THE VENTRAL TEGMENTAL AREA AND NUCLEUS ACCUMBENS SHELL AS A DISTRIBUTED BRAIN NETWORK FOR FEEDING ELICITED BY GABA-B RECEPTOR AGONISTS: MODULATORY ROLES OF GABA AND OPIOID RECEPTOR SUBTYPES IN RATS

By

PATRICIA KAVANAGH-MINER

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7/31/12	Dr. Richard J. Bodnar
Date	Chair of Examining Committee
7/31/12	Maureen O'Connor
Date	Executive Officer

Dr. Robert Ranaldi, Queens College

Dr. Andre Ragnauth, City College

Dr. Grace Rossi, Long Island University-Post

Dr. Jon Horvitz, City College

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

THE VENTRAL TEGMENTAL AREA AND NUCLEUS ACCUMBENS SHELL AS A DISTRIBUTED BRAIN NETWORK FOR FEEDING ELICITED BY GABA-B RECEPTOR AGONISTS: MODULATORY ROLES OF GABA AND OPIOID RECEPTOR SUBTYPES IN RATS

by

Patricia Kavanagh-Miner

Advisor: Doctor Richard Bodnar

Food intake is significantly increased following administration of GABA and opioid agonists into the nucleus accumbens (NAC) shell and ventral tegmental area (VTA) with receptor-selective antagonist pretreatment capable of blocking these responses within sites. To evaluate whether regional VTA and NAC shell interactions occur for GABA-mediated feeding, specifically meal size of chow intake, the first aim examined whether feeding elicited by the GABA-B agonist, baclofen, microinjected into the NAC shell or VTA dose dependently blocked pretreatment with either the GABA-B antagonist, saclofen, or the GABA-A antagonist, bicuculline, into the alternate site, VTA or NAC shell in rats. VTA and NAC shell saclofen dose-dependently and significantly blocked feeding elicited by baclofen injected into the NAC shell and VTA baclofen, respectively. Whereas VTA bicuculline significantly blocked the increased feeding elicited by NAC shell baclofen, NAC shell bicuculline reduced but did not block feeding elicited by VTA baclofen.

To evaluate whether regional VTA and NACs feeding interactions occur for opioid receptor modulation of GABA agonist-mediated feeding, the second and third aims examined whether

feeding elicited by the GABA-B agonist, baclofen microinjected into the NACs or VTA was dose-dependently blocked by pretreatment with general (naltrexone: NTX), mu (beta-funaltrexamine: BFNA), kappa (nor-binaltorphamine: NBNI) or delta (naltrindole: NTI) opioid antagonists into the alternate site, VTA or NAC shell in rats. VTA NTX significantly reduced NACs baclofen-induced feeding. Correspondingly, NACs NTX significantly reduced VTA baclofen-induced feeding. Whereas, the high VTA BFNA dose reduced NACs baclofen-induced feeding, NACs BFNA failed to affect VTA baclofen-induced feeding. Whereas VTA NBNI at both doses reduced NACs baclofen-induced feeding, only the high NACs NBNI dose significantly reduced VTA baclofen-induced feeding. Whereas VTA NTI transiently reduced NACs baclofen induced feeding, NACs NTI failed to affect VTA baclofen-induced feeding. Therefore, the present series of studies suggest that GABA employs a distributed brain network in mediating its ingestive effects that is dependent upon intact GABA and opioid receptor signaling with kappa opioid receptors more involved than mu and delta opioid receptors underlying these regional effects.

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I dedicate this dissertation to my mother who is a never ending role model for me and to my dad who died just a few months ago from end stage Alzheimer's disease but to the end he was one of my biggest fans always asking me "when are you going to finish that thing?"

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NACs by acting on GABA-B receptors (present results) on an opioid-containing cell (e.g., dynorphin and GABA (Nestler, 2001; Shippenberg & Rae, 1997; Spangler et al., 1996). (**3**) VTA baclofen-induced feeding is then mediated by a kappa-opioid receptor located in the NACs (present results) that acts to inhibit an output system to elicit feeding. Local opioid actions within the NACs upon food intake (e.g., Ragnauth et al., 2000) are not included in the model for simplicity's sake, but include potential disinhibitory actions. *Panel B*: (**1**) Baclofen administered into the NACs acts on GABA-B receptors to elicit feeding (Znamensky et al., 2001), and in this model acts on GABA-containing neurons projecting from the NACs either directly to the VTA or indirectly to the VTA through the ventral pallidum (VP) (Churchill et al., 1991; Churchill & Kalivas, 1994; Kalivas et al., 1993; Meredith et al., 1993; Van Bockstaele & Pickel, 1995; Zahm et al., 1985). (**2**) NACs baclofen-induced feeding is mediated by GABA released from these neurons into the VTA by acting on GABA-A and -B receptors (present study) presumably on an opioid-containing cell. (**3**) NACs baclofen-induced feeding is then mediated by a kappa-opioid receptor located in the VTA (present results) that acts to inhibit an output system to elicit feeding. Local opioid actions within the VTA upon food intake (e.g., Lamonte et al., 2002) are not included in the model for simplicity's sake, but include potential disinhibitory actions.

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Figure 12. (A and B) An empirically-based parsimonious model of opioid and GABA antagonist modulation of feeding elicited by the GABA-B agonist, opioid and GABA antagonist modulation of feeding elicited by the mu-opioid agonist, DAMGO administered into the VTA (Panel A) or into the NACs (Panel B). *Panel A*: **(1)** DAMGO administered into the VTA acts on mu and kappa opioid receptors to elicit feeding (Lamonte et al., 2002) that are located in this

model on a VTA GABA interneuron. (**2**) VTA DAMGO –induced feeding is reduced by GABA-A and GABA-B antagonist pretreatment (Echo et al., 2002). These GABA receptors are located on a VTA output neuron that does not contain GABA because GABA-A or GABA-B receptor antagonists in the NACs fail to block VTA DAMGO-induced feeding (Ackerman et al., 2003). This VTA output neuron would then functionally activate a NACs opioid-containing neuron (Meredith, 1999). (**3**) VTA DAMGO-induced feeding is then mediated by NACs mu- and delta-opioid receptors (Bodnar et al., 2005) that act to inhibit an output system to elicit feeding. Local opioid actions within the NACs upon food intake (e.g., Ragnauth et al., 2000) are not included in the model for simplicity's sake, but include potential disinhibitory actions.

Panel B: (**1**) DAMGO administered into the NACs acts on mu, delta and kappa opioid receptors to elicit feeding (Ragnauth et al., 2000) that are located in this model on a NACs GABA interneuron. (**2**). NACs DAMGO-induced feeding is blocked by NACs GABA-B antagonist pretreatment (Znamensky et al., 2001). This GABA receptor is located on a NACs output neuron that does not contain GABA because GABA-A or GABA-B receptor antagonists in the VTA fail to block NACs DAMGO-induced feeding (Ackerman et al., 2003). This NACs output neuron in this model projects from the NACs either directly to the VTA or indirectly to the VTA through the VP (Churchill et al., 1991; Churchill and Kalivas, 1994; Kalivas et al., 1993; Meredith et al., 1993; Van Bockstaele and Pickel, 1995; Zahm et al., 1985). (**3**) NACs DAMGO-induced feeding is then mediated by VTA mu- and kappa-opioid receptors (Bodnar et al., 2005) that act to inhibit an output system to elicit feeding. Local opioid actions within the VTA upon food intake (e.g., Lamonte et al., 2002) are not included in the model for simplicity's sake, but include potential disinhibitory actions.

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Glossary of Abbreviations

BAC – Baclofen
BFNA - Beta-funaltrexamine
BIC – Bicuculline
CeA – Central Nucleus of the Amygdala
CNS – Central Nervous System
DA - Dopamine
DAMGO – [D-Ala2, N-MePhe4, Gly-ol]-enkephalin
GABA – Gamma-aminobutyric acid
MFB – Medial Forebrain Bundle
NAC – Nucleus Accumbens
NACs - Nucleus Accumbens Shell
NBNI - Nor-binaltorphamine
NMDA - N-methyl-D-aspartate
NTI – Naltrindole
NTS – Nucleus Tractus Solitarius
NTX – Naltrexone
SAC- Saclofen
Veh – Vehicle treated
VTA – Ventral Tegmental Area

CHAPTER ONE

SIGNIFICANCE AND SPECIFIC AIMS

Obesity is currently a world-wide health problem, associated with the incidence of heart disease, diabetes and cancer, contributing to early mortality and rising health care costs. Multiple factors have been implicated including greater food availability of highly palatable foods, larger portion sizes and a defect or adaptation in the homeostasis or appetite regulation of the organism. These factors may affect meal frequency and/or meal size. An increase in meal size and meal frequency is defined as hyperphagia, leading to weight gain, and is considered a hallmark of obesity (Schwartz, 2004). An "obesity epidemic" has been declared, supported by data showing that the incidence of obesity in adults in the United States is greater than 20% in all but one (Colorado) of the 50 states. The prevalence of obesity in children has also sharply increased over the last 30 years from 5% to 12% for 2-5 year olds, and from 5% to 17% for 6 to 19 year olds (Obesity and Overweight, 2010). Although the relationship between individual meal size and resultant obesity is a source of great debate as explained in the research of Ello-Martin, Ledikwe, Rolls(2005) and Keith et al(2006), neuroanatomical and neurochemical modulation of individual meals can be informative in understanding basic physiological and homeostatic processes. Meal size is positively influenced by the orosensory aspects of food, particularly taste and smell, and negatively influenced by pre and post ingestive chemo and mechano-receptors in the gut, e.g., serotonin 1, 2, 3,and 4 receptor subtypes (Kim & Camilleri, 2000), which communicate with the hindbrain and sends relays to the midbrain and forebrain (Smith, 2000). Both the positive and negative feedback of food intake are influenced by neurochemicals within the midbrain and forebrain to control appetitive motivation. Therefore, understanding the neurochemical and

neuroanatomical controls of food intake may have eventual bearing on the appropriate treatment of obesity.

Research in the basic neuroscience of the organism has contributed to the understanding and potential treatments of obesity. To date, animal research has demonstrated roles of many orexigenic neurotransmitters, including GABA and opioids, in stimulating feeding following direct injections in neuroanatomically connected brain areas (see review; Arora & Anubhuti, 2006).

Two reciprocally-interconnected neuroanatomical structures have received particular attention in this regard; the nucleus accumbens shell (NACs) and ventral tegmental area (VTA). Both opioid (mu and delta) and GABA (A and B) receptor subtype agonists are capable of stimulating food intake following direct microinjection into the VTA or NAC (Echo, Lamonte, Ackerman, Bodnar, 2002; Khaimova et al, 2004, Lamonte, Echo, Ackerman, Christian, Bodnar, 2002; Ragnauth, Moroz, Bodnar, 2000; Stratford, Kelley, 1997; Znamensky et al, 2001), and the two systems interact with each other. Specifically within the NAC, mu and delta opioid agonist-induced feeding is reduced following pretreatment with general, mu, delta and kappa antagonists, whereas within the VTA, mu and delta agonist-induced feeding is blocked by general, mu and kappa antagonists (Echo, 2002; Khaimova, 2004; Znamensky, 2001). Feeding elicited by the GABA-B agonist, baclofen in the NAC is reduced by pretreatment with kappa and delta, but not mu, opioid antagonists, whereas baclofen-induced feeding elicited from the VTA is reduced by pretreatment with kappa and mu, but not delta antagonism. Feeding elicited by baclofen in either the NAC or VTA is blocked when GABA-B, but not GABA-A receptor antagonists administered to the same site. A major way to demonstrate the existence of a distributed brain network is to evaluate intersite interactions between the NAC and VTA by placing an antagonist in one site

followed by an agonist in the other site. Bidirectional regional interactions have been observed for mu agonist-induced feeding in the NAC when general, mu or kappa antagonists in the VTA blocked the response (Bodnar et al, 2005). Correspondingly, mu agonist-induced feeding in the VTA is blocked by general, mu, delta and to a lesser degree kappa antagonists in the NAC (Bodnar et al, 2005). These brain areas and neurotransmitters apparently modulate the orosensory, post-ingestive and incentive salience aspects of food intake, and thereby form a distributed brain network modulating the reward, energy homeostasis and motivation of food intake and consequently body weight (Bodnar, 2004; Kelley, 2004). Thus, the present series of studies will evaluate whether the feeding responses elicited by the GABA-B agonist, baclofen in the VTA and NAC is modulated by a regional distributed brain network involving GABA and opioid receptors.

Specific Aim 1 will examine whether GABA-A (bicuculline) or GABA-B (saclofen) antagonist pretreatment in the VTA dose-dependently reduce baclofen-induced feeding elicited from the NAC and does bicuculline or saclofen pretreatment in the NAC dose-dependently reduce baclofen-induced feeding elicited from the VTA. The following hypothesis is made. Pretreatment with the GABA-B antagonist (saclofen) or GABA-A (bicuculline) will dose dependently reduce GABA-B (baclofen) feeding bidirectionally.

Specific Aim 2 will examine whether general opioid (naltrexone) antagonist pretreatment in the VTA dose-dependently reduce baclofen-induced feeding elicited from the NAC and does naltrexone pretreatment in the NAC dose-dependently reduce baclofen-induced feeding elicited from the VTA. The following hypothesis is made. Pretreatment with the opioid antagonist (naltrexone) will dose dependently reduce GABA-B (baclofen) induced feeding bidirectionally.

Specific Aim 3 will examine whether mu (BFNA), kappa (NBNI) or delta (NTI) opioid receptor subtype antagonist pretreatment in the VTA reduce baclofen-induced feeding elicited from the NAC shell, and does mu, kappa or delta opioid receptor subtype antagonist pretreatment in the NAC shell reduce baclofen-induced feeding elicited from the VTA. The following hypothesis is made. Pretreatment with the opioid antagonists mu (BFNA), kappa (NBNI) or delta (NTI) will dose dependently reduce GABA-B (baclofen) induced feeding bidirectionally.

Background

A central premise of this dissertation is that GABA and the endogenous opioids act as inhibitory orexigenic agents utilizing two important neuroanatomical areas, the nucleus accumbens shell (NACs) and ventral tegmental area (VTA) to form a distributed brain network. Studies have examined these orexigenic agents in and of themselves, how they interact within sites but have not extensively studied the interaction of these neurochemicals between these two sites. Therefore, the background section is organized to initially cover the significance of studying the nucleus accumbens shell and the ventral tegmental area in the control of food intake. The second section addresses NACs and VTA within site control of food intake by GABA in and of themselves. The third section addresses NACs and VTA within site control of food intake by opioids in and of themselves. The fourth section addresses NACs and VTA within site control between GABA and opioids. The fifth section of the background will summarize the evidence supporting a distributed brain network in the control of food intake by reviewing the affect of GABA and opioid antagonists on mu agonist-induced specifically between the NACs, and VTA and other brain sites. This will be followed by a rationale of the Specific Aims and their hypotheses for the present set of proposed studies.

Significant Brain Sites Involved in the Control of Food Intake: NAC

The NAC is divided into a core and shell region receiving inputs from the prefrontal cortex (cognition), the amygdala (emotion), hippocampus (memory), thalamus (sensory information) and the VTA (motivational significance), information allowing the animal to be adaptive in its behavior (Kelley, 2004). The NAC has two distinct areas; the core and the shell with distinct afferents and efferents. NAC core efferents project to the basal ganglia motor circuits including the ventral pallidum, subthalamic nucleus and substantia nigra and its inputs include the hippocampus and prefrontal cortex. NAC shell efferents project to the VTA, ventral pallidum, hypothalamus and brainstem and afferents also include the hippocampus and prefrontal cortex though different subregions (Meredith, 1999). This supports Mogenson's (1980) initial proposal that the NAC acts as the interface between cognitive/emotional information and motor control. Therefore, the core appears more involved in voluntary motor function and the shell in motivational mechanisms particularly in regards to natural rewards which include food intake (Kelley, 2004). In vitro studies show the NAC contains receptors for dopamine, glutamate, acetylcholine and GABA. The majority of the NAC contains medium spiny projection neurons (90%) with 95% of the medium spiny neurons containing GABA but co-express for opioid peptides (enkephalin and dynorphin) (Meredith, 1999; Shi & Rayport, 1994). Based on autoradiography and *in situ* hybridization mu receptors are found postsynaptically and kappa receptors are found presynaptically (Mansour, Fox, Akil & Watson, 1995).

Reynolds and Berridge (2002) indicated a further demarcation of the NAC shell. When the GABA-A agonist (muscimol) was injected into the rostral NACs, rats showed a 400% increase in chow intake while injections into the caudal NACs resulted in food intake below vehicle levels. Defensive treading was elicited from caudal NACs muscimol injections but this behavior

was non-existent from more rostrally-placed injections. After being trained for conditioned place preference with muscimol injected into the rostral or caudal NACs, during the testing period, the rats showed positive place preference from rostral injections and aversive for caudal injections. Hedonic reactions for sucrose did show aversive reactions (i.e., gapes, headshakes, chin rubs) for sucrose when muscimol was injected into the caudal NACs and a decrease in positive reactions (i.e., tongue protrusions, paw licking) when muscimol was injected into the rostral NACs. The authors propose the dissociated effect of increased intake but decreased positive hedonics seen from rostrally-injected muscimol reveals GABA inducement of simply 'wanting' the food *without* 'liking' it (Reynolds & Berridge, 2002).

Injections of the mu opioid agonist (DAMGO) into the medial NACs showed increased food intake and an increased intake of a sucrose solution, indicating involvement of a positive hedonic influence suggesting opioid NACs contribution in 'liking' of appetitive substances (Pecina & Berridge, 2005).

Significant Brain Sites Involved in the Control of Food Intake: VTA

The seminal work by Olds and Milner (1954) initiated the vast amount of research on the medial forebrain bundle (MFB) which encompasses the neuronal pathways between the prefrontal cortex, septum, amygdala, hypothalamus, VTA, and NAC (Brain from top to bottom). Decades of research has purported the MFB to be predominantly involved in reward, reinforcement and motivation. Neurotransmitters found in the MFB as well as in other CNS areas include the orexigenics neuropeptide Y, galanin, norepinephrine, GABA and the endogenous opioids. The anorexigenics studied include cholecystokinin, bombesin, serotonin and melanocyte stimulating hormone (Arora & Anubhuti, 2006) while neuromodulators include dopamine and glutamate (Kelley & Berridge, 2002). Since dopamine (DA) is the major neurotransmitter released from the

VTA to the NAC (mesoaccumbens pathway) most of the research has investigated this monoamine. Horvitz (2000) discusses DA involvement in goal directed behavior which incorporates activation of DA mesolimbocortical neurons induced by salient environmental events. These include novel events, strong aversive events and intense visual and auditory stimuli. In regard to food, DA levels specifically increase under the salient condition of a novel or unexpected food presentation. Zellner and Ranaldi (2010) propose glutamate has a modulatory role on the DA mesolimbocortical reward system during the acquisition of conditioned stimuli (cues associated with reward). They suggest this occurs due to stimulation of NMDA receptors by the normally weak cue (i.e., tone) coupled with the reward signal (i.e., food) causing synaptic strengthening of VTA DA neurons.

Along with DA, evidence supports VTA neurons containing GABA and glutamate and NAC projections to the VTA apparently containing GABA, and the opioids, dynorphin and enkephalin (Fields, 2007; Kalivas, Churchill, Klitenick, 1993; Van Bockstaele & Pickel, 1995). Based on autoradiography and *in situ* hybridization kappa receptors are found postsynaptically (Mansour, Fox, Akil & Watson, 1995).

Kelley, Baldo, Pratt, & Will (2005) have proposed that CNS control of food intake involves GABA's involvement in energy homeostasis, the opioids in hedonics, and dopamine in arousal and reinforcement with the key neuroanatomical sites being the hypothalamus, NAC and VTA. Therefore, this set of studies focuses on the intercommunication of GABA and the opioids between the NAC and VTA.

Within-site Control of GABA Induced Feeding: NACs

Based on dose response curves GABA-A and GABA-B agonists (muscimol; 175, 438, 876 pmol and baclofen; 37, 94, 188 ng) injected into the NACs significantly increased food intake

(Khaimova et al, 2004; Stratford & Kelley, 1997; Znamensky et al, 2001), although no effects were found when the agonists were injected into the nucleus accumbens core (Stratford & Kelley, 1997). Further findings showed baclofen (188ng/ul) microinjected into the NACs of non-food deprived rats significantly increased standard chow food intake, but did not increase water intake, intake of a palatable liquid (glucose/saccharin) or chewing on wood chucks (Stratford & Kelley, 1997; Ward, Somerville, & Clifton, 2000). In non-food deprived rats, microinjections of muscimol into the NACs doubled food intake although this was accompanied by increased agitation (Soderpalm, & Berridge, 2000). Zhang, Balmadrid & Kelley (2003) showed muscimol injection into the NACs was ineffective in inducing instrumental learning.

Elimination of NACs GABA agonist-induced feeding was observed with co-administration of the antagonist of the same receptor type but not with the antagonist of a different receptor type; GABA-A antagonist (bicuculline) eliminated GABA-A, but not GABA-B agonist induced intake and GABA-B antagonist (saclofen) eliminated GABA-B but not GABA-A agonist induced intake (Stratford, & Kelley, 1997; Znamensky et al, 2001).

GABA-A and GABA-B receptor antagonists (bicuculline and saclofen) injected into the NACs also significantly reduced food intake induced by food deprivation and lipoprivation (preventing fatty acid oxidation) but not with glucoprivation (which prevents cellular glucose uptake). This was effective short term (1-4 hours) after injection but not long term (24-48 hours) (Kandov et al., 2006).

In conclusion, NACs GABA-A and GABA-B receptors are involved in the control of meal size, specifically of standard chow whether under conditions of food deprivation, lipoprivation or ad libitum eating but not of water, highly palatable liquids, or simply the activity of chewing nor do GABA NACs receptors appear to participate in instrumental learning.

Within-site control of GABA-induced feeding: VTA

Whereas both VTA muscimol and baclofen are found to increase feeding, they are differentially affected by GABA and opioid antagonists. Within the VTA, naltrexone was unable to diminish muscimol-induced feeding but did diminish baclofen-induced feeding. Saclofen also decreased baclofen-induced feeding but showed neither an aversive nor positive influence on conditioned flavor preference, whereas, bicuculline failed to effect baclofen-induced feeding (Echo, Lamonte, Ackerman, & Bodnar, 2002). Bicuculline and saclofen injected into the VTA did reduce food intake induced by lipoprivation but did not affect food deprivation or glucoprivation (Kandov et al., 2006).

Within-site Control of Opioid Induced Feeding: Introduction

Research on opioid involvement in control of feeding was instigated when Holtzman (1974) showed systemic injection of the general opioid antagonist (naloxone) reduced food intake in food deprived rats. Further research has gone on to investigate receptor selective opioid agonists and antagonists within specific sites of the CNS including the VTA and NAC supporting specific opioid involvement in inducing food intake.

Within-site control of opioid-induced feeding: NAC

The mu opioid receptor agonists, morphine and DAMGO, as well as the $delta_2$ opioid agonist (deltorphin) injected into the NAC were found to significantly increase food intake (Ragnauth, Moroz, & Bodnar, 2000; Soderpalm, & Berridge, 2000. Morphine and DAMGO and the delta opioid agonist (DPEN) enhanced intake of the palatable sucrose solution while the kappa agonist (U50488H) had no effect (Zhang, & Kelley, 1997). Not unexpectedly, receptor- specific opioid antagonists injected into the NAC are shown to decrease intake of a highly palatable sucrose solution (10%) as well as under food deprivation and glucoprivic conditions, all of which, under

normal conditions, would induce hyperphagia. General opioid antagonist naltrexone (NTX) and mu opioid antagonist beta-funaltrexamine (BFNA) decreased food intake under deprivation, glucoprivic and palatable conditions, with mu_1 opioid antagonist naloxonazine having no effect when tested with food deprivation. Autoradiographic studies for BFNA have shown a reduction in the affinity of DAMGO and the number of mu opioid receptors thereby decreasing palatable sucrose intake by 40% (Martin, Dworkin & Smith, 1995; Ward, Nicklous, Aloyo & Simansky, 2006). The kappa antagonist, nor-binaltorphamine (NBNI) also decreased intake under food deprivation and glucoprivation but not under palatable condition (Bodnar, Glass, Ragnauth, & Cooper, 1995). Kelley, Bless & Swanson (1996) also found similar results but they did not find naltrexone nor NBNI to decrease food intake. They also tested the delta antagonist naltrindole (NTI) and found decreased food and sucrose intake. Mu and δ_1 opioid agonist-induced food intake is reduced by μ, δ_1, δ_2, κ but not μ_1 receptor antagonism. In contrast, δ_2 opioid agonist-induced feeding is increased by μ and κ antagonists, and unaffected by δ_2 receptor antagonism (Ragnauth, Moroz & Bodnar, 2000).

These results indicate opioid receptors; μ, particularly μ_2 ; δ, particularly δ_1, and possibly κ receptors are responsible for modulation of food intake, although the κ receptor does not appear to be involved in affecting response to a palatable carbohydrate solution.

Within-site Control of Opioid-Induced Feeding: VTA

DAMGO or morphine and δ opioid agonists (DPDPE) injected into the VTA dose dependently increased the speed of eating but did not decrease the latency to eat. Kappa (U-50488H) agonist did not increase speed of eating (Noel & Wise, 1993/1995). VTA naltrexone and δ opioid antagonists (NTI) decrease food intake under conditions of food deprivation, glucoprivation and palatable sucrose (Ragnauth, Ruegg, & Bodnar, 1997). These results implicate μ and δ receptors

involvement in VTA control of food intake with δ receptors also involved in VTA palatable sucrose intake.

Within-site Opioid-GABA Interactions: NACs

Opioids exert differential effects on feeding elicited by GABA receptor subtypes. Agonist induced feeding elicited by either GABA- A or B receptor subtype was reduced by NBNI. Whereas, naltrexone and BFNA reduced GABA-A mediated feeding they had no effect on GABA-B mediated feeding. In contrast, NTI reduced GABA-B induced, but not GABA-A induced feeding. (Khaimova et al, 2004; Znamensky et al, 2001).

Within-site Opioid-GABA Interactions: VTA

Muscimol induced feeding was reduced by κ but enhanced by μ or δ opioid antagonist pretreatment. Baclofen-induced feeding was reduced by κ or μ but was not effected by δ antagonism (Khaimova et al., (2004). These results show a VTA within-site interaction of opioid and GABA feeding responses with the κ receptor being responsible for feeding elicited by both GABA receptor subtypes, but μ and δ receptors exerting differential effects. In turn, VTA DAMGO induced feeding was decreased dose dependently by VTA GABA-A or GABA-B receptor antagonists though seizures occurred with GABA-A antagonist (bicuculline) (Echo, Lamonte, Ackerman & Bodnar, 2002).

Mu Opioid Agonist-Induced Feedings: Between NAC and VTA

DAMGO injected into the NAC can increase fat intake by 300% that is blocked by muscimol treatment in the VTA (Will, Franzblau, & Kelley, 2003). In contrast, NAC DAMGO-induced feeding is not mitigated by bicuculline or saclofen pretreatment in VTA, and correspondingly, VTA DAMGO-induced feeding was unaffected by NAC bicuculline or saclofen pretreatment (Ackerman, Lamonte, & Bodnar, 2003). Thus, although GABA antagonists suppress mu opioid-

induced feeding within the NACs or VTA, these results show NACs mu opioid-induced feeding is not dependent on GABAergic projections from the VTA, and VTA mu-opioid induced feeding is not dependent on GABAergic projections from the NAC.

MacDonald, Billington, & Levine (2003) found bilateral but not unilateral, injection of the naltrexone into the VTA suppressed NACs DAMGO induced eating and naltrexone into the NACs suppressed VTA DAMGO induced eating. Bodnar et al (2004) showed unilateral injection of naltrexone, βFNA, and NBNI, but not NTI into the VTA suppressed bilateral injections of the NAC DAMGO induced eating over a 2-4 hour time period. Whereas, naltrexone, βFNA and NTI and less so with kappa (at 4 hours but not at 2 hours) suppressed unilateral injection of the VTA DAMGO induced feeding.

Mu Opioid Agonist-Induced Feedings: Between Other Brain Sites

Opioid antagonists injected into one CNS site has been shown to reduce mu opioid agonist (DAMGO) induced food intake injected in other CNS site; DAMGO induced food intake within the NACs has been shown to be reduced by general naltrexone (NTX) injected into nucleus tractus solitarius (NTS) (Kim, Quinn, Spanswick, O'Hare, 2009) or central nucleus of the amygdala (Kim, Quinn, Levine, O'Hare, 2004). Concurrently, VTA DAMGO induced food intake is reduced by NTX injected into the NTS (Kim, Quinn, Spanswick, O'Hare, 2009) and paraventricular nucleus of the hypothalamus (Kim, Quinn, Levine, O'Hare, 2004). These results support evidence of a distributed brain network in the control of food intake.

Rationale, Specific Aims and Hypotheses

The foregoing sections presented a series of studies that evaluated GABA and opioid neurotransmitter modulation within and between the NACs and VTA of spontaneous food intake in sated rats or conditions of food deprivation, glucoprivation, lipoprivation and palatability.

Antagonist-agonist injections in different sites were also presented establishing a preliminary understanding of a distributed brain network in the control of food intake. Further understanding of the complexity and possible redundancy of neurotransmitters in multiple brain sites in inducing food intake will expand the breadth of our understanding of human recalcitrance in losing and maintaining weight loss. Based on this objective and the previous studies reviewed, three Specific Aims are proposed.

- **Specific Aim 1:** NACs and VTA GABA agonist induced feeding has been found to be reduced by co-administration of the antagonist of the same receptor subtype but not with the antagonist of a different receptor subtype. Hence, within the NACs GABA-A antagonist (bicuculline) eliminated GABA-A (muscimol), but not GABA-B agonist (baclofen) induced intake and GABA-B antagonist (saclofen) eliminated GABA-B but not GABA-A agonist induced intake (Znamensky et al, 2001). The same effect was found within the VTA (Echo, Lamonte, Ackerman, Bodnar, 2002; Stratford, & Kelley, 1997).

  Specific Aim 1 hypothesizes that GABA-B agonist (baclofen) induced intake in the NACs or VTA will dose dependently be reduced by the GABA-B antagonist (saclofen) or GABA-A (bicuculline) when injected into the opposing site, VTA or NACs. Therefore, a bidirectional dependency between the NACs and VTA for GABA-B will be shown. As of yet a GABA neuronal dependency for food intake between the NACs and VTA has not been shown.

- **Specific Aim 2**: The general opioid antagonist (naltrexone) has been shown to differentially effect GABA agonist induced feeding depending on the site and receptor subtype. Hence, within the NAC shell naltrexone significantly reduced GABA-A agonist

induced feeding but not GABA-B agonist induced feeding (Znamensky, 2001) and within the VTA naltrexone significantly reduced GABA-B agonist induced feeding but did not reduce GABA-A agonist induced feeding (Echo, Lamonte, Ackerman, Bodnar, 2002). Specific Aim 2 hypothesizes pretreatment with the opioid antagonist (naltrexone) in the NACs or VTA will dose dependently reduce GABA-B (baclofen) induced feeding into the opposing site, VTA or NACs. Therefore a bidirectional dependency for food intake between the NACs and VTA will be shown. As of yet a GABA neuronal dependency on opioid involvement for food intake between the NACs and VTA has not been shown.

- **Specific Aim 3**: Within the NACs the mu opioid antagonist (beta-funaltrexamine -BFNA) has been shown to significantly decrease food intake under the following conditions; food deprivation, glucoprivation, palatability (sucrose), mu agonist induced intake, delta $_1$ agonist-induced intake and GABA-A agonist-induced intake (Bodnar, Glass, Ragnauth, Cooper, 1995; Ragnauth, Moroz, Bodnar, 2000; Khaimova et al, 2004), whereas in the VTA BFNA was found to reduce mu and GABA-B agonist induced intake while enhancing GABA-A agonist induced intake (Ragnauth, Ruegg, Bodnar, 1997; Lamonte, Echo, Ackerman, Christian, Bodnar, 2002; Khaimova et al, 2004). Within the NACs the kappa antagonist (NBNI) has been shown to significantly decrease food intake under the following conditions; food deprivation, glucoprivation, mu agonist induced intake, delta$_{1,}$ GABA-A and GABA-B agonist-induced intake (Bodnar, Glass, Ragnauth, Cooper, 1995; Ragnauth, Moroz, Bodnar, 2000; Khaimova et al, 2004), whereas in the VTA NBNI was found to reduce mu, GABA-A and GABA-B agonist induced intake (Ragnauth, Ruegg, Bodnar, 1997; Lamonte, Echo, Ackerman, Christian, Bodnar, 2002; Khaimova et al, 2004). Within the NACs the delta antagonist (NTI) or the delta$_2$

antagonist (naltrindole isothyiocyanate) has been shown to significantly decrease food intake under the following conditions; mu, delta $_{1,}$ GABA-A and GABA-B agonist-induced intake (Ragnauth, Moroz, Bodnar, 2000; Khaimova et al, 2004), whereas in the VTA NTI was found to significantly decrease food intake under the following conditions; food deprivation, glucoprivation, palatability (sucrose) while enhancing GABA-A agonist induced intake (Ragnauth, Ruegg, Bodnar, 1997; Lamonte, Echo, Ackerman, Christian, Bodnar, 2002; Khaimova et al, 2004).

Specific Aim 3 hypothesizes pretreatment with the opioid antagonists (BFNA, NBNI, NTI) will dose dependently reduce GABA-B (baclofen) induced feeding bidirectionally. As of yet a GABA neuronal dependency on opioid involvement for food intake between the NACs and VTA has not been shown.

CHAPTER TWO

GENERAL METHODS

Subjects

Adult male albino Sprague–Dawley rats (Charles River Laboratories, Kingston, NY; 80–120 days of age) were individually housed in wire-mesh cages and maintained on a 12/12-h light–dark cycle with Purina rat chow and water available ad libitum. All experimental protocols were approved by the Queens College Institutional Animal Care and Use Committee certifying that all subjects and procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Surgery

Each rat was pretreated with chlorpromazine (3 mg/kg ip) and anesthetized with ketamine HCl (120 mg/kg, im). For the opioid antagonist-GABA agonist studies, two pairs of stainless steel guide cannulae (26 gauge, Plastics One, Roanoke, VA) were aimed stereotaxically (Kopf Instruments, Tujunga, CA) at bilateral placements using the following coordinates: NACs (incisor bar (+ 5 mm), 3.1 mm anterior to the bregma suture, 1.7 mm lateral and angled 10^{o} towards each side of the sagittal suture and 6.8 mm from the top of the skull), and VTA (incisor bar (+ 5 mm), 5.6 mm posterior to the bregma suture, 2.2 mm lateral to and angled 10^{o} towards the sagittal suture and 8.4 mm from the top of the skull. For the opioid antagonist studies, one pair of guide cannulae was aimed bilaterally at the NACs or VTA. The cannulae were secured to the skull by four anchor screws with dental acrylic. To allow full drug clearance, all animals were allowed at least 1 week to recover from stereotaxic surgery before behavioral testing began.

Drugs

Baclofen, Bicuculline, NTX, BFNA and NBNI (Sigma Chemical Company, St. Louis, MO) were

dissolved in distilled water, whereas saclofen and NTI were dissolved in 0.1 M NaOH. NTX, NBNI and NTI were administered 0.5 h prior to agonist administration, whereas BFNA was administered 24 h prior to agonist administration. All microinjections were administered bilaterally in 0.5-ul volumes over 30 s through stainless internal cannulae (33-gauge, Plastics One) connected by polyethylene tubing to a Hamilton microsyringe. This relatively high injection volume is necessary because of limited solubility of the antagonists. Antagonist-agonist conditions were presented randomly at weekly intervals to minimize potential order and carry-over effects.

Dependent Measure: Food Intake

In all experiments, each rat was initially tested for spontaneous cumulative food intake after 1, 2 and 4 h at 3–10 h into the light cycle when minimal amounts of intake are typically observed. Pre-weighed food pellets were placed on the cage floor to allow easy access. All intakes were adjusted for spillage collected by paper towels placed under the wire mesh cages; water was available in all test sessions for all protocols. Four baseline sessions established consistently low intakes and minimized the occurrence of novelty-induced feeding.

Statistical Analyses

A three-way split-plot analysis of variance was performed to evaluate differences in baclofen-induced feeding in the two sites with VTA and NAC shell microinjections as a between-subject variable, baseline and baclofen treatments as one within-subject variable, and the three intake time course points as a second within-group variable.

Specific Aim 1: Four separate two-way repeated measures analyses of variance were performed to evaluate antagonist-agonist effects at each pair of sites with six paired injection conditions as one within-subject variable and the three intake time course points as the second within-subject

variable. Tukey comparisons ($P < .05$) were used to assess significant alterations in both agonist-induced feeding in each site relative to corresponding control conditions and in antagonist pretreatment upon agonist-induced feeding in each site.

Specific Aims 2-3 : Eight separate two-way repeated measures analyses of variance were performed to evaluate antagonist-agonist effects at each pair of sites with the paired injection conditions as one within-subject variable and the three intake time course points as the second within-subject variable. Tukey comparisons ($p < .05$) were used to assess significant alterations in both GABA-B agonist-induced feeding in each site relative to corresponding control conditions and in opioid antagonist pretreatment upon GABA-B agonist-induced feeding in each site. A separate three-way randomized block analysis of variance was also performed to evaluate antagonist effects alone with the sites as a between-subjects variable, the five injection conditions as one within-subject variable and the three intake time course points as the second within-subject variable. Tukey comparisons ($p < .05$) were used to assess any significant alterations in feeding following opioid antagonist treatment in each site relative to the corresponding control conditions.

Histological Verification and Analysis

At the completion of testing, all animals received an overdose of Euthasol (Del Marva Laboratories; 390 mg/ml sodium pentobarbital; 50 mg/ml sodium phenytoin; 0.05 ml/kg). Transcardiac perfusions were performed with 0.9% normal saline followed by 10% buffered formalin. Only animals with confirmed bilateral cannulae placements in each site were included in the experimental groups described in the protocols, and subject to data analysis. A stereotaxic atlas (Paxinos and Watson, 2009) was used to evaluate the localization of the tips of the cannula placements.

CHAPTER THREE

GABA RECEPTORS MEDIATE GABA-B AGONIST-INDUCED FEEDINGS: BETWEEN THE NACs and VTA

Introduction

Recent data have suggested that many feeding responses elicited from GABA and opioid agonist administration into specific brain sites activate a distributed brain network (e.g., Pecina and Berridge, 2000, 2005; Smith and Berridge, 2007; Stratford, 2005; Stratford and Kelley, 1999; Will et al., 2003), suggesting regional interactions between neuroanatomical loci. One paradigm used to study regional interactions in feeding studies is to administer an antagonist in one site of interest prior to administration of the feeding-active agonist in a second site of interest to determine if this antagonist blocked agonist-induced feeding, thereby demonstrating functional relationships between the two sites. This approach was initially employed for opioid–opioid feeding interactions, and revealed unilateral interactions between the hypothalamic paraventricular nucleus (PVN) and the central nucleus of the amygdala (CeA) (Giraudo et al., 1998a), and bidirectional interactions between the nucleus of the solitary tract (NTS) and the CeA (Giraudo et al., 1998b), the PVN and VTA (Quinn et al., 2003), the NAC shell and CeA (Kim et al., 2004), and the VTA and NAC shell (Bodnar et al., 2005; MacDonald et al., 2003). Regional opioid-dopamine (MacDonald et al., 2004), opioid-orexin (Sweet et al., 2004) and opioid-melanocortin (Beckman et al. 2009), but not opioid-ghrelin (Naleid et al., 2005), feeding interactions have also been observed. Finally, DAMGO-induced feeding elicited from the VTA was unaffected by GABA-A or GABA-B antagonist pretreatment in the NAC shell, and NAC shell DAMGO-induced feeding was unaffected by VTA GABA-A or GABA-B antagonism (Ackerman et al., 2003). This latter failure to observe a regional opioid-GABA interaction

occurred despite the ability of GABA-B antagonism to block DAMGO-induced feeding within the NAC shell (Znamensky et al., 2001), and the ability of both GABA-A and GABA-B antagonists to block DAMGO-induced feeding within the VTA (Echo et al., 2002). Although GABA-A and GABA-B antagonists respectively and receptor-selectively block GABA-A and GABA-B agonist-induced feeding responses within the NAC shell and within the VTA (Echo et al., 2002; Stratford and Kelley, 1997; Znamensky et al., 2001), it is unknown whether there are regional GABA-GABA feeding interactions between the NAC shell and the VTA, whether they are receptor-subtype-selective, and whether they are bidirectional. Therefore, to evaluate these issues, the present study examined whether feeding elicited by the GABA-B agonist, baclofen microinjected into the NAC shell was dose-dependently blocked by pretreatment with either the GABA-B antagonist, saclofen or the GABA-A antagonist, bicuculline into the VTA, and then whether VTA baclofen-induced feeding was dose-dependently blocked by NAC shell pretreatment of either saclofen or bicuculline in rats.

Experimental Procedure

Subjects, Surgery, Drugs, Statistics and Histology

The subjects, surgery, drugs, statistics and histology are described in the General Methods section.

Protocols

Four protocols were followed evaluating the effect on spontaneous food intake: 1) VTA GABA-B receptor (saclofen) antagonism affect on NAC shell GABA-B agonist (baclofen)-induced feeding; 2) NAC shell GABA-B receptor antagonism affect on VTA GABA-B agonist-induced feeding; 3) VTA GABA-A (bicuculline) receptor antagonism affect on NAC shell GABA-B agonist-induced feeding; and 4) NAC shell GABA-A receptor antagonism affect on VTA

GABA-B agonist-induced feeding. In each protocol, six conditions were tested: (a) baseline values, (b) bilateral baclofen (200 ng, 100 ng each side) microinjections in the agonist site, and (c-f) bilateral saclofen (0.5, 1.5, 3, 5 ug, 0.25, 0.75, 1.5, 2.5 ug each side) or bicuculline (7.5, 75, 150, 300 ng, 3.75, 37.5, 75, 150 ng each side) in the antagonist site followed by bilateral baclofen (200 ng, 100 ng each side) microinjections in the agonist site. GABA-A or GABA-B antagonist treatments preceded GABA-B agonist treatments by 20 min in all protocols, and cumulative intakes were assessed at 1, 2 and 4 h following the agonist microinjection. The NAC and VTA baclofen (total: 200 ng) dose was chosen because it was used previously in the analysis of GABA receptor antagonist effects within the NAC shell (Znamensky et al., 2001) and the VTA (Echo et al., 2002). The saclofen and bicuculline dose ranges were chosen on the basis of their respective effectiveness in reducing baclofen-induced and muscimol-induced feeding within the NAC shell or VTA (Echo et al., 2002; Znamensky et al., 2001).

Results

Histological Verification

Figures 1 and 2 respectively display the bilateral locations of the tips of the cannula placements in the NAC shell and VTA in the 32 animals that successfully completed the study. Proper and overlapping bilateral NAC shell cannula placements were observed for the 16 rats receiving baclofen (circles), the eight rats receiving bicuculline (squares), and the eight rats receiving saclofen (squares) in this site (Figure 1). In turn, the two NAC shell antagonist groups showed considerable overlap with each other. There was considerable overlap in NAC shell placements in agonist-treated animals treated with bicuculline (n=9) or saclofen (n=7) in the VTA as well as in animals receiving bicuculline (n=8) or saclofen (n=8) and tested with baclofen in the VTA. Proper and overlapping bilateral VTA cannula placements were observed for the 16 rats

receiving baclofen (circles), the nine rats receiving bicuculline (squares), and the seven rats receiving saclofen (squares) in this site (Figure 2). In turn, the two VTA antagonist groups showed considerable overlap with each other. There was considerable overlap in VTA placements in agonist-treated animals treated with bicuculline (n=8) or saclofen (n=8) in the NAC shell as well as in animals receiving bicuculline (n=9) or saclofen (n=7) and tested with baclofen in the NAC shell.

Figure 1. Schematic representation of bilateral NAC shell cannula placements for 32 rats receiving the GABA-B agonist, baclofen (n=16, circles), the GABA-A antagonist, bicuculline (n=8, squares) or the GABA-B antagonist, saclofen (n=8, squares). The three panels (Figures 14, 16 and 20) are from the atlas of Paxinos and Watson (2009), and are respectively 2.28, 2.04 and 1.56 mm anterior to the bregma suture. All cannulae tips were localized within the NAC shell, and there was considerable overlap in NAC shell placements in agonist-treated animals treated with bicuculline (n=9) or saclofen (n=7) in the VTA. There was also considerable overlap in NAC shell placements in animals receiving bicuculline (n=8) or saclofen (n=8) and tested with baclofen in the VTA.

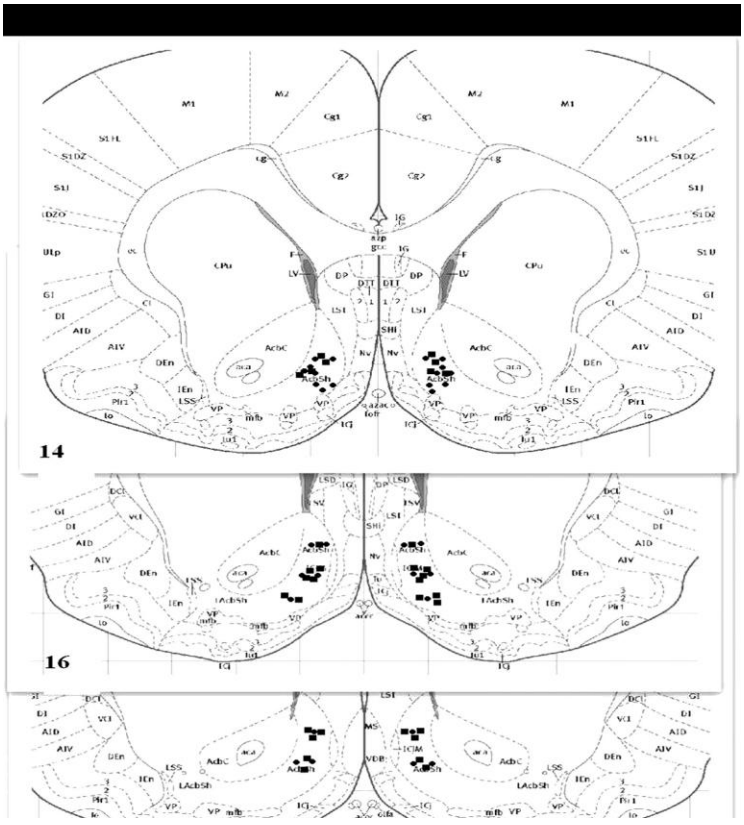
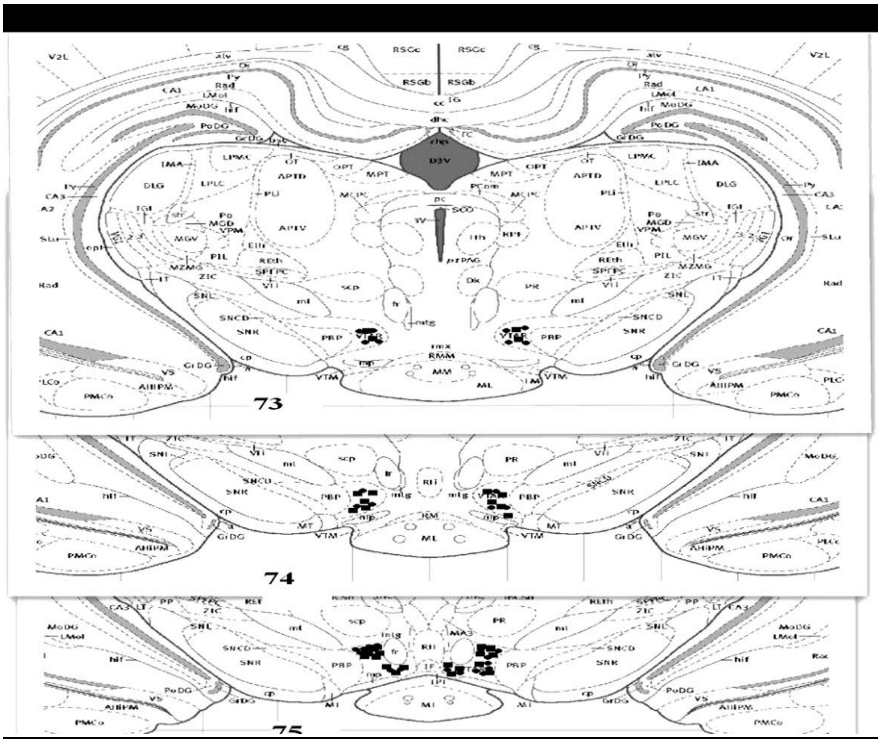


Figure 2. Schematic representation of bilateral VTA cannula placements for 32 rats receiving baclofen (n=16, circles), bicuculline (n=9, squares) or saclofen (n=7, squares). The three panels (Figures 73, 74 and 75) are from the atlas of Paxinos and Watson (2009), and are respectively 4.80, 4.92 and 5.04 mm posterior to the bregma suture. All cannulae tips were localized within the VTA, and there was considerable overlap in VTA placements in agonist-treated animals treated with bicuculline (n=8) or saclofen (n=8) in the NAC shell. There was also considerable overlap in VTA placements in animals receiving bicuculline (n=9) or saclofen (n=7) and tested with baclofen in the NAC shell.



GABA-B Agonist-Induced Feeding in the VTA and NAC shell

Sixteen rats with bilateral VTA cannulae and 16 rats with bilateral NAC shell cannulae were assessed for baclofen-induced feeding relative to baseline intake. Significant differences in food intake were observed between baseline and baclofen conditions ($F(1,15)= 123.99$, $p<0.0001$), across test times ($F(2,30)= 54.52$, $p<0.0001$), and for the interaction between conditions and times ($F(2,30)= 4.60$, $p<0.018$), but not between sites ($F(1,15)= 1.01$, ns), or for the interactions between sites and conditions ($F(1,15)= 2.58$, ns), between sites and times ($F(2,30)= 1.06$, ns), or among sites, conditions and times ($F(2,30)= 0.14$, ns). Food intake was significantly increased above baseline levels after 1, 2 and 4 h following baclofen administration into the NAC shell or VTA. The magnitude of baclofen-induced feeding between the NAC shell (4 h: 4.8 g) and the VTA (4 h: 5.1 g) failed to differ from each other across the time course. Therefore, any regional antagonist effects upon GABA-B agonist-induced feeding in the VTA or NAC shell were not due to intrinsic differences in the magnitude of the agonist's ability to differentially elicit feeding in the two sites.

VTA GABA-B Antagonism and NAC shell GABA-B Agonist-Induced Feeding

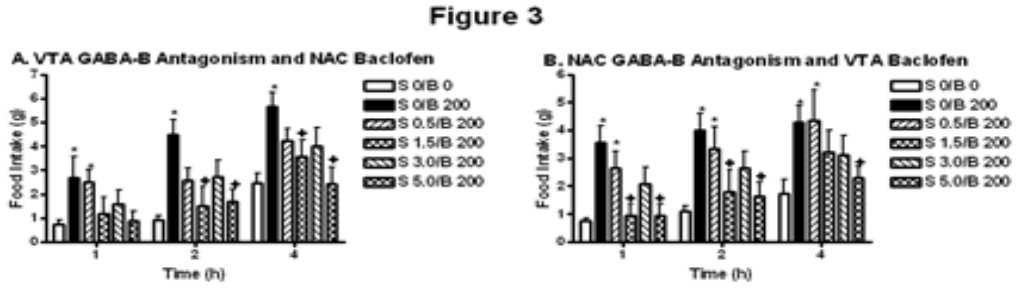
Significant differences in food intake were observed across saclofen doses ($F(5,36)= 4.20$, $p<0.004$) and across test times ($F(2,72)= 45.22$, $p<0.0001$), but not for the interaction between doses and times ($F(10,72)= 1.04$, ns). Baclofen (200 ng) in the NAC shell significantly increased food intake relative to baseline treatment across the time course (Figure 3A). In the presence of VTA saclofen doses of 0.5, 1.5, 3 and 5 ug, NAC baclofen failed to significantly increase food intake above baseline values. Moreover, pretreatment with VTA saclofen doses of 1.5 and 5 ug significantly decreased NAC baclofen-induced feeding after 2 and 4 h (Figure 3A). Thus, the

ability of baclofen administered into the NAC shell to elicit feeding depended upon the integrity of VTA GABA-B receptors, indicating the presence of a regional interaction.

NAC shell GABA-B Antagonism and VTA GABA-B Agonist-Induced Feeding

Significant differences in food intake were observed across saclofen doses ($F(5,42)= 3.43$, $p<0.011$) and across test times ($F(2,84)= 28.45$, $p<0.0001$), but not for the interaction between doses and times ($F(10,84)= 0.88$, ns). Baclofen (200 ng) in the VTA significantly increased food intake relative to baseline treatment across the time course (Figure 3B). In the presence of NAC shell saclofen doses of 1.5, 3 and 5 ug, VTA baclofen failed to significantly increase food intake above baseline values. However, the pairing of NAC shell saclofen at the lowest (0.5 ug) dose with VTA baclofen still resulted in significantly increased food intake. Moreover, pretreatment with NAC shell saclofen doses of 1.5 (1-2 h) and 5 (1-4 h) ug significantly decreased VTA baclofen-induced feeding (Figure 3B). Thus, the ability of baclofen administered into the VTA to elicit feeding depended upon the integrity of NAC shell GABA-B receptors, indicating the presence of a regional interaction as well as bidirectional regional interactions for GABA-B antagonist-agonist interactions.

Figure 3. Alterations in food intake (g, +S.E.M.) following administration of the GABA-B agonist, baclofen (200 ng) into the NAC shell (Panel A) or VTA (Panel B) 20 min following administration of the GABA-B antagonist, saclofen at doses of 0.5, 1.5, 3 and 5 ug into the other site. The asterisks (*) in this and the subsequent figure indicate significant increases in food intake following agonist administration relative to baseline treatment, whereas the crosses (+) indicate significant decreases in agonist-induced food intake following antagonist administration.



VTA GABA-A Antagonism and NAC shell GABA-B Agonist-Induced Feeding

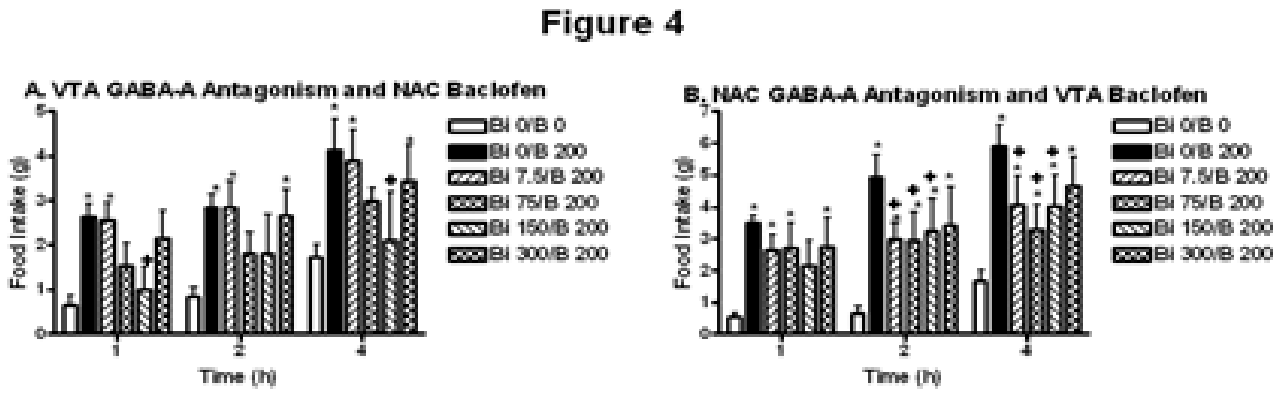
Significant differences in food intake were observed across bicuculline doses ($F(5,43)= 2.79$, $p<0.029$) and across test times ($F(2,86)= 30.51$, $p<0.0001$), but not for the interaction between doses and times ($F(10,86)= 0.42$, ns). Baclofen (200 ng) in the NAC shell significantly increased food intake relative to baseline treatment across the time course (Figure 4A). In the presence of VTA bicuculline doses of 75 and 150 ng, NAC baclofen failed to significantly increase food intake above baseline values. However, the pairing of VTA bicuculline at the lowest (7.5 ng: 1-4 h) and highest (300 ng: 2-4 h) doses with NAC baclofen still resulted in significantly increased food intake. Moreover, pretreatment with VTA bicuculline dose of 150 ng significantly decreased NAC baclofen-induced feeding after 1 and 4 h (Figure 4A). Thus, the ability of baclofen to elicit feeding following injections into the NAC shell depended in part upon the integrity of VTA GABA-A receptors, although not to the same extent as VTA GABA-B receptors.

NAC shell GABA-B Antagonism and VTA GABA-B Agonist-Induced Feeding

Significant differences in food intake were observed across bicuculline doses ($F(5,42)= 3.04$, $p<0.02$) and across test times ($F(2,84)= 39.97$, $p<0.0001$), but not for the interaction between doses and times ($F(10,84)= 1.43$, ns). Baclofen (200 ng) in the VTA significantly increased food intake relative to baseline treatment across the time course (Figure 4B). NAC shell bicuculline at the highest 300 ng dose failed to affect VTA baclofen-induced feeding. Although the 7.5, 75 and 150 ng doses of NAC shell bicuculline significantly reduced feeding effects induced by VTA baclofen after 2 and 4 h, the agonist was still able to elicit significant increases in food intake relative to baseline values (Figure 4B). Thus, the ability of baclofen to elicit feeding following

injections into the VTA was only marginally affected by blockade of NAC shell GABA-A receptors.

Figure 4. Alterations in food intake (g, +S.E.M.) following administration of the GABA-B agonist, baclofen (200 ng) into the NAC shell (Panel A) or VTA (Panel B) 20 min following administration of the GABA-A antagonist, bicuculline at doses of 7.5, 75, 150 and 300 ng into the other site.



Discussion

The present data indicated that bilateral microinjection of the GABA-B receptor agonist, baclofen, significantly increased feeding in the VTA and NACs, confirming previous reports of this effect (Arnt and Scheel-Kruger, 1979; Echo et al., 2002; Khaimova et al., 2004; Klitenick and Wirtshafter, 1988; Soderpalm and Berridge, 2000; Stratford and Kelley, 1997; Ward et al., 2000; Znamensky et al., 2001). The significant increases in food intake following baclofen administration into the VTA and NACs were comparable between sites, and thus, any site-specific changes in antagonist effects could not be attributed to any potential intrinsic site-specific differences in the magnitude of feeding responses elicited by baclofen.

Previous studies (Echo et al., 2002; Stratford and Kelley, 1997; Znamensky et al., 2001) demonstrated that baclofen-induced feeding elicited from the VTA or NACs was significantly dose-dependently reduced by saclofen pretreatment in the same site, indicating GABA-B receptor specific effects within a given site. Examining between-site interactions and summarized in Table 1, the present study found that pretreatment with the GABA-B antagonist, saclofen into the VTA at a dose range from 0.5 to 5 ug blocked the significant increases in food intake elicited by baclofen administered into the NACs with the 1.5 and 5 ug VTA saclofen doses reducing NACs baclofen-induced feeding to levels observed during vehicle treatment. Correspondingly, pretreatment with saclofen in the NAC shell (1.5-5 ug) blocked the significant increases in food intake elicited by baclofen administered into the VTA with the 1.5 and 5 ug NACs saclofen doses reducing VTA baclofen-induced feeding to levels observed during vehicle treatment. Thus, the ability of baclofen administered into either the VTA or NACs to elicit feeding depended upon the integrity of GABA-B receptors in the other site, supporting the presence of a bidirectional regional interaction.

Table 1. Protocols evaluating GABA-B agonist and GABA- B or GABA-A antagonist feeding interactions between the ventral tegmental area (VTA) and the nucleus accumbens (NAC) shell.

Protocol	**Sample**	**Antagonist**	**Agonist**	
	Size	**Site/Drug**	**Site/Drug**	**Summary of Effects**
1	**7**	**VTA Saclofen**	**NAC Baclofen**	**All VTA antagonist doses blocked NAC agonist feeding**
2	**8**	**NAC Saclofen**	**VTA Baclofen**	**Three highest VTA antagonist doses blocked NAC agonist feeding**
3	**9**	**VTA Bicuculline**	**NAC Baclofen**	**Two middle NAC antagonist doses blocked VTA agonist feeding**
4	**8**	**NAC Bicuculline**	**VTA Baclofen**	**Three lowest NAC antagonist doses significantly reduced but did not block VTA agonist feeding**

Previous studies have shown the ability of GABA antagonists to block GABA agonist-induced feeding within the VTA or NACs is dependent upon receptor specificity (Echo et al., 2002; Stratford and Kelley, 1997; Znamensky et al., 2001) with baclofen-induced feeding elicited from the VTA or NACs selectively blocked by within-site pretreatment with GABA-B (saclofen), but not GABA-A (bicuculline) antagonism. Also summarized in Table 1, the present study indicated that VTA pretreatment with bicuculline at a dose range from 75 to 300 ng blocked the significant increases in food intake elicited by baclofen administered into the NACs with the 150 ng VTA bicuculline dose reducing NACs baclofen-induced feeding to levels observed during vehicle treatment. Hence, the ability of NACs baclofen to elicit feeding also depended on the integrity of VTA GABA-A receptors, indicating a regional interaction between the two GABA receptor subtypes. In contrast, NACs bicuculline pretreatment failed to block the significant increases in food intake induced by VTA baclofen, but significantly reduced the magnitude of the agonist effect following the three lowest bicuculline doses. Unlike saclofen's dose-dependent effects, bicuculline effects were not dose-dependent, particularly for the general lack of effects at the 300 ng dose. There is no apparent pharmacodynamic or pharmacokinetic reason for this lack of effect, but it cannot be attributed to order or carry-over effects given the random presentation of antagonist-agonist pairings. Thus, although there were bidirectional feeding interactions, blockade of GABA-A receptors in the VTA had far greater effects on baclofen-induced feeding elicited from the NACs than blockade of GABA-A receptors in the NACs had on baclofen-induced feeding elicited from the VTA. That said, the between-site interactions between GABA-A receptor antagonists particularly in the VTA in reducing baclofen-induced feeding stands in marked contrast to the inability of GABA-A antagonism in the VTA or NACs to block baclofen-

induced feeding elicited from the same site (Echo et al., 2002; Stratford & Kelley, 1997; Znamensky et al., 2001), again supporting the presence of a bidirectional regional interaction. One caveat that should be noted is that blockade of GABA receptors in the VTA or NAC shell may have produced a generalized state of behavioral suppression that may not be specific to food intake, thereby producing a state incompatible with feeding. This might be addressed in future studies by including a measure of locomotor activity during the test to assess potential non-specific sedating effects of the antagonists or by evaluating a measure of taste reactivity to assess potential malaise-inducing properties of the antagonists. That said, behavioral suppression therefore, cannot account for the failure of NACs bicuculline pretreatment to alter NAC shell baclofen-induced feeding, of NACs saclofen pretreatment to alter NACs muscimol-induced feeding, of VTA bicuculline pretreatment to alter VTA baclofen-induced feeding, and of VTA saclofen pretreatment to alter VTA muscimol-induced feeding (Echo et al., 2002; Stratford and Kelley, 1997; Znamensky et al., 2001).

The GABA-B agonist, baclofen was chosen for study in this paradigm even though the GABA-A agonist, muscimol also can elicit feeding from both sites (Echo et al., 2002; Khaimova et al., 2004; Klitenick and Wirtshafter, 1988; Soderpalm and Berridge, 2000; Stratford and Kelley, 1997; Znamensky et al., 2001). First, whereas baclofen produced robust dose-dependent feeding responses in both the NACs and VTA over a wide dose range, muscimol produced a more muted, but significant feeding response in both sites over a very limited dose range (Echo et al., 2002; Khaimova et al., 2004; Znamensky et al., 2001). Second, seizure or pre-seizure activity was noted for muscimol doses just outside the effective feeding dose range. Third, whereas baclofen effects upon feeding were invariably described as an agonist action at GABA-B receptors, the differential cellular localization of GABA-A and GABA-B receptors resulted in

the more universal inhibition of cells by muscimol. That said, muscimol effects are also informative about GABA feeding. Region-specific effects in the NACs are observed following muscimol with increased feeding, visits to food, place preferences and positive hedonic reactions after administration into the rostral NACs, and defensive paw-treading and burial behaviors after administration into the caudal NACs (Reynolds and Berridge, 2001, 2002). Muscimol in the NAC shell activates Fos-like immunoreactivity in the lateral hypothalamus, lateral septum, paraventricular hypothalamus, VTA, substantia nigra and nucleus of the solitary tract (Stratford and Kelley, 1999), stimulates intake of both carbohydrates and fats regardless of whether the macronutrients are presented singly or together, and increases intake of sucrose, but not water, saccharin or saline (Basso and Kelley, 1999). However, muscimol administered into the NACs failed to alter operant lever-pressing for food (Hanlon et al., 2004) and failed to increase the breaking point in a food reinforced progressive ratio paradigm (Zhang et al., 2003). Finally, muscimol administration into the central amygdala reduces GABA-A-agonist-induced feeding elicited from the NAC shell (Baldo et al., 2005).

What are some potential mechanisms of action by which bidirectional GABA-B antagonism in the other site significantly reduces GABA-B-induced feeding from the VTA and NACs and by which VTA GABA-A antagonism significantly reduces GABA-B-induced feeding from the NACs. First, GABA receptors in the VTA appear to be innervated by GABA projections from the NAC and ventral pallidum (Churchill et al., 1991; Kalivas et al., 1993; Van Bockstaele and Pickel, 1995; Zahm et al., 1985). Second, GABA-A and GABA-B receptors originate from separate and discrete afferent neuronal pools (Sugita et al., 1992) with the former found primarily on non-dopamine neurons (Churchill et al., 1992) and the latter found on dopamine neurons (Margreta-Mitrovic et al., 1999; Wirtshafter & Sheppard, 2001). In this regard, VTA

GABA-A receptor inhibition produces rewarding conditioned place preferences independent of dopaminergic neural motivational systems (Laviolette & van der Kooy, 2001). Further, a number of the non-dopaminergic VTA cells receiving GABA-A receptor innervation contain GABA, and project to the NAC (Van Bockstaele & Pickel, 1995). Third, the selectivity and specificity of GABA-A- and GABA-B-mediated activity in the VTA is supported by the findings that GABA-A synaptic potentials were never accompanied by GABA-B synaptic potentials within the VTA, and that serotonin reduced GABA-B, but not GABA-A IPSPs in the VTA (Sugita et al., 1992). Fourth, GABA is found in medium-sized spiny neurons in the NAC (Meredith, 1999) that form reciprocal projections among the NAC, ventral pallidum or VTA (Carr & Sesack, 2000a; Churchill and Kalivas, 1994; Churchill et al., 1991; Kalivas et al., 1993; Meredith et al., 1993; Van Bockstaele & Pickel, 1995). From these data, blockade of either GABA-A or GABA-B receptors in the VTA would reduce baclofen-induced feeding elicited from the NACs by interfering with activity of: a) descending GABA (or other) fibers from the NACs or ventral pallidum to the VTA, b) interneurons within the VTA, and/or, c) ascending dopaminergic (or other) fibers ascending from the VTA to the NACs. Correspondingly, blockade of GABA-B receptors in the NACs would reduce baclofen-induced feeding elicited from the VTA by interfering with activity of: a) descending GABA (or other) fibers from the NACs or ventral pallidum to the VTA, b) interneurons within the NACs, and/or c) ascending dopaminergic (or other) fibers ascending from the VTA to the NACs.

The next chapter evaluates whether GABA-B-mediated feeding in the NACs and VTA are under regional control of opioid receptors thus providing additional understanding of the functional neuroanatomical links between these two orexigenic systems.

CHAPTER FOUR

OPIOID RECEPTORS MEDIATE GABA-B AGONIST-INDUCED FEEDING: BETWWEN THE NACs AND VTA

Introduction

Using within-site pharmacological feeding studies, a relationship between opioid and GABA systems has been established such that GABA agonist-induced feeding elicited from the VTA and NACs were influenced by opioid receptor antagonism, and opioid agonist-induced feeding elicited from the VTA and NACs were influenced by GABA receptor antagonism. Thus, feeding elicited by the mu-opioid receptor agonist, DAMGO in the NACs was significantly and respectively reduced and enhanced by GABA-B and GABA-A receptor antagonist pretreatment (Znamensky et al., 2001). Moreover, pretreatment with GABA-A or GABA-B antagonists in the VTA significantly reduced DAMGO-induced feeding elicited from that site (Echo et al., 2002). In turn, pretreatment with the general opioid antagonist, NTX blocked feeding elicited by GABA-B, but not GABA-A agonists when both antagonist and agonist were administered into the VTA (Echo et al., 2002), but for GABA-A, but not GABA-B agonists when both antagonist and agonist were administered into the NACs (Znamensky et al., 2001). Moreover, baclofen-induced feeding elicited from the VTA or NACs was significantly reduced by the kappa opioid antagonist, NBNI administered into the same site. Yet, whereas baclofen-induced feeding elicited from the VTA was significantly reduced by mu-, but not delta-opioid antagonists, baclofen-induced feeding elicited from the NACs was significantly reduced by delta-, but not mu-opioid antagonists (Khaimova et al., 2004).

Opioid agonists elicit feeding across a range of brain sites, including the VTA and NACs (see review: Bodnar, 2004). Unlike the receptor selectivity observed for GABA antagonism of

GABA agonist-induced feeding effects within sites, multiple opioid receptor subtype antagonists can block opioid mu and delta agonist-induced feeding responses elicited from the VTA and NACs when both antagonist and agonist are administered into a common site. Thus, mu-opioid agonist-induced feeding with DAMGO in the NACs was significantly reduced by accumbal pretreatment with mu, delta, kappa but not mu-1 opioid receptor subtype antagonists (Ragnauth et al., 2000). Correspondingly, DAMGO-induced feeding elicited from the VTA was significantly reduced by general, mu, kappa, but not delta opioid receptor subtype pretreatment in the VTA (Lamonte et al., 2002). Further findings using selective opioid receptor subtype antagonists, general, mu and kappa, but not delta, opioid receptor antagonist pre-treatment in the VTA reduced DAMGO-induced feeding elicited from the NACs. Correspondingly, general, mu and delta, and to a lesser degree kappa, opioid receptor antagonist pre-treatment in the NACs reduced DAMGO-induced feeding elicited from the VTA. Thus, multiple opioid receptor subtypes mediate opioid–opioid feeding interactions between the NACs and VTA regions (Bodnar et al., 2005).

Therefore, there has been substantial evaluation of feeding interactions in the VTA and NACs for local GABA antagonist-GABA agonist effects, for local opioid antagonist-opioid agonist effects, for local GABA antagonist-opioid agonist effects, for local opioid antagonist-GABA agonist effects, and for regional opioid antagonist-opioid agonist effects (Bodnar et al., 2005; Echo et al., 2002; Lamonte et al., 2002; Ragnauth et al., 2000; Stratford and Kelley, 1997; Znamensky et al., 2001). An analysis of regional interactions between the VTA and NACs examining GABA-A and GABA-B antagonist pretreatment effects in one site upon DAMGO-induced feeding elicited from a second site failed to show regional GABA-induced mediation of mu-opioid agonist-induced feeding (Ackerman et al., 2003). The present study was designed to

answer the last remaining question in this series of studies, namely whether feeding elicited by the GABA-B agonist, baclofen microinjected into the NACs was dose-dependently blocked by pretreatment with opioid antagonists blocking all (NTX), mu (BFNA), kappa (NBNI) or delta (NTI) opioid receptor subtypes in the VTA, and whether baclofen-induced feeding elicited from the VTA was dose-dependently blocked by pretreatment with NTX, BFNA, NBNI or NTI in the NACs.

Experimental Procedure

Subjects, Surgery, Drugs, Statistics and Histology

The subjects, surgery, drugs, statistics and histology are described in the General Methods Section.

Protocols

Eight protocols were followed evaluating the effect on spontaneous food intake: 1) VTA NTX and NACs baclofen-induced feeding; 2) NACs NTX and VTA baclofen-induced feeding; 3) VTA BFNA and NACs baclofen-induced feeding; 4) NACs BFNA and VTA baclofen-induced feeding; 5) VTA NBNI and NACs baclofen-induced feeding; 6) NACs NBNI and VTA baclofen-induced feeding; 7) VTA NTI and NACs baclofen-induced feeding; and 8) NACs NTI and VTA baclofen-induced feeding. In the first two NTX protocols, five conditions were tested: (a) baseline vehicle values, (b) bilateral baclofen (200 ng, 100 ng each side) microinjections in the agonist site, and (c-e) bilateral NTX (0.1, 1, 5 ug, 0.05, 0.5, 2.5 ug each side) in the antagonist site followed by bilateral baclofen (200 ng, 100 ng each side) microinjections in the agonist site. In the subsequent six specific opioid receptor subtype protocols, four conditions were tested: (a) baseline vehicle values, (b) bilateral baclofen (200 ng) microinjections in the agonist site, and (c-d) bilateral BFNA (0.4, 4 ug, 0.2, 2 ug each side), bilateral NBNI (0.6, 6 ug,

0.6, 3 ug each side) or bilateral NTI (0.4, 4 ug, 0.2, 2 ug each side) in the antagonist site followed by bilateral baclofen (200 ng) microinjections in the agonist site. Cumulative intakes were assessed at 1, 2 and 4 h following the agonist microinjection. The NACs and VTA baclofen (total: 200 ng) dose was chosen because it was used previously in the analysis of GABA receptor antagonist effects within the NACs and the VTA (Echo et al., 2002; Znamensky et al., 2001). The opioid antagonist injection intervals and test doses were chosen to produce maximal antagonist effects in previous studies (e.g., Bodnar et al., 2005; Khaimova et al., 2004; Lamonte et al.,2002; Ragnauth et al., 2000), and to allow comparisons of effects across studies.

Results

Histological Verification

Figure 5 and 6 displays the bilateral locations of the tips of the cannula placements in the NACs (Figure 5) and VTA (Figure 6) in the 66 animals that successfully completed the GABA-B agonist-opioid antagonist study. Proper and overlapping bilateral NACs cannula placements were observed for the 33 rats receiving baclofen (circles) and the 33 rats receiving opioid antagonists (squares: NTX (n=8); BFNA (n=8), NBNI (n=9), NTI (n=8). In turn, the four NACs opioid antagonist groups showed considerable overlap with each other. Proper and overlapping bilateral VTA cannula placements were observed for the 33 rats receiving baclofen (circles) and the 33 rats receiving opioid antagonists (squares: NTX (n=8); BFNA (n=9), NBNI (n=8), NTI (n=8). In turn, the four VTA opioid antagonist groups showed considerable overlap with each other.

Figure 5. Schematic representation of bilateral NACs cannula placements for 33 rats receiving the GABA-B agonist, baclofen (circles) and the 33 rats receiving opioid antagonists (squares: NTX (n=8); BFNA (n=8), NBNI (n=9), NTI (n=8). The three panels (Figures 14, 16 and 20) are from the atlas of Paxinos and Watson (2009), and are respectively 2.28, 2.04 and 1.56 mm anterior to the bregma suture. All cannulae tips were localized within the NACs, and there was considerable overlap in NACs placements in agonist-treated animals treated with opioid antagonists (NTX (n=8); BFNA (n=9); NBNI (n=8); NTI (n=8)) in the VTA. There was also considerable overlap in NACs placements in animals receiving NTX (n=8), BFNA (n=8), NBNI (n=9) or NTI (n=8) and tested with baclofen in the VTA.

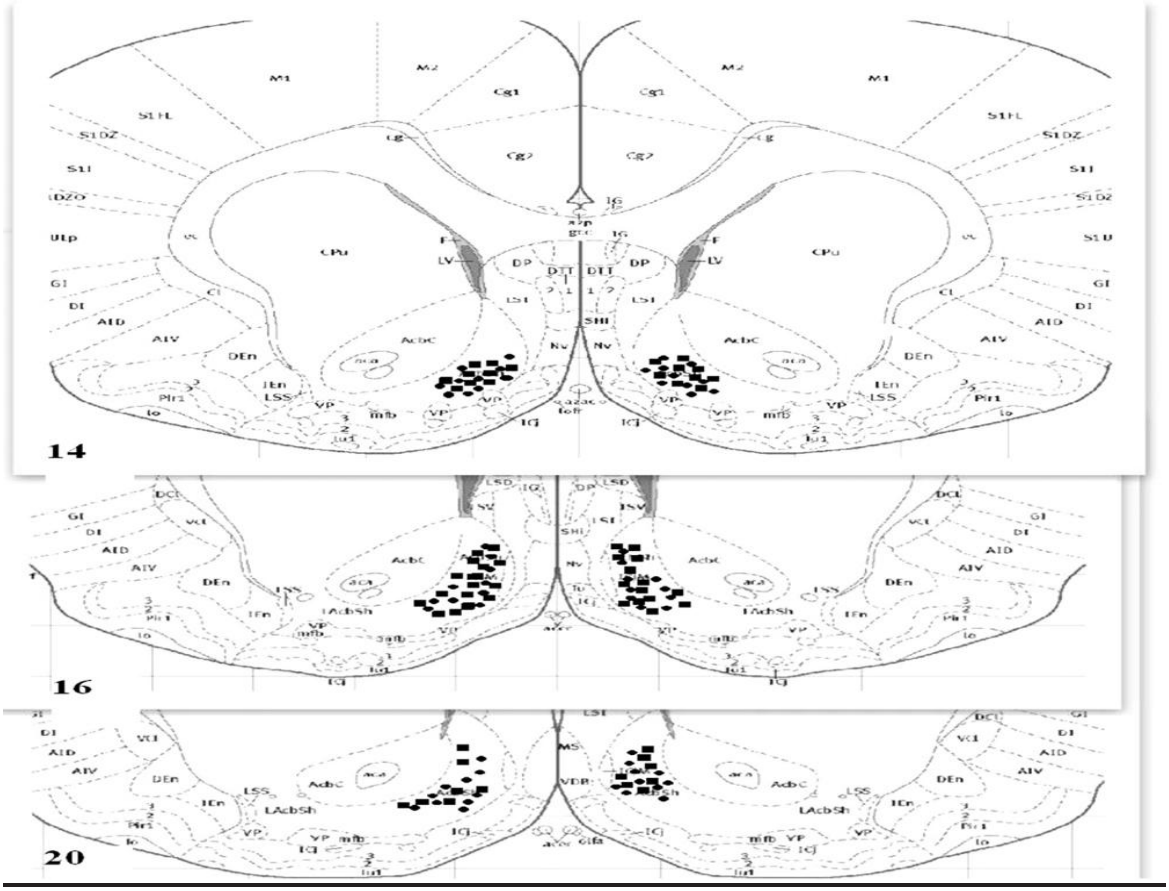
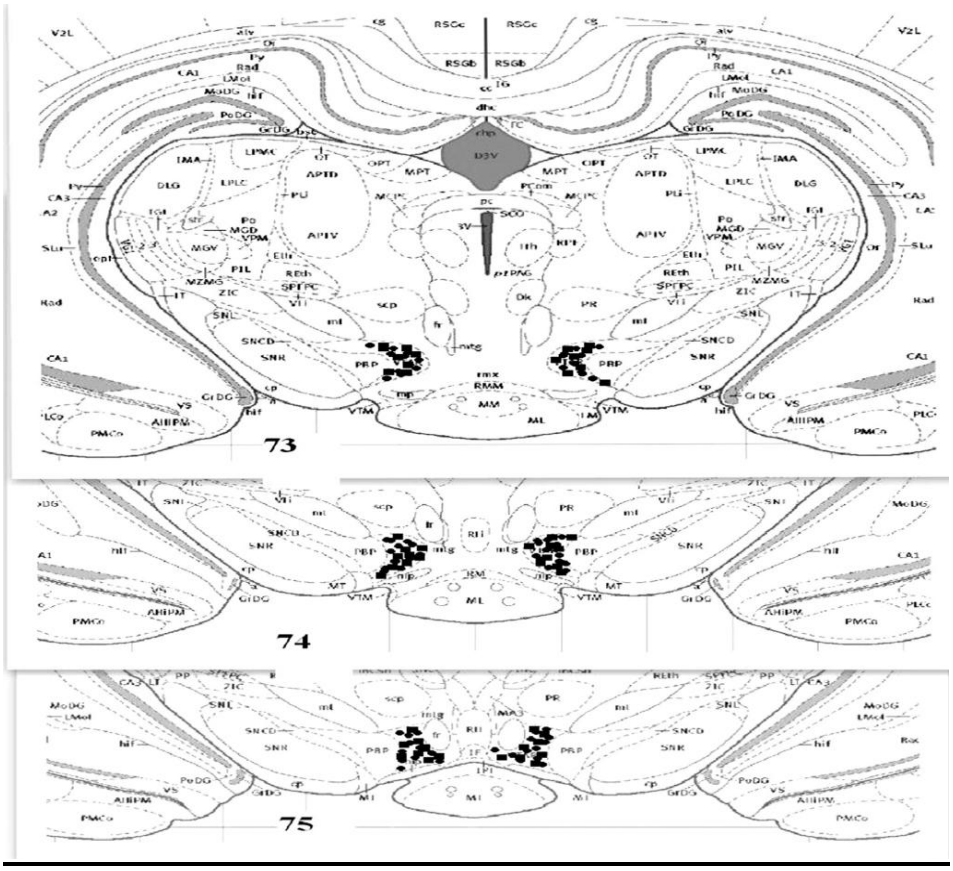


Figure 6. Schematic representation of bilateral VTA cannula placements for 33 rats receiving baclofen (circles) and the 33 rats receiving opioid antagonists (squares: NTX (n=8); BFNA (n=9), NBNI (n=8) or NTI (n=8). The three panels (Figures 73, 74 and 75) are from the atlas of Paxinos and Watson (2009), and are respectively 4.80, 4.92 and 5.04 mm posterior to the bregma suture. All cannulae tips were localized within the VTA, and there was considerable overlap in VTA placements in agonist-treated animals treated with opioid antagonists (NTX (n=8); BFNA (n=8), NBNI (n=9), NTI (n=8)) in the NACs. There was also considerable overlap in VTA placements in animals receiving NTX (n=8); BFNA (n=9), NBNI (n=8) or NTI (n=8) and tested with baclofen in the NACs. The cannula placements of rats receiving opioid antagonists alone in either the NACs (n=8) or VTA (n=8) showed considerable overlap with the rats used in the GABA-B agonist-opioid antagonist study (data not shown).



GABA-B Agonist-Induced Feeding in the VTA and NAC

Thirty-three rats with bilateral VTA cannulae and 33 rats with bilateral NACs cannulae were assessed for baclofen-induced feeding relative to baseline intake. Significant differences in food intake were observed between baseline and baclofen conditions ($F(1,32)= 108.22$, $p<0.0001$), across test times ($F(2,64)= 119.56$, $p<0.0001$), and for the interactions between sites and times ($F(2,64)= 5.77$, $p<0.005$), conditions and times ($F(2,64)= 41.90$, $p<0.0001$), or among sites, conditions and times ($F(2,64)= 5.91$, $p<0.0004$) but not between sites ($F(1,32)= 0.36$, ns), or for the interaction between sites and conditions ($F(1,32)= 0.37$, ns). Food intake was significantly increased above baseline levels after 1, 2 and 4 h following baclofen administration into the NACs or VTA. Whereas the magnitude of baclofen-induced feeding between the NACs (2 h: 3.6 g; 4 h: 4.6 g) and the VTA (2 h: 3.6 g; 4 h: 5.1 g) failed to differ from each other, baclofen-induced food intake was initially significantly higher after 1 h following NACs (2.7 g) relative to VTA (1.9 g) administration. Therefore in general, any regional opioid antagonist effects upon GABA-B agonist-induced feeding in the VTA or NACs were not due to intrinsic differences in the magnitude of the agonist's ability to differentially elicit feeding in the two sites; this pattern was observed in the previous protocol.

VTA NTX Antagonism and NACs GABA-B Agonist-Induced Feeding

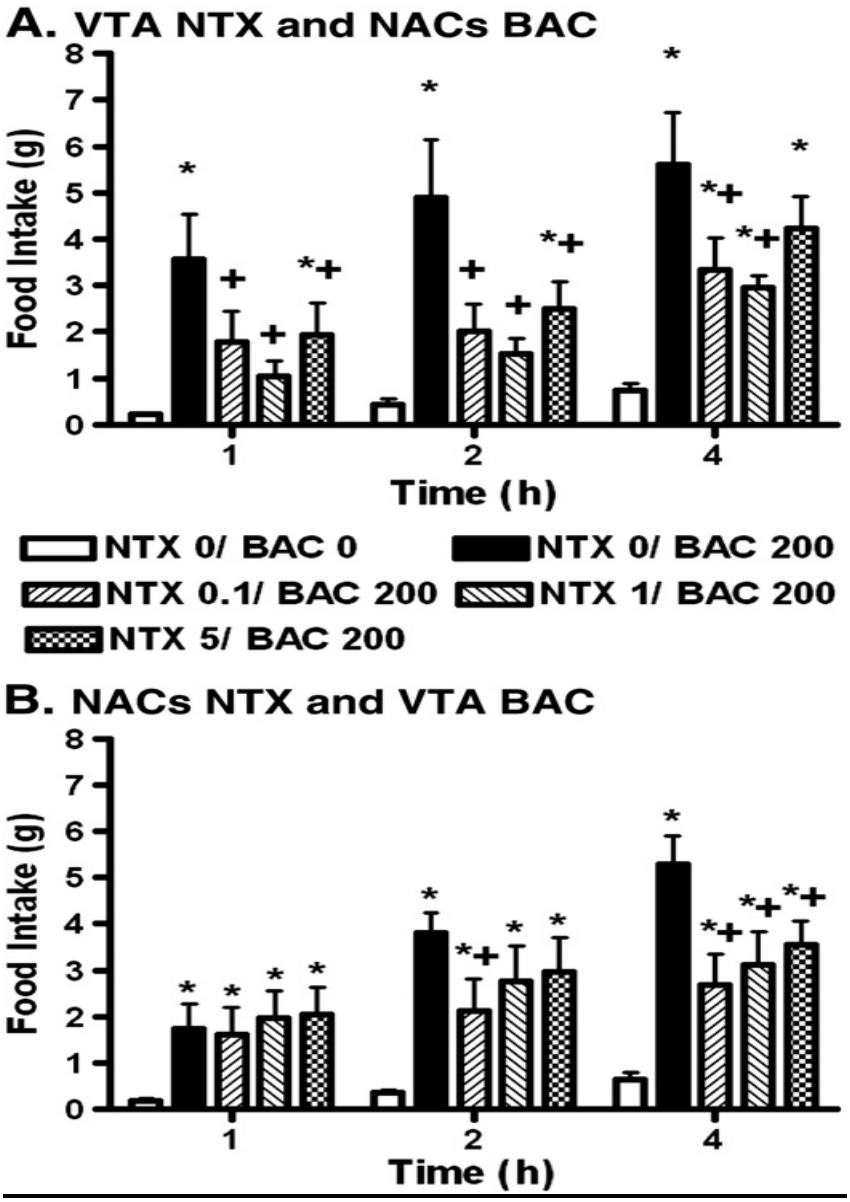
Significant differences in food intake were observed across NTX doses ($F(4,32)= 5.90$, $p<0.001$) and across test times ($F(2,64)= 38.79$, $p<0.0001$), and approached significance for the interaction between doses and times ($F(8,64)= 1.93$, $p=0.072$). Baclofen in the NACs significantly increased food intake relative to baseline treatment across the time course (Figure 7A). In the presence of VTA NTX, NACs baclofen significantly increased food intake above baseline values only after 4 h following the 0.1 and 1 ug NTX doses, and these VTA NTX doses significantly

decreased NACs baclofen-induced feeding across the time course (Figure 7A). In the presence of the highest (5 ug) VTA NTX dose, NACs baclofen significantly increased food intake over baseline values across the time course, but the magnitude of this effect was significantly reduced after 1 and 2 h by the antagonist(Figure 7A). Thus, the ability of baclofen administered into the NACs to elicit feeding depended upon the integrity of all VTA opioid receptors, indicating the presence of a regional interaction.

NACs NTX Antagonism and VTA GABA-B Agonist-Induced Feeding

Significant differences in food intake were observed across NTX doses ($F(4,35)= 5.87$, $p<0.001$), across test times ($F(2,70)= 30.98$, $p<0.0001$), and for the interaction between doses and times ($F(8,70)= 3.57$, $p<0.016$). Baclofen in the VTA significantly increased food intake after 2-4 h relative to baseline treatment (Figure 7B). VTA baclofen also significantly increased food intake above baseline values in the presence of all NTX doses in the NACs. However, the magnitude of VTA baclofen-induced feeding was significantly decreased by NACs NTX doses of 0.1 (2-4 h), 1 (4 h) and 5 (4 h) ug (Figure 7B). Thus, the ability of baclofen administered into the VTA to elicit feeding depended upon the integrity of all NACs opioid receptors, indicating the presence of a regional interaction as well as bidirectional regional interactions for opioid antagonist-GABA-B agonist interactions.

Figure 7. Alterations in food intake (g, +S.E.M.) following administration of the GABA-B agonist, baclofen (200 ng) into the NACs (Panel A) or VTA (Panel B) 30 min following administration of the general opioid antagonist, naltrexone (NTX) at doses of 0.1, 1 and 5 ug into the other site. The asterisks (*) in this and the subsequent figures indicate significant increases in food intake following agonist administration relative to baseline treatment, whereas the crosses (+) indicate significant decreases in agonist-induced food intake following antagonist administration.



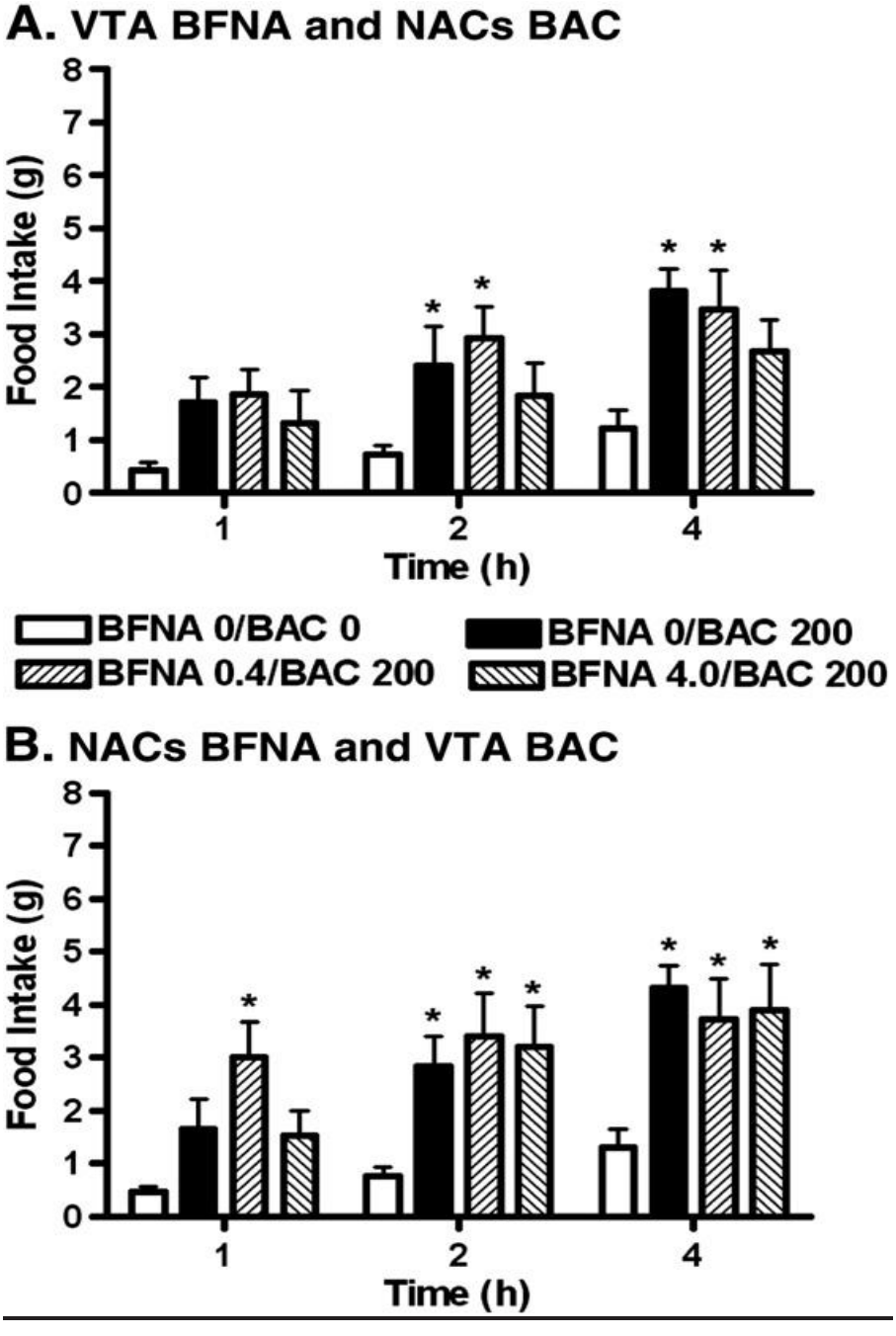
VTA BFNA Antagonism and NACs GABA-B Agonist-Induced Feeding

Significant differences in food intake were observed across BFNA doses ($F(3,32)= 2.92$, $p<0.049$) and across test times ($F(2,64)= 26.38$, $p<0.0001$), but not for the interaction between doses and times ($F(6,64)= 1.22$, ns). Baclofen in the NACs significantly increased food intake relative to baseline treatment after 2 and 4 h (Figure 8A). Significant NACs baclofen-induced feeding was observed 2 and 4 h following the low, but not high VTA BFNA dose (Figure 8A). However, neither dose of VTA BFNA significantly reduced the magnitude of NACs baclofen-induced feeding (Figure 8A). Thus, the ability of baclofen to elicit feeding following injections into the NACs depended only in part upon the integrity of VTA mu-opioid receptors, although not to the same extent as general (NTX) antagonism of all VTA opioid receptors.

NACs BFNA Antagonism and VTA GABA-B Agonist-Induced Feeding

Significant differences in food intake were observed across BFNA doses ($F(3,28)= 3.53$, $p<0.028$), across test times ($F(2,56)= 22.79$, $p<0.0001$), and for the interaction between doses and times ($F(6,56)= 2.37$, $p<0.041$). Baclofen in the VTA significantly increased food intake relative to baseline treatment after 2 and 4 h (Figure 8B). VTA baclofen significantly increased food intake over baseline following accumbal pretreatment with the 0.4 (1-4 h) and 4 (2-4) ug BFNA doses, and neither NACs mu antagonist dose significantly affected the magnitude of VTA baclofen-induced feeding (Figure 8B). Thus, the ability of baclofen to elicit feeding following injections into the VTA was unaffected by blockade of NACs mu opioid receptors, thereby indicating only a mild unidirectional (VTA mu antagonist- NACs GABA-B agonist) regional interaction.

Figure 8. Alterations in food intake (g, +S.E.M.) following administration of the GABA-B agonist, baclofen (200 ng) into the NACs (Panel A) or VTA (Panel B) 24 h following administration of the mu-selective opioid antagonist, beta-funaltrexamine (BFNA) at doses of 0.4 and 4 ug into the other site.



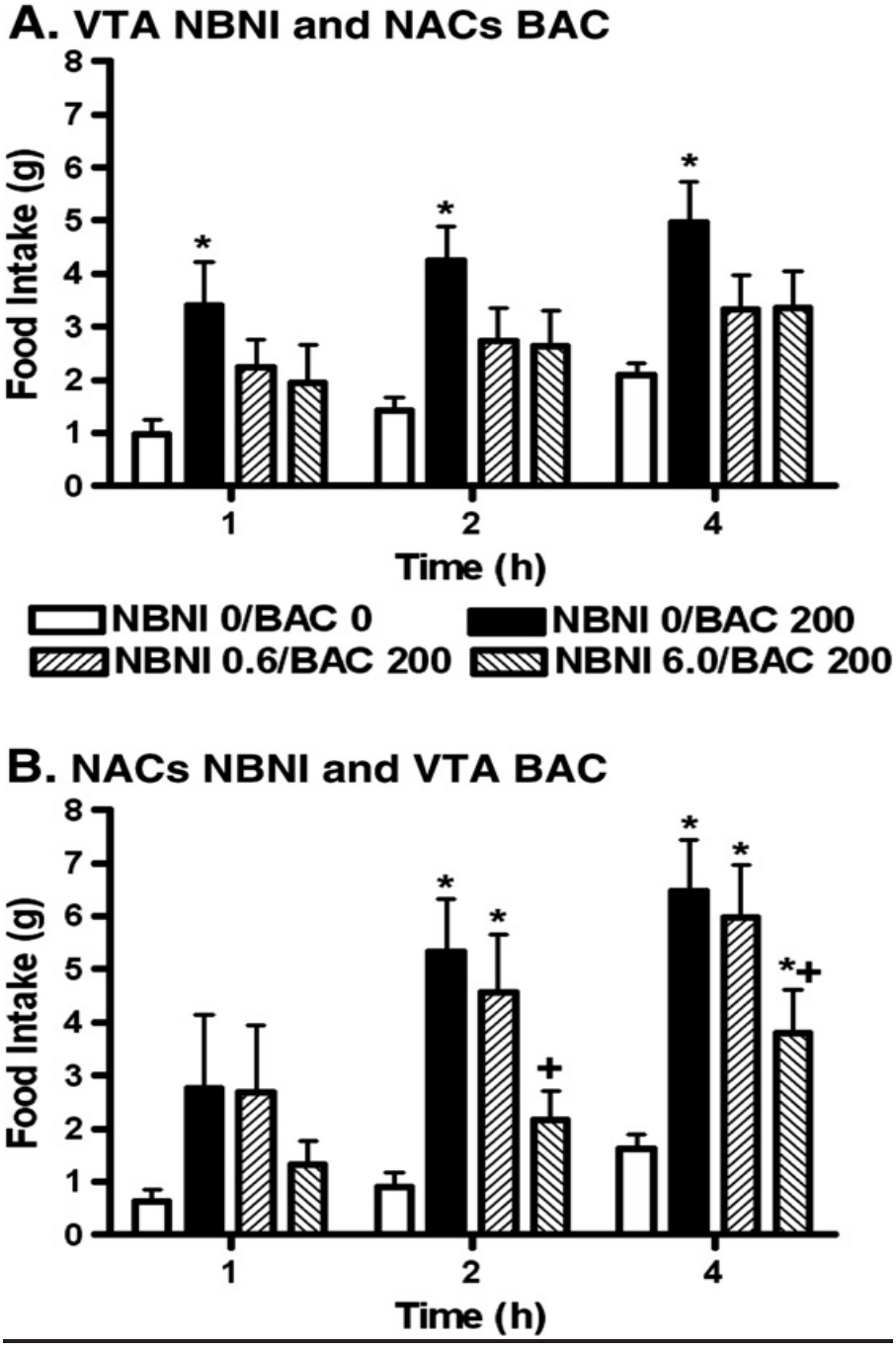
VTA NBNI Antagonism and NACs GABA-B Agonist-Induced Feeding

Significant differences in food intake were observed across NBNI doses ($F(3,28)= 4.15$, $p<0.015$) and across test times ($F(2,56)= 16.94$, $p<0.0001$), but not for the interaction between doses and times ($F(6,56)= 0.16$, ns). Baclofen in the NACs significantly increased food intake relative to baseline treatment across the time course (Figure 9A). However, NACs baclofen-induced feeding failed to be observed at any time point in the presence of the low or high VTA NBNI dose. Yet neither dose of VTA NBNI significantly reduced the magnitude of NACs baclofen-induced feeding (Figure 9A). Thus, the ability of baclofen to elicit feeding following injections into the NACs depended in part upon the integrity of VTA kappa-opioid receptors, although not to the same extent as general (NTX) antagonism of all VTA opioid receptors.

NACs NBNI Antagonism and VTA GABA-B Agonist-Induced Feeding

Significant differences in food intake were observed across NBNI doses ($F(3,31)= 4.90$, $p<0.007$), across test times ($F(2,62)= 39.32$, $p<0.0001$), and for the interaction between doses and times ($F(6,62)= 2.54$, $p<0.029$). Baclofen in the VTA significantly increased food intake relative to baseline treatment after 2 and 4 h (Figure 9B). VTA baclofen significantly increased food intake over baseline following accumbal pretreatment with the 0.6 (2-4 h) and 6 (4 h) ug NBNI doses with the magnitude of this feeding response significantly reduced by the high NACs NBNI dose after 2 and 4 h (Figure 9B). Thus, the ability of baclofen to elicit feeding following injections into the VTA was dose-dependently reduced by blockade of NACs kappa opioid receptors, thereby indicating a bidirectional regional interaction which approached the effect of general (NTX) antagonism of all NACs opioid receptors.

Figure 9. Alterations in food intake (g, +S.E.M.) following administration of the GABA-B agonist, baclofen (200 ng) into the NACs (Panel A) or VTA (Panel B) 30 min following administration of the kappa-selective opioid antagonist, nor-binaltorphamine (NBNI) at doses of 0.6 and 6 ug into the other site.



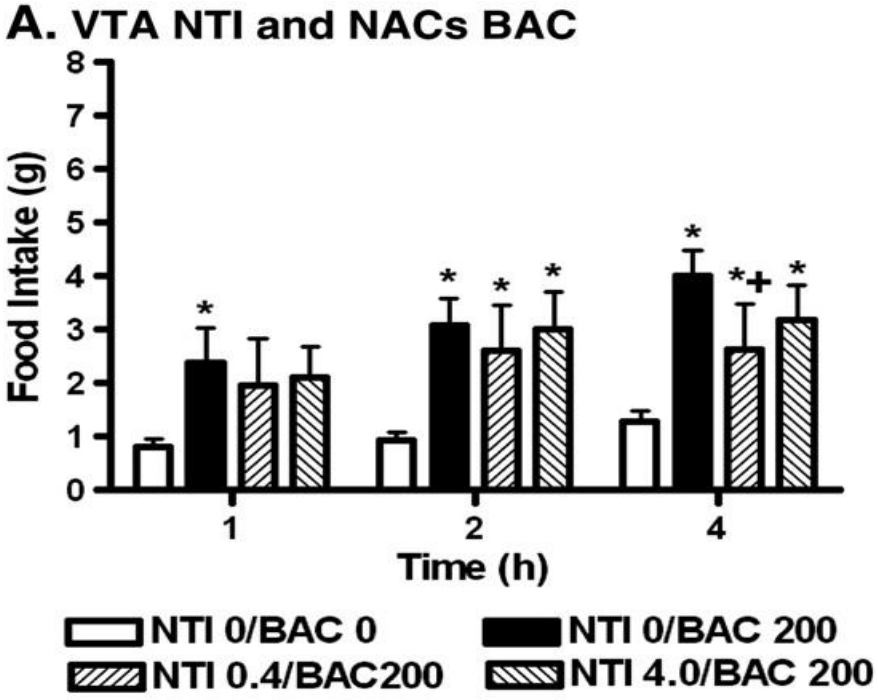
VTA NTI Antagonism and NACs GABA-B Agonist-Induced Feeding

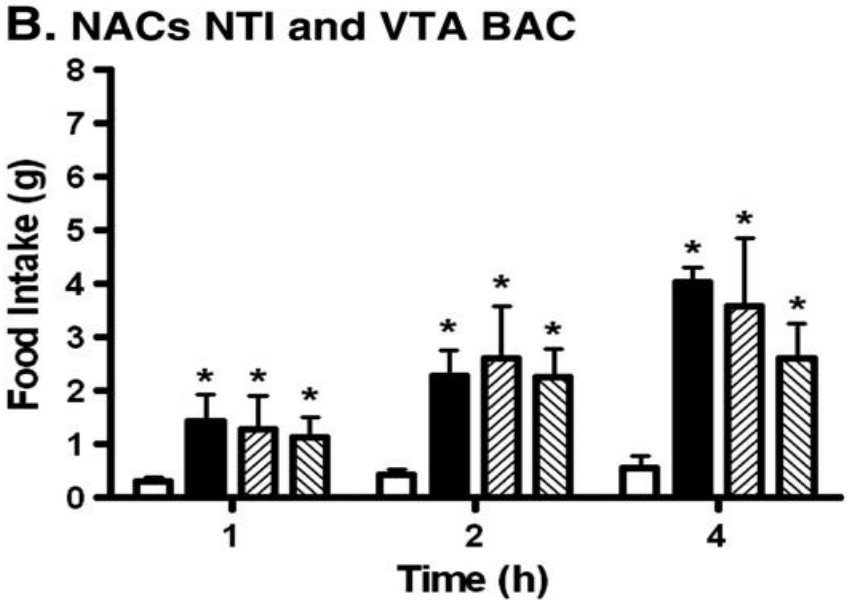
Significant differences in food intake were observed across test times ($F(2,56)= 15.41$, $p<0.0001$), but not across NTI doses ($F(3,28)= 2.64$, $p=0.069$) or for the interaction between doses and times ($F(6,56)= 1.47$, ns). Baclofen in the NACs significantly increased food intake relative to baseline treatment across the time course (Figure 10A). Significant NACs baclofen-induced feeding was observed 2 and 4 h in the presence of both VTA NTI doses with the magnitude of the feeding response significantly, but only transiently (4 h) reduced by the low VTA NTI dose (Figure 10A). Thus, the ability of baclofen to elicit feeding following injections into the NACs depended minimally upon the integrity of VTA delta-opioid receptors, and certainly not to the same extent as general (NTX) antagonism of all VTA opioid receptors.

NACs NTI Antagonism and VTA GABA-B Agonist-Induced Feeding

Significant differences in food intake were observed across NTI doses ($F(3,28)= 3.21$, $p<0.038$) and across test times ($F(2,56)= 21.28$, $p<0.0001$), but not for the interaction between doses and times ($F(6,56)= 1.67$, ns). Baclofen in the VTA significantly increased food intake relative to baseline treatment across the time course as well as in the presence of both accumbal NTI doses with neither accumbal antagonist dose affecting the magnitude of the feeding response (Figure 10B). Thus, the ability of baclofen to elicit feeding following injections into the VTA was unaffected by blockade of NACs delta opioid receptors, and the unidirectional (VTA delta antagonist-NACs GABA-B agonist) regional interaction was far less than that observed following general (NTX) antagonism.

Figure 10. Alterations in food intake (g, +S.E.M.) following administration of the GABA-B agonist, baclofen (200 ng) into the NACs (Panel A) or VTA (Panel B) 30 min following administration of the delta-selective opioid antagonist, naltrindole isothiocyanate (NTI) at doses of 0.4 and 4 ug into the other site.





Discussion

Bilateral microinjection of the GABA-B receptor agonist, baclofen, significantly increased feeding in the VTA and NACs, confirming numerous previous reports (Arnt and Scheel-Kruger, 1979; Echo et al., 2002; Khaimova et al., 2004; Klitenick and Wirtshafter, 1988; Soderpalm and Berridge, 2000; Stratford and Kelley, 1997; Ward et al., 2000; Znamensky et al., 2001). The significant increases in food intake following baclofen administration into the VTA and NACs were comparable between sites, confirming our previous finding. Thus, any site-specific changes in opioid antagonist effects could not be attributed to any potential intrinsic site-specific differences in the magnitude of feeding responses elicited by baclofen.

Previous studies (Echo et al., 2002; Khaimova et al., 2004) demonstrated that baclofen-induced feeding elicited from the VTA was significantly reduced by NTX, BFNA, NBNI and NTI pretreatment in the same site, indicating inclusive opioid receptor involvement within the VTA. Whereas, within the NACs baclofen-induced feeding was reduced by NBNI and NTI (Khaimova et al., 2004; Znamensky et al., 2001), indicating only kappa and delta receptor involvement within the NACs. Examining between-site interactions the present study found that pretreatment with a) NTX in the VTA or NACs significantly reduced baclofen-induced feeding in the alternate site, NACs or VTA, respectively, *unlike the within site NTX affect only found within the VTA,* b) BFNA in the VTA significantly reduced NACs baclofen-induced feeding, whereas BFNA in the NACs failed to affect VTA baclofen-induced feeding, *same as within site findings,* c) NBNI in the VTA significantly reduced NACs baclofen-induced feeding and NBNI in the NACs significantly reduced VTA baclofen-induced feeding, *unlike the within site affect of NBNI to reduce baclofen-induced feeding in the VTA and NACs,* d) NTI in the VTA failed to affect NACs baclofen-induced feeding although, NTI in the NACs significantly reduced VTA

baclofen-induced feeding, *unlike within site affect of NTI to reduce baclofen-induced feeding in the VTA and NACs*. Collectively, these results show a difference in results of between site opioid antagonist pretreatment compared to within site antagonist pretreatment on baclofen-induced feeding, thereby indicating a regional interaction between the VTA and NACs involving the GABA-B and specific opioid receptors. The following chapter will discuss how these regional interactions support the hypothesis of a distributed brain network for feeding elicited by the GABA-B receptor agonist and modulated by GABA and opioid receptor subtypes in rats.

CHAPTER FIVE

GENERAL DISCUSSION

This final chapter will initially review the findings of GABA-A and GABA-B antagonist effects in the NACs and VTA upon GABA-B agonist induced feeding within the alternate site, VTA or NACs in regards to the hypothesis for Specific Aim 1 followed by the findings of opioid antagonist (general, mu, kappa and delta) effects in the NACs and VTA upon GABA-B agonist induced feeding within the alternate site, VTA and NACs in regards to the hypotheses for Specific Aim 2 and 3. This will be followed by a general discussion of a potential empirically based anatomical and neurochemical model mediating GABA antagonist-GABA agonist and opioid antagonist-GABA agonist feeding interactions with the VTA and NACs specifically addressing: (1) the nature of opioid-GABA feeding interactions in general, (2) the regional mediation of GABA-B agonist-induced feeding following general opioid antagonism, (3) the regional mediation of GABA-B agonist-induced feeding following mu-selective antagonism, (4) the regional mediation of GABA-B agonist-induced feeding following delta--selective antagonism, (5) the regional mediation of GABA-B agonist-induced feeding following kappa-selective opioid antagonism, and (6) a potential anatomical and neurochemical empirically-based model mediating opioid-GABA feeding interactions within the VTA and NACs.

GABA-A and GABA-B antagonism in the NACs and VTA effect on GABA-B agonist induced feeding in the alternate site, VTA and NACs (Specific Aim 1): Thus, the goal of **Specific Aim 1** was to examine whether GABA-A (bicuculline) or GABA-B (saclofen) antagonist pretreatment in the VTA dose-dependently reduce baclofen-induced feeding elicited from the NAC and does bicuculline or saclofen pretreatment in the NAC dose-dependently

reduce baclofen-induced feeding elicited from the VTA. The stated hypothesis that pretreatment with the antagonist (bicuculline or saclofen) will dose dependently reduce GABA-B (baclofen) induced feeding bidirectionally was only *partially confirmed.* The present data indicate that robust bidirectional interactions are observed between the VTA and NAC shell in mediating GABA-B-induced feeding with saclofen being as effective in blocking baclofen-induced feeding elicited from the VTA or NAC shell when it is administered into the alternate site. These results correspond to GABA-B antagonist effect on GABA-B agonist-induced feeding within the same site (Echo et al., 2002; Stratford and Kelley, 1997; Znamensky et al., 2001). Whereas GABA-A antagonist pretreatment failed to alter GABA-B-mediated feeding when both antagonist and agonist were administered into the same site (Echo et al., 2002; Stratford and Kelley, 1997; Znamensky et al., 2001), GABA-A antagonist pretreatment in the VTA blocked baclofen-induced feeding elicited from the NACs while GABA-A antagonist pretreatment in the NACs failed to significantly reduce VTA baclofen-induced feeding compared to baseline, indicating the existence of a robust unidirectional interaction.

Opioid antagonism in the NACs and VTA effect on GABA-B agonist induced feeding in the alternate site, VTA and NACs (Specific Aim 2 and 3): Thus, the goal of **Specific Aim 2** was to examine whether general opioid (naltrexone) antagonist pretreatment in the VTA dose-dependently reduce baclofen-induced feeding elicited from the NACs and does naltrexone pretreatment in the NAC dose-dependently reduce baclofen-induced feeding elicited from the VTA. The stated hypothesis that pretreatment with the opioid antagonist (naltrexone) will dose dependently reduce GABA-B (baclofen) induced feeding bidirectionally was *confirmed*. The present data indicate that robust bidirectional interactions are observed between the VTA and NACs in mediating GABA-B-induced feeding with naltrexone. These results correspond to

within site VTA naltrexone blockage of NACs baclofen-induced feeding (Echo et al., 2002) although, this does not correspond to within site NACs baclofen-induced feeding where no effect was found by naltrexone (Znamensky et al., 2001). **Specific Aim 3** was to examine whether mu (beta-funaltrexamine), kappa (nor-binaltorphamine) or delta (naltrindole) opioid receptor subtype antagonist pretreatment in the VTA reduce baclofen-induced feeding elicited from the NACs, and does mu, kappa or delta opioid receptor subtype antagonist pretreatment in the NACs reduce baclofen-induced feeding elicited from the VTA.

The stated hypothesis that pretreatment with the opioid antagonists (beta-funaltrexamine, nor-binaltorphamine, naltrindole) will dose dependently reduce GABA-B (baclofen) induced feeding bidirectionally was only *partially confirmed.* A bidirectional interaction between the NACs and VTA was found only for NBNI, the kappa antagonist. Whereas VTA NTI transiently reduced NACs baclofen-induced feeding, no other significant reduction in baclofen-induced feeding for BFNA or NTI was shown.

1. Nature of Opioid-GABA Feeding Interactions: Changes in feeding responses elicited by the GABA-B agonist, baclofen administered into the VTA or NACs by pretreatment with GABA or opioid antagonists administered into the alternative site could be caused by two different and distinct processes. The first hypothesis proposed by this and a series of previous (Bodnar et al., 2005; Echo et al., 2002; Khaimova et al., 2004; Lamonte et al., 2002; MacDonald et al., 2003; Ragnauth et al., 2000; Stratford and Kelley, 1999; Will et al., 2003; Znamensky et al., 2001) studies would argue that opioid and GABA systems within and between the VTA and NACs act as part of an integrated distributed brain system producing dynamic feeding interactions. Activation of distributed brain networks is also been suggested by studies showing feeding responses elicited by central administration of orexigenic modulators (e.g., Pecina and Berridge,

2000, 2005; Smith and Berridge, 2007; Stratford, 2005; Stratford and Kelley, 1999; Will et al., 2003), and thereby regional interactions between neuroanatomical loci. This has been most actively demonstrated in opioid antagonist–opioid agonist feeding interactions between the NTS and CeA (Giraudo et al., 1998b), the PVN and VTA (Quinn et al., 2003), and the NAC shell and CeA (Kim et al., 2004). Other feeding neuromodulators have been shown to interact between sites with opioid systems. Feeding elicited by orexin-A administered into the LH was blocked by NTX pretreatment in the NAC shell (Sweet et al., 2004), whereas DAMGO-induced feeding elicited from the CeA was blocked by PVN co-treatment with the melanocortin agonist, melanotan II (Beckman et al., 2009). Bidirectional opioid-opioid regional feeding interactions have also been noted between the NAC shell and VTA (Bodnar et al., 2005; MacDonald et al., 2003). Regional opioid-dopamine (MacDonald et al., 2004), opioid-orexin (Sweet et al., 2004) and opioid-melanocortin (Beckman et al. 2009, but not opioid-ghrelin (Naleid et al., 2005), feeding interactions have also been observed.

A specific aspect of this hypothesis was tested in the present series of studies, namely that baclofen-induced feeding in each of the sites is dependent upon GABA or opioid-receptor dependent signaling in the alternative site. However, a second separate hypothesis explaining such data is that the interaction between baclofen and the GABA and opioid receptor antagonists is merely the result of summing two independent drug effects in which baclofen increases food intake and saclofen or NTX (and/or the other GABA and selective opioid antagonists) concomitantly decreases food intake.

As indicated by the second hypothesis, specific decreases in baclofen-induced feeding elicited from one site by GABA or opioid antagonism in a second site could be the result of merely offsetting baclofen-induced increases in intake with GABA or opioid antagonist-induced

decreases in intake. To assess whether the general and specific opioid antagonists administered into the NACs or VTA could significantly reduce spontaneous intake our laboratory investigated whether food intake was altered by NTX, BFNA, NBNI or NTI administered in the VTA or NACs, during the light cycle, when rats typically display minimal consumption of standard laboratory chow. Two groups of eight rats each were stereotaxically implanted with bilateral VTA and NACs cannulae and tested in the early- to mid-phase of the light cycle under the following five conditions: (a) baseline vehicle values, (b) bilateral NTX (5 ug), BFNA (4 ug), NBNI (6 ug) and NTI (4 ug). Cumulative intakes (1, 2 and 4 h) were assessed 0.5 h following NTX, NBNI or NTI administration and 24 h following BFNA administration. The cannula placements in the NACs or VTA showed considerable overlap with the rats used in the GABA-B agonist-opioid antagonist study. The data indicated that vehicle baseline intakes elicited from the VTA and NACs failed to differ from each other (Table 2). VTA NTX significantly reduced baseline intake at 4, but not at 1 or 2 h, producing a 22% reduction. Similarly, VTA BFNA significantly reduced baseline intake at 4, but not at 1 or 2 h, producing a 20% reduction. In contrast, neither NBNI nor NTI administered into the VTA significantly reduced baseline intake across the time course (Table 2). NACs NTX significantly reduced baseline intake at 1 and 4, but not at 2h, producing a 21% reduction. Similarly, NACs BFNA significantly reduced baseline intake at 1 and 4, but not at 2 h, producing a 26% reduction. In contrast, neither NBNI nor NTI administered into the NACs significantly reduced baseline intake across the time course (Table 2). Thus, general and mu, but not delta or kappa opioid antagonists administered into the NACs or VTA produced small, but significant decreases in spontaneous food intake.

Table 2. Alterations (Mean, ±SEM) in spontaneous food intake following vehicle, general (naltrexone: NTX, 5 ug), mu (beta-funaltrexamine: BFNA, 4 ug), kappa (nor-binaltorphamine: NBNI, 6 ug) or delta (naltrindole: NTI, 4 ug) opioid antagonist treatment into the ventral tegmental area (VTA) or nucleus accumbens shell (NACs).

Condition	**Intake (1 h)**	**Intake (2 h)**	**Intake (4 h)**
VTA			
Vehicle Baseline	0.34 (0.03)	0.44 (0.05)	0.69 (0.05)
NTX (5 ug)	0.30 (0.03)	0.40 (0.05)	0.54 (0.03)*
BFNA (4 ug)	0.26 (0.04)	0.38 (0.05)	0.55 (0.05)*
NBNI (6 ug)	0.33 (0.04)	0.44 (0.03)	0.61 (0.05)
NTI (4 ug)	0.29 (0.04)	0.43 (0.05)	0.60 (0.03)
NACs			
Vehicle Baseline	0.38 (0.05)	0.49 (0.06)	0.73 (0.07)
NTX (5 ug)	0.26 (0.03)*	0.40 (0.04)	0.58 (0.04)*
BFNA (4 ug)	0.25 (0.04)*	0.41 (0.03)	0.54 (0.07)*
NBNI (6 ug)	0.34 (0.05)	0.53 (0.05)	0.66 (0.06)
NTI (4 ug)	0.30 (0.03)	0.43 (0.04)	0.63 (0.07)

One limitation of this paradigm is the issue of "floor" effects. Our laboratory and others have conducted a wide range of opioid antagonist studies in the NACs and VTA evaluating feeding paradigms with robust intake following food deprivation, glucoprivation and exposure to a palatable sucrose solution (Bodnar et al., 1995; Kelley et al., 1996; Ragnauth et al., 1997). As indicated in Table 3, deprivation-induced intake is significantly reduced following general, mu and kappa, but not delta opioid antagonists administered into the NACs with the magnitude of inhibition (31-55%) which is greater than the significant results observed following general (21%) and delta (19%) antagonists administered into the VTA. A similar pattern is observed for significant inhibition of glucoprivic intake by general, mu and kappa, but not delta opioid antagonists administered into the NACs (75-100%) relative to general (64%) and delta (27%) antagonists administered into the VTA. Finally, sucrose intake is significantly, but more marginally inhibited to a similar degree by general (39%) and delta (25%) opioid antagonists in the VTA, and by general (27%) and mu (37%) opioid antagonists in the NACs. Therefore, there are differential actions of the opioid antagonists depending on the receptor subtype, the site of action and the feeding paradigm, that appears to be indicative of specific reductions rather than countervailing overall intake reductions. How these opioid-mediated effects on specific feeding paradigms compare with regional interactions with GABA-B agonist-induced feeding is discussed in the following sections.

Another argument against a mere summing of two independent drug effects (baclofen-induced increases countervailed by opioid antagonist-induced decreases) is the ability of NTX and the mu-opioid antagonist, BFNA to basically eliminate feeding elicited by the mu-opioid agonist, DAMGO when both are administered into the NACs or the VTA (Table 3; Bakshi and Kelley, 1993; Lamonte et al., 2002; Ragnauth et al., 2000). Again site-specific effects are observed for

other selective antagonists in which kappa, but not delta opioid antagonism significantly suppresses feeding elicited by DAMGO in the VTA, and in which both kappa and delta antagonists suppress feeding elicited by DAMGO in the NACs. The presence of regional opioid-opioid feeding interactions between the VTA and NACs (Bodnar et al., 2005; MacDonald et al., 2003) as well as opioid-opioid interactions between the PVN and CeA (Giraudo et al., 1998a), the NTS and CeA (Giraudo et al., 1998b), the PVN and VTA (Quinn et al., 2003) and the NACs and CeA (Kim et al., 2004) appears to be predicated more on the receptor selectivity of the opioid antagonist acting on an agonist-stimulated system in a distributed brain network (hypothesis 1), relative to independent and countervailing opioid antagonist-opioid agonist effects (hypothesis 2).

Table 3. Reductions (%) in food intake elicited under spontaneous, food deprivation, glucoprivic (2-deoxy-D-glucose) and palatable (10% sucrose) conditions or following the mu-opioid agonist, DAMGO or the GABA-B agonist baclofen into the ventral tegmental area (VTA) or nucleus accumbens shell (NACs) in animals pretreated with general (naltrexone, NTX), mu (beta-funaltrexamine, BFNA), kappa (nor-binaltorphamine, NBNI) or delta (naltrindole, NTI) opioid antagonists in the VTA or NAC.

Condition	**Spontaneous Intake**	**Food Deprivation**	**Glucoprivic Intake**	**Sucrose Intake**	**NAC DAMGO**	**VTA DAMGO**	**NAC Baclofen**	**VTA Baclofen**
VTA					**Regional**	**Within-Site**	**Regional**	**Within-Site**
NTX	**22%***	**21%*[1]**	**64%*[1]**	**39%*[1]**	**100%*[6]**	**100%*[7]**	**58-78%***	**40%*[9]**
BFNA	**20%***	0%[1]	0%[1]	0%[1]	**100%*[6]**	**80%*[7]**	24-30%	**100%*[10]**
NBNI	12%	5%[1]	20%[1]	9%[1]	**50%*[6]**	**90%*[7]**	**30-36%***	**60%*[10]**
NTI	13%	**19%*[1]**	**27%*[1]**	**25%*[1]**	15%[6]	10%[7]	34%	**50%*[10]**
NACs					**Within-Site**	**Regional**	**Within-Site**	**Regional**
NTX	**21%***	**44%*[2]**	**79%*[2]**	**27%*[2]**	**100%*[5]**	**100%*[6]**	0%[8]	**46-56%***
BFNA	**26%***	**55%*[2]**	**100%*[2]**	**37%*[2]**	**100%*[4]**	**100%*[6]**	20%[10]	14%
NBNI	10%	**31%*[2]**	**75%*[2]**	0%[2]	**50%*[4]**	**60%*[6]**	**100%*[10]**	**40-47%***
NTI	14%	0%[3]	N/A	0%[3]	**100%*[4]**	**100%*[6]**	**50%*[10]**	35%

Data derived from: [1]Ragnauth et al., 1997; [2]Bodnar et al., 1995; [3]Kelley et al., 1996; [4]Ragnauth et al., 2000; [5]Bakshi and Kelley, 1993; [6]Bodnar et al., 2005; [7]Lamonte et al., 2002; [8]Znamensky et al., 2001; [9]Echo et al., 2002; [10]Khaimova et al., 2004; N/A: not available. *Significant reductions are depicted in **bold**.

2. Regional General Opioid Antagonist Effects upon GABA-B Agonist-induced Feeding: Whereas baclofen-induced feeding elicited from the VTA was significantly, but not completely reduced by NTX pretreatment in the same site (Echo et al., 2002), baclofen-induced feeding elicited from the NACs was insensitive to NTX pretreatment in the same site (Znamensky et al., 2001) (Table 2). The present study demonstrates that robust bidirectional feeding interactions occur between opioid and GABA-B receptor systems in the VTA and NACs such that general opioid antagonism in the VTA mitigates the ability of baclofen to elicit feeding when administered into the NACs, and correspondingly that general opioid antagonism in the NACs mitigates the ability of baclofen to elicit feeding when administered into the VTA. Given that NTX pretreatment interferes with VTA baclofen-induced feeding when the antagonist is administered into the same site (VTA) or another site (NACs) (see Table 3), one cannot distinguish between local opioid and GABA-B receptor circuits mediating this interaction. However, given that VTA NTX pretreatment interferes with the full expression of baclofen-induced feeding elicited from the NACs but within site NACs NTX does not (see Table 3), this suggests that the synaptic circuitry underlying opioid effects upon GABA-B receptor function differs as a function of the sites into which the opioid antagonist is administered. Thus, this regional general opioid antagonist-GABA-B agonist feeding interaction between the VTA and NACs is consistent with the first hypothesis of a conceptualized distributed brain network mediating ingestive effects employing multiple sites and neuromodulator candidates (e.g., Pecina and Berridge, 2000, 2005; Smith and Berridge, 2007; Stratford, 2005; Stratford and Kelley, 1999; Will et al., 2003). The use of NTX as a general opioid antagonist and DAMGO as a mu-opioid agonist has identified previous regional feeding interactions between the NTS and CeA (Giraudo et al., 1998b), the PVN and VTA (Quinn et al., 2003), the NACs and CeA (Kim et al.,

2004) and the NACs and VTA (MacDonald et al., 2003). Further, feeding elicited by orexin-A administered into the LH was blocked by NTX pretreatment in the NAC shell (Sweet et al., 2004). All of these data are consistent with and typically explained by the existence of an integrated distributed brain network controlling feeding (hypothesis 1).

As indicated in Table 3, NTX alone in the NACs (79%) and VTA (64%) robustly reduced glucoprivic intake, and produced more modest though significant decreases in sugar intake and deprivation-induced intake when administered into these sites (Bodnar et al., 1995; Kelley et al., 1996; Ragnauth et al., 1997). Thus, this general opioid antagonist could conceivably be acting by non-specifically decreasing food intake in one of the sites, and countervailing the baclofen-induced feeding response elicited from the second site (hypothesis 2). Thus, based on general opioid antagonism alone, one cannot effectively differentiate between the two hypotheses.

3. Regional Mu-Selective Opioid Antagonist Effects upon GABA-B Agonist-induced Feeding: Selective antagonism of mu opioid receptors in the NACs failed to affect VTA baclofen-induced feeding. Moreover, VTA pretreatment with the mu opioid antagonist weakly reduced NACs baclofen-induced feeding, indicating at best a unidirectional mu opioid and GABA-B receptor feeding interaction. This weaker role for mu opioid receptors within the VTA and NACs in mediating GABA-B agonist-induced feeding from the other site is quite surprising given that robust feeding responses are observed following NACs or VTA administration of mu opioid agonists (Badiani et al., 1995; Bakshi and Kelley, 1993, 1994; Evans and Vaccarino, 1990; Majeed et al., 1986; Mucha and Iversen, 1986; Nencini and Stewart, 1990; Noel and Wise, 1993, 1995). Baclofen-induced feeding elicited from the VTA, but not from the NACs was significantly reduced by within site mu opioid antagonists (Khaimova et al., 2004) (see Table 3). Whereas VTA BFNA failed to block feeding responses to food deprivation, glucoprivation and

palatable sucrose solutions (Ragnauth et al., 1997), accumbal BFNA significantly reduced food intake following deprivation and glucoprivation, and palatable sucrose solutions (Bodnar et al., 1995; Table 3) as well as suppressing the development of dietary obesity in rats (Lenard et al., 2010). Further, selective mu opioid antagonism within the NACs significantly reduced feeding responses induced by accumbal mu and delta-1 opioid agonists (Ragnauth et al., 2000), and mu opioid antagonism blocked feeding elicited by mu opioid agonist administration in the VTA (Lamonte et al., 2002). Moreover, mu opioid antagonist pretreatment in the VTA reduced accumbal mu-opioid agonist-induced feeding, and VTA mu-opioid agonist-induced feeding was reduced by accumbal mu opioid antagonism (Bodnar et al., 2005). The relatively weak regional feeding interactions between mu-opioid antagonists and GABA-B receptor agonists appears consistent with the inability of GABA-A or GABA-B antagonist pretreatment to block mu-opioid agonist-induced feeding in interaction studies between the VTA and NACs (Ackerman et al., 2003). Thus, this relative lack of a mu-opioid antagonist-GABA-B agonist feeding interaction does not appear to support hypothesis 2.

4. Regional Delta-Selective Opioid Antagonist Effects upon GABA-B Agonist-induced Feeding: Whereas selective antagonism of delta opioid receptors in the NACs failed to affect VTA baclofen-induced feeding. VTA pretreatment with a delta opioid antagonist weakly reduced NACs baclofen-induced feeding, indicating at best a unidirectional delta opioid and GABA-B receptor feeding interaction. As with mu opioid antagonism, this weaker role for delta opioid receptors within the VTA and NACs (e.g., Mansour et al., 1987, 1995*;* Tempel & Zukin, 1987) in mediating GABA-B agonist-induced feeding from the other site is quite surprising given that robust feeding responses are observed following NACs or VTA administration of delta-opioid agonists (Cador et al., 1986; Majeed et al., 1986; Mucha and Iversen, 1986; Noel and Wise,

1993, 1995). Baclofen-induced feeding elicited from the NACs, but not the VTA was significantly reduced by within-site delta-opioid antagonism (Khaimova et al., 2004). VTA NTI also blocked feeding responses to food deprivation, glucoprivation and palatable sucrose solutions (Ragnauth et al., 1997). Selective delta opioid antagonism within the NACs, but not the VTA significantly reduced feeding responses induced by within-site mu and delta-1 opioid agonists (Lamonte et al., 2002; Ragnauth et al., 2000). VTA mu-opioid agonist-induced feeding was reduced by accumbal delta opioid antagonism, whereas VTA NTI failed to affect feeding elicited by NACs DAMGO (Bodnar et al., 2005).

The mild and unidirectional delta-opioid/antagonist/GABA-B agonist feeding interaction demonstrated by VTA NTI pretreatment weakly reduced NACs baclofen-induced feeding appears to be best explained by the existence of a distributed brain network (hypothesis 1), indicating at best a unidirectional delta opioid and GABA-B receptor feeding interaction. If hypothesis 2 was correct, one would logically expect that NTI in one site should produce similar countervailing effects upon baclofen-induced feeding elicited from the second site.

5. Regional Kappa-Selective Opioid Antagonist Effects upon GABA-B Agonist-induced Feeding: Insight regarding the relative strengths of the two potential hypotheses, activation of a distributed brain network (hypothesis 1) or countervailing antagonist-induced reductions and agonist-induced increases upon intake (hypothesis 2), appear to be answered in regional kappa-opioid antagonist effects upon GABA-B agonist-induced feeding from the alternate site. Blockade of kappa opioid receptors with both the low (0.6 ug) and high (6 ug) doses NBNI in the VTA effectively interfered with the ability of baclofen to elicit feeding when administered into the NACs, and NACs NBNI pretreatment with the higher 6 ug dose effectively interfered with VTA baclofen-induced feeding. Of the three opioid receptor subtypes, it appeared surprising that

kappa opioid receptor involvement appeared more pronounced than mu or delta opioid receptor involvement. Interestingly, this putative role of kappa opioid receptors in the NACs and VTA to bidirectionally and regionally mediate the stimulation of feeding by local GABA-B receptor agonists occurs in the relative absence of feeding responses following administration of kappa-selective agonists into either the VTA (Nencini & Stewart, 1990; Noel & Wise, 1993, but see Hamilton & Bozarth, 1988) or NACs (Bakshi & Kelley, 1993; Zhang & Kelley, 1997). Thus, it is therefore highly unlikely that this blockade by kappa receptor antagonism upon the regional feeding action of GABA-B receptor activation is due to kappa opioid receptors to activate feeding circuits directly in the NACs and VTA. Rather, it would appear that kappa receptors are playing a modulatory and/or permissive role both within and between the VTA and NACs to allow GABA-B receptor activation within these sites to induce feeding.

Importantly, the reductions in baclofen-induced feeding in one site by pretreatment with NBNI in the other site could not be explained by comparable reductions in spontaneous intake induced by the kappa opioid antagonist, since NBNI alone failed to significantly alter spontaneous food intake following administration into the NACs (10%) and VTA (12%). Thus, reduced support for hypothesis 2 is shown. Kappa-selective opioid antagonists have also displayed mixed effects in modulating other forms of feeding responses in the NACs and VTA. Thus, baclofen-induced feeding elicited from the VTA was significantly reduced by kappa opioid antagonists (Khaimova et al., 2004), but NBNI in the VTA failed to block feeding responses to food deprivation, glucoprivation or palatable sucrose solutions (Ragnauth et al., 1997) (see Table 3).

Yet accumbal NBNI significantly reduced food intake elicited by accumbal baclofen (Khaimova et al., 2004) as well as following food deprivation and glucoprivation, but failed to affect palatable sucrose intake (Bodnar et al., 1995; Kelley et al., 1996) (see Table 3). Selective kappa

antagonism within the NACs significantly reduced feeding responses induced by accumbal mu and delta-1 opioid agonists (Ragnauth et al., 2000), and NBNI pretreatment blocked feeding elicited by mu opioid agonist administration in the VTA (Lamonte et al., 2002) (see Table 3). Moreover, VTA NBNI pretreatment dose-dependently reduced accumbal mu-opioid agonist-induced feeding, whereas VTA mu-opioid agonist-induced feeding was reduced to a lesser degree by accumbal NBNI (Bodnar et al., 2005). These data would suggest that intrinsic kappa receptors within the VTA and NACs (e.g., Mansour et al., 1987, 1995; Tempel & Zukin, 1987) not only mediate opioid-opioid feeding interactions, but opioid-GABA feeding interactions as well potentially through a distributed brain system (hypothesis 1).

6. A Potential Empirically-Based Anatomical and Neurochemical Model Mediating GABA-GABA and Opioid-GABA Feeding Interactions Within the VTA and NACs

Anatomical studies demonstrate that both GABA and enkephalin are found together in spiny cells in the NACs (Meredith, 1999), and form reciprocal projections between both the NACs and the ventral pallidum (VP) as well as between the NACs and the VTA (Churchill et al., 1991; Churchill and Kalivas, 1994; Kalivas et al., 1993; Meredith et al., 1993; Van Bockstaele and Pickel, 1995; Zahm et al., 1985) with mu-opioid receptor-like immunoreactivity found on GABA-containing neurons in the NACs (Svingos et al., 1997). Anatomical colocalization of mu-opioid receptors and GABA receptors has also been observed in the NACs, ventral pallidum and VTA region with functional consequences (e.g., Churchill et al., 1991, 1992; Klitenick et al., 1992). Within the NAC, a class of GABAergic cells co-express the opioid peptide, dynorphin, and produce presynaptic and postsynaptic inhibition upon incoming dopamine terminals and targets (see reviews: Nestler, 2001; Shippenberg & Rae, 1997; Spangler et al., 1996), and other instances of reciprocal opioid-GABA inhibition has been noted (Chieng & Williams, 1998;

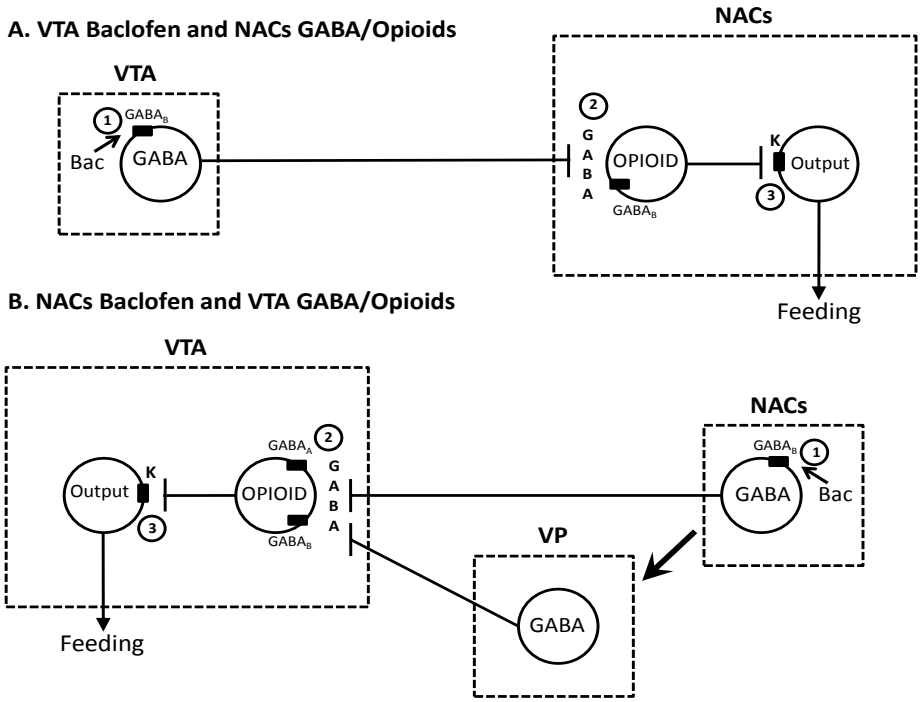
Napier & Mitrovic, 1999). Correspondingly, GABA cell bodies within the VTA project to the NACs and pre-frontal cortex (Carr and Sesack, 2000b; Van Bockstaele and Pickel, 1995), and both mu and kappa agonists decrease the inhibitory effects of VTA GABA on ventral pallidal neurons (Mitrovic and Napier, 2002).

Figure 11 provides a potential model of opioid-GABA feeding interactions based upon empirical findings gathered from our laboratory, and paired with the cited neuroanatomical evidence. In these models, it is assumed that baclofen and DAMGO in respectively binding with their receptors on cells in the VTA and NACs are exerting typical inhibitory effects. Therefore, feeding elicited by baclofen and DAMGO will result as functional disinhibitory actions. For the GABA-B agonist feeding effects, baclofen acts at GABA-B, but not GABA-A receptors in the VTA (Figure 11A: Echo et al., 2002) and NACs (Figure 11B: Znamensky et al., 2000) to induce feeding. In this model, these activated GABA-B receptors will inhibit GABA-ergic cells in the VTA that project directly to the NACs (Figure 11A: Carr and Sesack, 2000b) and will inhibit GABA-ergic cells in the NACs that project either directly to the VTA or indirectly to the VTA through VP GABA-ergic interneurons (Figure 11B: Churchill et al., 1991; Churchill and Kalivas, 1994; Kalivas et al., 1993; Meredith et al., 1993; Van Bockstaele and Pickel, 1995; Zahm et al., 1985). In the case of VTA baclofen-induced feeding (Figure 11A), the cells in the NACs that receive this GABA-ergic input act through GABA-B receptors; this is based on the ability of GABA-B, but not GABA-A receptor antagonist pretreatment in the NACs to significantly reduce baclofen-induced feeding elicited from the VTA (Specific Aim 1). In the case of NACs baclofen-induced feeding (Figure 11B), the cells in the VTA that receive NACs or VP GABA-ergic input act through both GABA-A and GABA-B receptors; this is based on the ability of both GABA-A and GABA-B receptor antagonist pretreatment to significantly reduce baclofen-induced feeding

elicited from the NACs (Specific Aim 1). In this parsimonious data-based model, the relevant NACs GABA-B receptors inhibit opioidergic (presumably dynorphin: Nestler, 2001; Shippenberg and Rae, 1997; Spangler et al., 1996)-containing neurons in the NACs (Figure 11A). In turn, these NACs opioidergic neurons activate a kappa opioid receptor thereby inhibiting NACs output neurons which control a feeding response (Figure 11A). This is based upon the present data indicating that kappa opioid antagonist pretreatment in the NACs significantly reduces feeding elicited by baclofen in the VTA. Correspondingly, the relevant VTA GABA-A and GABA-B receptors inhibit opioidergic-containing neurons in the VTA (Figure 11B). In turn, these VTA opioidergic neurons activate a kappa opioid receptor thereby inhibiting VTA output neurons which control a feeding response (Figure 11B). This is also based upon the present data indicating that kappa opioid antagonist pretreatment in the VTA significantly reduces feeding elicited by baclofen in the NACs. Local opioid actions within the VTA (e.g., Lamonte et al., 2002) and NACs (e.g., Ragnauth et al., 2000) upon food intake are not included in the model for simplicity's sake, but include potential disinhibitiory actions within these output neurons (Figures 11A and 11B).

Figure 11. (A and B). An empirically-based parsimonious model of opioid and GABA antagonist modulation of feeding elicited by the GABA-B agonist, baclofen administered into the VTA (Panel A) or into the NACs (Panel B). The arrows in the figures represent either an excititory action of the agonist (e.g., baclofen) on its receptor, or a functional output behavior (e.g., feeding) or connection. The symbol (T rotated 90^{o} to the right) represents an inhibitory action of one neuron upon another. *Panel A*: (**1)** Baclofen administered into the VTA acts on GABA-B receptors to elicit feeding (Echo et al., 2002), and in this model acts on GABA-containing neurons projecting from the VTA to the NACs (Van Bockstaele and Pickel, 1995). (**2**) VTA baclofen-induced feeding is mediated by GABA released from these neurons into the NACs by acting on GABA-B receptors (present results) on an opioid-containing cell (e.g., dynorphin and GABA (Nestler, 2001; Shippenberg & Rae, 1997; Spangler et al., 1996). (**3**) VTA baclofen-induced feeding is then mediated by a kappa-opioid receptor located in the NACs (present results) that acts to inhibit an output system to elicit feeding. Local opioid actions within the NACs upon food intake (e.g., Ragnauth et al., 2000) are not included in the model for simplicity's sake, but include potential disinhibitory actions. *Panel B*: (**1**) Baclofen administered into the NACs acts on GABA-B receptors to elicit feeding (Znamensky et al., 2001), and in this model acts on GABA-containing neurons projecting from the NACs either directly to the VTA or indirectly to the VTA through the ventral pallidum (VP) (Churchill et al., 1991; Churchill & Kalivas, 1994; Kalivas et al., 1993; Meredith et al., 1993; Van Bockstaele & Pickel, 1995; Zahm et al., 1985). (**2**) NACs baclofen-induced feeding is mediated by GABA released from these neurons into the VTA by acting on GABA-A and -B receptors (present study) presumably on an opioid-containing cell. (**3**) NACs baclofen-induced feeding is then mediated by a kappa-opioid receptor located in the VTA (present results) that acts to inhibit an output system to elicit

feeding. Local opioid actions within the VTA upon food intake (e.g., Lamonte et al., 2002) are not included in the model for simplicity's sake, but include potential disinhibitory actions.



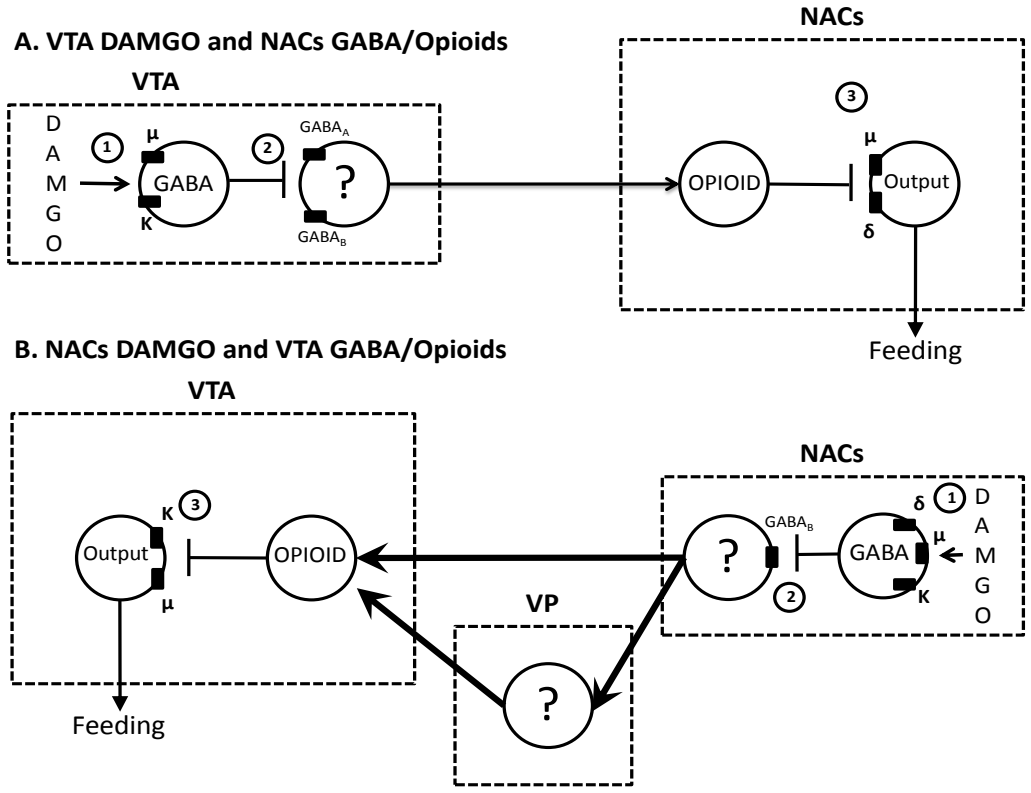
For mu-opioid feeding effects, DAMGO acts at mu and kappa, but not delta opioid receptors in the VTA (Figure 12A: Lamonte et al., 2002) and at mu, delta and kappa opioid receptors in the NACs (Figure 12B: Ragnauth et al., 2000) to induce feeding. In this model, these activated opioid receptors will inhibit local GABA-ergic interneurons in the VTA and NACs. They will not act on long GABAergic projection neurons from the VTA to the NACs because GABA-A and GABA-B receptor antagonist pretreatment in the NACs failed to affect feeding elicited by DAMGO in the VTA (Ackerman et al., 2003). Correspondingly, they will not act on long GABAergic projection neurons from the NACs to the VTA because GABA-A and GABA-B receptor antagonist pretreatment in the VTA failed to affect feeding elicited by DAMGO in the NACs (Ackerman et al., 2003). Local GABAergic neurons in the VTA will act at GABA-A and GABA-B receptors on an unidentified (Figure 12A) projection neuron to the NACs; this is based on the ability of VTA GABA-A and GABA-B receptor antagonist pretreatment to significantly reduce VTA DAMGO-induced feeding (Echo et al., 2002). Correspondingly, local GABAergic neurons in the NACs will act at GABA-B receptors on an unidentified (Figure 12B) projection neuron to the VTA (and VP); this is based on the ability of NACs GABA-B receptor antagonist pretreatment to significantly reduce NACs DAMGO-induced feeding (Znamensky et al., 2000). Finally, the respective projections from VTA to NACs and from NACs/VP to VTA will synapse onto an opioid neuron in each structure (Figures 12A and 12B). The NACs opioid neuron will act through mu and delta opioid receptors thereby inhibiting NACs output neurons which control a feeding response (Figure 12A); this is based upon the ability of mu and delta opioid antagonist pretreatment in the NACs to significantly reduce feeding elicited by DAMGO in the VTA (Bodnar et al., 2005). Correspondingly, the VTA opioid neuron will act through mu and kappa opioid receptors thereby inhibiting VTA output neurons which control a feeding response

(Figure 12B); this is based upon the ability of mu and kappa opioid antagonist pretreatment in the VTA to significantly reduce feeding elicited by DAMGO in the NACs (Bodnar et al., 2005). Local opioid actions within the VTA (e.g., Lamonte et al., 2002) and NACs (e.g., Ragnauth et al., 2000) upon food intake are not included in the model for simplicity's sake, but include potential disinhibitory actions within these output neurons (Figures 12A and 12B).

Figure 12. (A and B) An empirically-based parsimonious model of opioid and GABA antagonist modulation of feeding elicited by the GABA-B agonist, opioid and GABA antagonist modulation of feeding elicited by the mu-opioid agonist, DAMGO administered into the VTA (Panel A) or into the NACs (Panel B). *Panel A*: **(1)** DAMGO administered into the VTA acts on mu and kappa opioid receptors to elicit feeding (Lamonte et al., 2002) that are located in this model on a VTA GABA interneuron. (**2**) VTA DAMGO –induced feeding is reduced by GABA-A and GABA-B antagonist pretreatment (Echo et al., 2002). These GABA receptors are located on a VTA output neuron that does not contain GABA because GABA-A or GABA-B receptor antagonists in the NACs fail to block VTA DAMGO-induced feeding (Ackerman et al., 2003). This VTA output neuron would then functionally activate a NACs opioid-containing neuron (Meredith, 1999). (**3**) VTA DAMGO-induced feeding is then mediated by NACs mu- and delta-opioid receptors (Bodnar et al., 2005) that act to inhibit an output system to elicit feeding. Local opioid actions within the NACs upon food intake (e.g., Ragnauth et al., 2000) are not included in the model for simplicity's sake, but include potential disinhibitory actions.

Panel B: (**1**) DAMGO administered into the NACs acts on mu, delta and kappa opioid receptors to elicit feeding (Ragnauth et al., 2000) that are located in this model on a NACs GABA interneuron. (**2**). NACs DAMGO-induced feeding is blocked by NACs GABA-B antagonist pretreatment (Znamensky et al., 2001). This GABA receptor is located on a NACs output neuron that does not contain GABA because GABA-A or GABA-B receptor antagonists in the VTA fail to block NACs DAMGO-induced feeding (Ackerman et al., 2003). This NACs output neuron in this model projects from the NACs either directly to the VTA or indirectly to the VTA through the VP (Churchill et al., 1991; Churchill and Kalivas, 1994; Kalivas et al., 1993; Meredith et al., 1993; Van Bockstaele and Pickel, 1995; Zahm et al., 1985). (**3**) NACs DAMGO-induced feeding

is then mediated by VTA mu- and kappa-opioid receptors (Bodnar et al., 2005) that act to inhibit an output system to elicit feeding. Local opioid actions within the VTA upon food intake (e.g., Lamonte et al., 2002) are not included in the model for simplicity's sake, but include potential disinhibitory actions.



In conclusion, these results support a distributed brain network between the VTA and NACs for feeding elicited by GABA-B receptor agonists with GABA and opioid receptor subtypes performing a modulatory role in rats. The theory of a distributed brain network is corroborated by the differential results of *alternate site* antagonist reductions of baclofen-induced intake compared to *within site* reductions of baclofen induced feeding. If a synergistic effect of baclofen induced feeding in one site was simply off-set by antagonist reduction in feeding in the other site similar significant reductions in baclofen-induced feeding should have been seen between within site and alternate site antagonist injections. The theory of a distributed brain network is also supported by the findings that a) mu-selective opioid antagonists administered into the VTA or NACs significantly reduce spontaneous intake to the same degree as NTX, yet fail to appreciably affect feeding responses elicited by baclofen administered into the other site, b) the absence of kappa-selective opioid antagonist effects in the VTA or NACs upon spontaneous feeding paired with the bidirectional ability of kappa-selective opioid antagonist pretreatment significantly reducing food intake elicited by baclofen administered into the alternate site.

Future Directions

GABA and opioid signaling within and between the VTA and NACs are clearly implicated in the determining ingestion of meal size implicating their role in a distributed brain network in the control of food intake. Further research should investigate the interrelationship between GABA and opioid receptors in other neuroanatomical loci; using the same protocols, investigate brain areas such as, the prefrontal cortex with the VTA or NACs, and ventral pallidum with the VTA or NACs. Due to the lack of research presently available on the functional roles of $GABA_B$ phasic and tonic inhibition on the control of food intake it is another intriguing area of research

to pursue. Another series of studies could investigate the affect of physical activity on meal size starting with its effect on baclofen and opioid-induced intake. Such studies would not only expand our knowledge about the nature of the distributed brain network mediating meal size, but expand the list of neuroanatomical and environmental participants affecting the control of food intake.

Implications

As discussed in the introduction of this dissertation obesity is currently a world-wide health problem with a significant increase in incidence in our children. Since obesity is associated with co-morbidities which include heart disease, diabetes and cancer this puts our population and healthcare system at risk of future crisis. Multiple factors have been implicated in the causation of obesity, one of which is availability of larger portion sizes contributing to larger meal size and consequently obesity. The protocols tested in this dissertation contribute to understanding central neurotransmitter systems and brain loci involved in determining meal size. Understanding the neurotransmitters involved in the natural reward system, which encompasses the VTA and NAC, will elucidate our understanding of potential preventions and treatments of obesity.

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