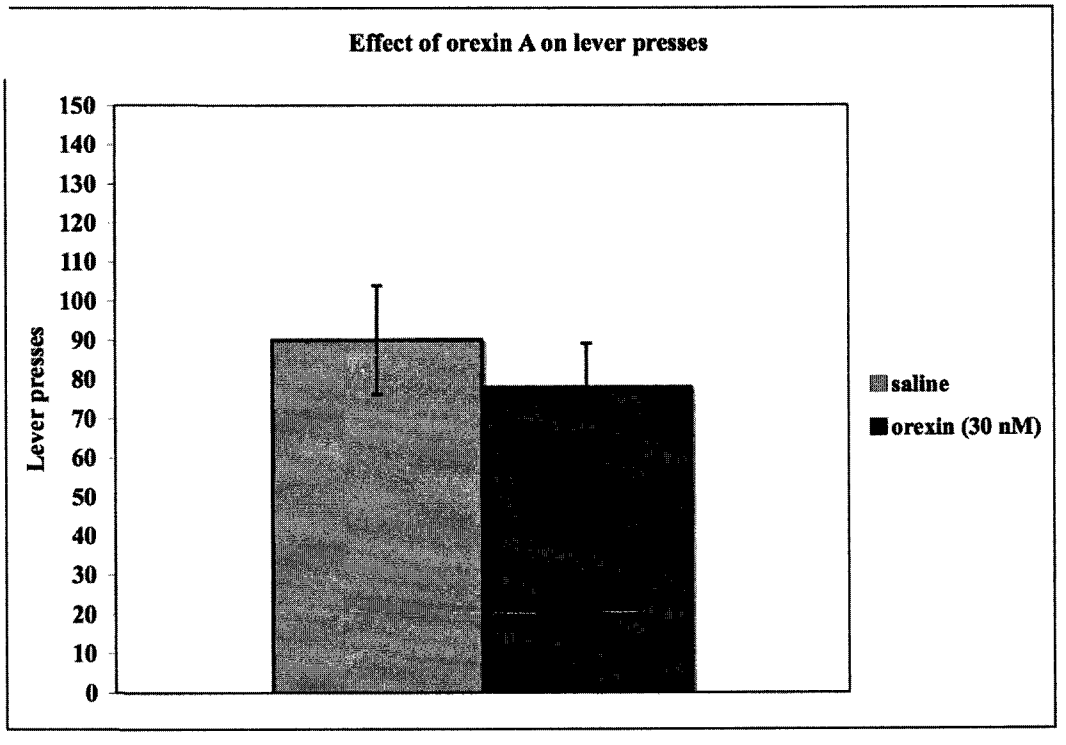

Effect of orexin A on lever presses
Lever presses
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
saline
orexin (30 nM)

Figure 3.

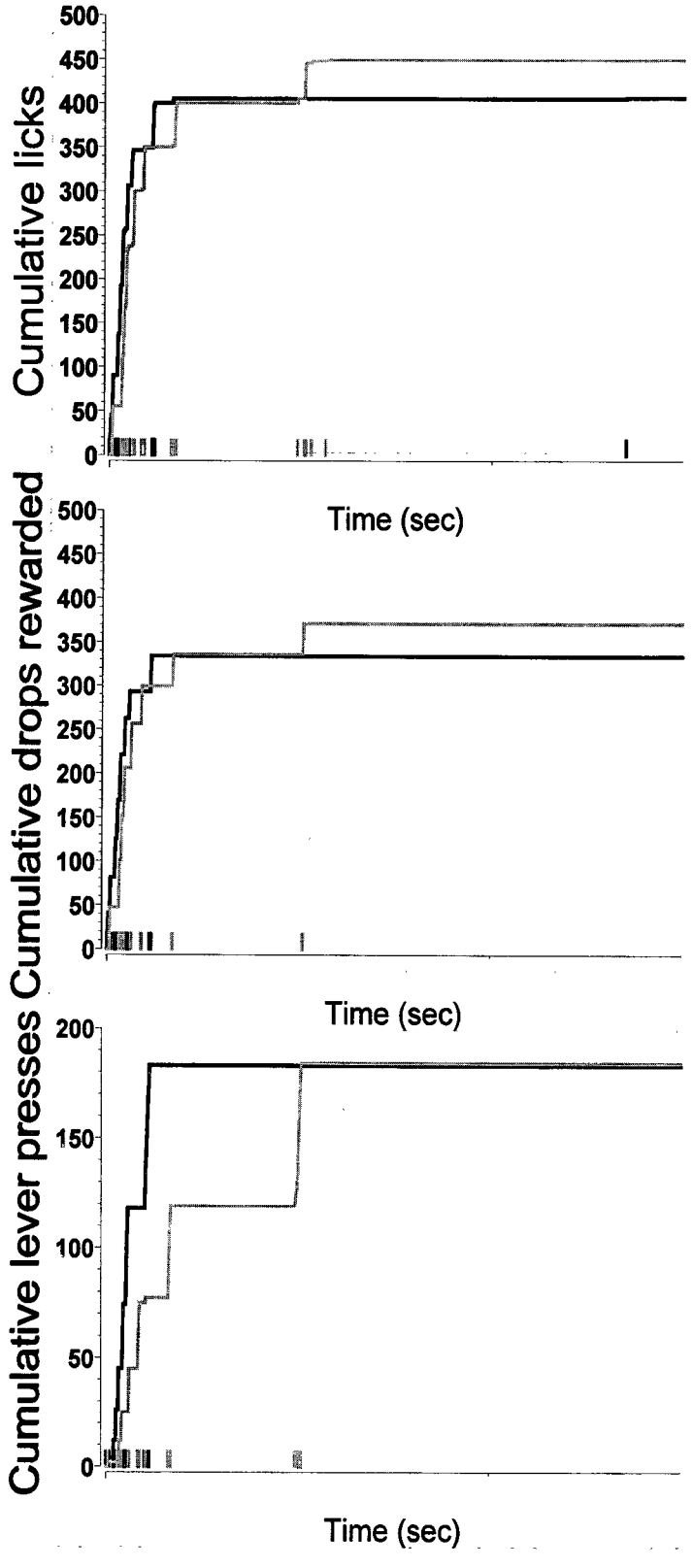

A)
B)
C)
Cumulative licks
Cumulative drops rewarded
Cumulative lever presses
500
450
400
350
300
250
200
150
100
50
0
Time (sec)
Time (sec)
Time (sec)

Figure 4.

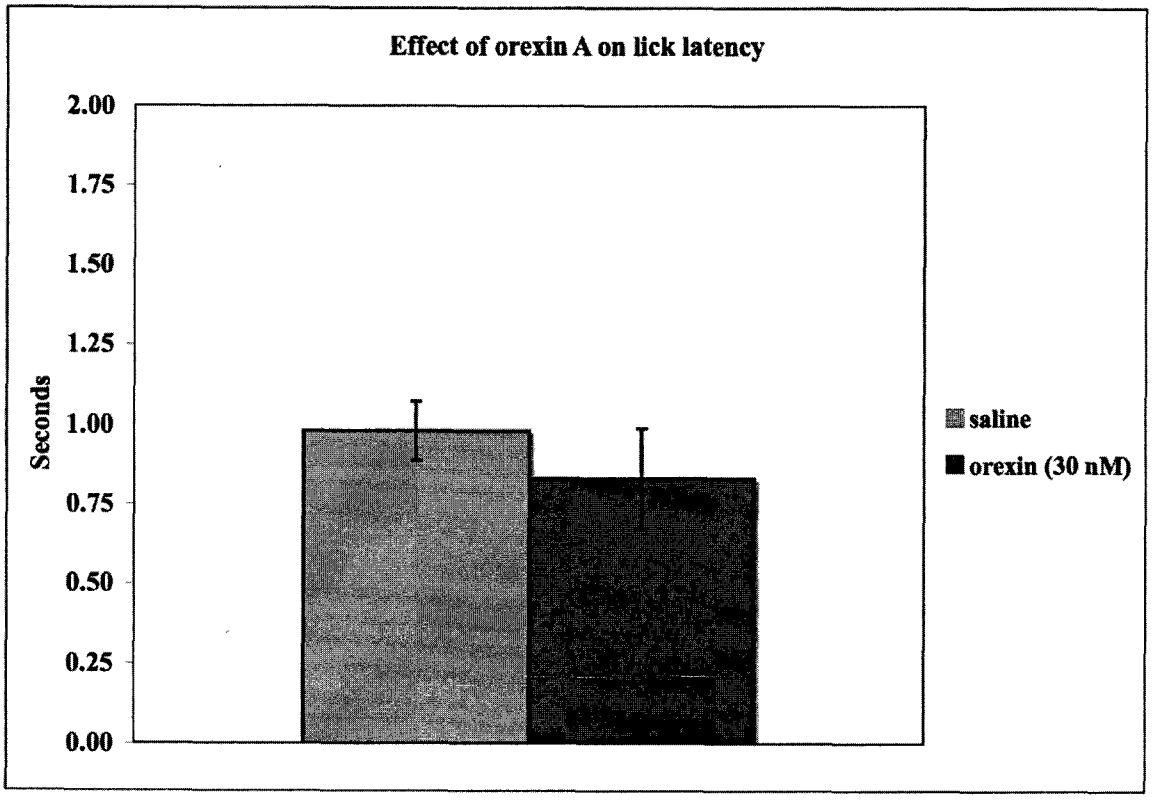

Effect of orexin A on lick latency
Seconds
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
saline
orexin (30 nM)

Figure 5.

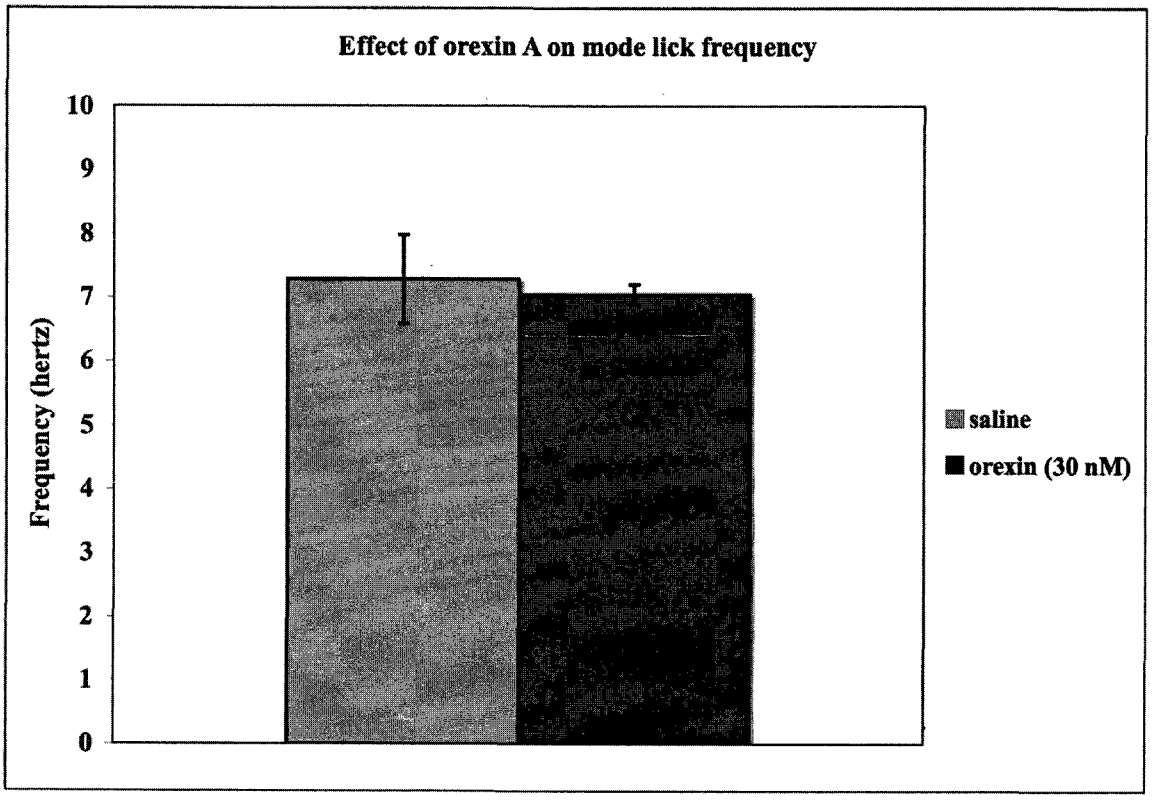

Effect of orexin A on mode lick frequency
Frequency (hertz)
10
9
8
7
6
5
4
3
2
1
0
saline
orexin (30 nM)

Figure 6.

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