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Regulation of Ventral Tegmental Area Dopamine Neuron Activity by Feeding-related Hypothalamic Neuropeptides

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REGULATION OF VENTRAL TEGMENTAL AREA DOPAMINE NEURON ACTIVITY BY FEEDING-RELATED HYPOTHALAMIC NEUROPEPTIDES

by

KATHERINE M. S. WEST

Under the Direction of Aaron Roseberry, Ph.D.

ABSTRACT

The prevalence of obesity has doubled worldwide since the 1980s, and having a high body mass index contributes to more deaths worldwide than being underweight. Over the past 20 years, consumption of calorie-dense foods has increased, and this is considered one of the major causes of the rapid rise in obesity. Thus, understanding the neural control of food intake is important for the development of new and effective treatments of obesity. Two important brain regions that regulate food intake are the hypothalamus and the mesocorticolimbic dopamine system. The hypothalamus is essential for the homeostatic control of feeding and body weight, while the mesocorticolimbic dopamine system, also known as the reward system, is the primary circuit for reward and motivated behavior. The reward system also regulates food intake and

food reward, and there is increasing evidence that hypothalamic feeding-related neuropeptides alter dopamine neuron activity to affect feeding. Nevertheless, how these neuropeptides interact with the reward system to regulate feeding is not fully understood. For example, centrally delivered neurotensin and neuropeptide-Y (NPY) increase dopamine release in the nucleus accumbens, but cause opposite effects on food reward. In addition, injection of the hypothalamic neuropeptides neurotensin, NPY, or alpha-melanocyte-stimulating hormone (α-MSH) into the ventral tegmental area (VTA), where reward-related dopamine neurons are located, alters multiple aspects of feeding, but how these neuropeptides interact with the reward system to alter feeding at both the circuit and cellular levels is not fully understood. In these studies, I have used whole cell patch-clamp electrophysiology in acute brain slices from mice to examine how neurotensin, α-MSH, and NPY affect VTA dopamine neuron activity. I have demonstrated that these neuropeptides use multiple mechanisms to alter VTA dopamine neuron activity, including both pre- and post-synaptic mechanisms. Neurotensin and α-MSH increased dopamine neuron activity, while NPY had both excitatory and inhibitory effects on dopamine neuron activity. Overall, these studies provide an important advancement in our understanding of the different mechanisms utilized by hypothalamic neuropeptides to alter VTA dopamine neuron activity and how hypothalamic neuropeptides interact with the mesocorticolimbic dopamine system to control food intake and food reward.

INDEX WORDS: Dopamine, Ventral tegmental area, Neurotensin, Alpha-melanocyte stimulating hormone, Neuropeptide-Y, MC3R, GIRK

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DEDICATION

I dedicate this dissertation to my family: to my parents Jack and Donna Stuhrman, my sister and brother-in-law Grace and Stephen Noh, and my husband Alex West. Thank you for the tremendous love and support you gave me throughout the time I spent working on this dissertation.

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LIST OF ABBREVIATIONS

Alpha-melanocyte-stimulating hormone, α-MSH

Acetylcholine, Ach

Agouti-related protein, AgRP

AMPA receptor, AMPAR

Artificial cerebral spinal fluid, aCSF

Body mass index, BMI

Calcium, Ca^{2+}

Central nervous system, CNS

Coefficient of variation, CV

Cycopiazonic acid, CPA

Dopamine, DA

Dopamine D1 receptor, D1R

Dopamine D2 receptor, D2R

Enhanced yellow fluorescent protein, EYFP

Excitatory post-synaptic current, EPSC

$GABA_A$ receptor, $GABA_AR$

$GABA_B$ receptor, $GABA_BR$

G-coupled inward rectifying potassium

Hyperpolarization-activated cation current, H-current

Inhibitory post-synaptic current, IPSC

Intra-cerebroventricular, icv

Intracranial self-stimulation, ICSS

Lateral habenula, LHb

Lateral hypothalamus, LH

Long-term depression, LTD

Long-term potentiation, LTP

Melanocortin-3 receptor, MC3R

Melanocortin-4 receptor, MC4R

Metabotropic glutamate receptor, mGluR

NMDA receptor, NMDAR

Neuropeptide-y, NPY

Neuropeptide-y Y1 receptor, Y1R

Neurotensin, NT

Neurotensin receptor 1, NTS1 receptor

Nucleus accumbens, NAc

Paired-pulse ratio, PPR

Prefrontal cortex, PFC

Potassium gluconate, KGluconate

Potassium methyl sulfate, $KMeSO_4$

pro-opiomelanocortin, POMC

Small-conductance Ca^{2+}-dependent K^+ channels, sK channels

Substantia nigra *pars compacta,* SNc

Tetrodotoxin, TTX

Transient receptor potential C channel, TrpC channel

Ventral tegmental area, VTA

1 INTRODUCTION AND LITERATURE REVIEW

Obesity is a worldwide epidemic and global health crisis that is on the rise. Obesity rates have more than doubled worldwide since 1980[1,2]. Obesity and a high body mass index (BMI) are detrimental to one's health. A high BMI (overweight: BMI ≥25, obese: BMI ≥30) is a major risk factor for several diseases such as musculoskeletal disorders, diabetes mellitus, chronic kidney disease, stroke, cancer, and cardiovascular disease[2]. Remarkably, being overweight or obese contributes to more deaths than being underweight worldwide[1], and a high BMI contributed to 4 million deaths and a 120 million disability-adjusted life-years among adults around the world in 2015[2]. Obesity is not only a substantial health burden but also an economic burden. An estimated $147 billion was spent on obesity related medical costs in the United States in 2008[3]. Thus, the global rise in BMI is a substantial problem.

The rise in body weight over the last couple of decades is largely due to an increase in food consumption[4-7]. The availability of food has increased over the past several decades, and calorie-dense foods such as chocolate, cakes, and chips are cheap and abundant[4-7]. This has lead to an environment that promotes overeating and weight gain[4-7]. Understanding the neural mechanisms that drive the over consumption of food is significantly important for the development of methods, policies, and treatments that can be used to prevent and reverse weight gain and obesity.

Two biological systems that regulate food intake are the homeostatic energy system and the reward system. The homeostatic system comprises hypothalamic and brainstem circuits, numerous neuropeptides, and hormonal gastrointestinal and fat signals that work together to control calorie intake and expenditure to maintain energy balance[8]. Thus, proper function of the homeostatic energy system should prevent people from gaining weight, and yet in the U.S.,

there are more overweight and obese people than people with a healthy BMI[9-11]. In contrast to the homeostatic system, the reward system or mesocorticolimbic dopamine system, regulates feeding and body weight by reinforcing behaviors that result in food intake. This especially holds true for intake of food that is high in fat and sugar, as these foods are highly rewarding and reinforcing. It has been hypothesized that the reward system overrides the homeostatic system in an environment rich in rewarding calorie-dense foods, leading to a net positive of calories consumed over calories burned[12]. Put simply, people are highly motivated to eat calorie-dense, sugary/fatty foods even as the homeostatic brain system signals for one to stop eating. Thus, it is important to understand how the brain's reward system regulates food intake, weight gain, and food reward-related behaviors and how the reward and homeostatic systems interact to regulate these behaviors. The homeostatic control of energy balance encompasses numerous circuits, hormones, and neuropeptides, and although the homeostatic energy system is often considered independent of the reward system, the homeostatic energy system does interact with the reward system to control food intake and body weight. Yet how these two systems interact is not completely understood. Here I describe our studies testing how three distinct hypothalamic feeding neuropeptides alter the activity of mesocorticolimbic dopamine neurons to regulate food intake, body weight, and reward-related behaviors.

1.1 Overview of the mesocorticolimbic dopamine system

Dopamine is a key neurotransmitter that regulates many different behaviors and physiological processes including motor learning, incentive motivation, reward and reinforcement, learning, aversion, and food intake and body weight[13-18]. Furthermore, disruptions in dopamine circuits are implicated in psychiatric disorders such as Parkinson's

disease, schizophrenia, depression, and drug addiction, as well as obesity. The two most studied populations of dopamine neurons that are involved in these disorders are the dopamine neurons of the substantia nigra *pars compacta* (SNc) and of the ventral tegmental area (VTA). These two populations of neurons are located in the midbrain, and the SNc laterally borders the VTA with some overlap between the two areas. SNc dopamine neurons are part of the mesostriatal dopamine system, and predominantly regulate movement and motor coordination and project to dorsal striatum. VTA dopamine neurons are part of the mesocorticolimbic dopamine system and predominantly regulate reward and reinforcement behavior and project to ventral striatum. In addition, as mentioned above, the mesocorticolimbic dopamine system also regulates food intake, food reward, and body weight, so the studies described here examine how hypothalamic feeding-related neuropeptides specifically affect VTA dopamine neurons.

The mesocorticolimbic dopamine system comprises not only dopamine neurons in the VTA but also the afferents inputs to the VTA and the projection targets of the dopamine neurons. VTA dopamine neurons receive afferent inputs from numerous brain areas including but not limited to the lateral hypothalamus (LH), ventral pallidum, amygdala, nucleus accumbens (NAc), cortex, and dorsal raphe nucleus[19,20]. In addition, dopamine neurons are innervated by local VTA inhibitory GABA neurons and excitatory glutamate neurons[21-23]. VTA dopamine neurons send efferent projections to many brain regions such as the NAc, olfactory tubercle, hippocampus, amygdala, bed nucleus of the stria terminalis, and prefrontal cortex (PFC). VTA GABA and glutamate neurons send long-range projections to both distinct and parallel dopamine neuron efferent sites[24-27]. The mesocorticolimbic dopamine system is truly a complex circuit with broad connections.

The VTA is made up primarily of dopamine neurons (~55-65%), which are defined as neurons that express tyrosine hydroxylase, the enzyme for catecholamine production[28,29]. The rest of the VTA is comprised of GABA and glutamate neurons. Approximately 20-35% are identified as GABA neurons and 2-3% as glutamate neurons[28,30,31]. Interestingly, a small portion of VTA neurons co-release both glutamate and GABA[32-34]. Dopamine neurons were once thought to be a uniform population, but recent studies show that the VTA contains a heterogeneous dopamine neuron population. Distinct subpopulations of dopamine neurons have been identified by their power to control distinct behaviors and by a variety of distinct characteristics such as projection site, electrophysiological properties, pharmacological properties, and molecular markers[16,17,35,36]. In addition, a subpopulation of VTA dopamine neurons co-releases GABA[37,38], and another population co-releases glutamate[39-41]. Thus, VTA dopamine neurons are not one distinct neuronal group but a mixture of multiple neuronal groups that express dopamine.

1.1.1 The mesocorticolimbic dopamine system and reward behavior

The role of dopamine in reward and reinforcement behavior is well established. Our first knowledge of the brain circuitry regulating reward behavior came from Olds and Milner's famous 1954 study demonstrating that rats will learn to lever press for electrical stimulation of specific brain regions (intracranial self-stimulation or ICSS)[42]. Several studies in the 1970s revealed the importance of dopamine for the regulation of reward behavior as injection of dopamine receptor antagonists decreases ICSS in rats[43-48]. After these initial studies, a detailed description of how dopamine regulates reward behavior soon emerged from a number of experiments (for review see[14,15,18,49-51]).

Dopamine acts as a salient signal or learning cue that reinforces or "stamps in" behaviors and responses that lead to rewards, and these learned behaviors are repeated until that behavior no longer leads to an obtained reward[14,15,49]. Dopamine acts as a learning cue, because dopamine neuron activity and dopamine release increase in response to a reward. Dopamine neurons fire tonically, but in response to an unexpected reward such as food, water, or sex dopamine neurons fire in bursts causing an increase in dopamine release[14,15,18,49-51]. If a cue consistently predicts a reward, then the timing of dopamine neuron burst firing will shift to the cue predicting the reward[18,50,51]. Dopamine neuron firing and dopamine release also increase if a reward is greater than predicted, but there is a decrease or pause in firing and dopamine release if an expected reward is less than predicted or not obtained[18,50,51]. Thus, dopamine acts as a reward prediction error that encodes the deviation between what is predicted and what is actually obtained[18,50,51].

1.1.2 Dopamine neuron tonic and burst firing

Grace and Bunny first characterized the activity states of dopamine neurons 35 years ago and identified three different activity states of midbrain dopamine neurons: silent and hyperpolarized, slow pacemaker firing (2-10 Hz)/irregular firing, and phasic burst firing[52-58]. These first studies were primarily conducted on anesthetized and unanesthetized paralyzed rats *in vivo*, and were later confirmed in unanesthetized behaving rats[59,60]. The slow pacemaker firing or "tonic firing" of dopamine neurons causes tonic release of dopamine at efferent target sites, and burst firing causes a phasic increase in dopamine release[61-63] which is triggered by rewards and cue-associated rewards[18,50,51]. This phasic release of dopamine is what encodes the reward prediction error discussed above. Since the first studies by Grace and Bunney illuminating the firing characteristic of dopamine neuron activity, extensive research has been

conducted aimed at understanding how dopamine neuron activity is controlled and how dopamine neurons transition from a tonic to a bursting state.

Spontaneous pacemaker firing in VTA dopamine neurons is primarily dependent on two Na^+ currents: a tetrodotoxin (TTX) insensitive Na^+ leak current, and a TTX-sensitive voltage-dependent Na^+ current[64,65]. In contrast, spontaneous pacemaker firing in SNc dopamine neurons is primarily dependent on Ca^{2+} currents and a Ca^{2+}-activated K^+ current, while pacemaker firing in VTA dopamine neurons is comparatively unaffected by Ca^{2+} channel blockers[64-66]. The transition from slow pacemaker firing to burst firing is regulated by afferent inputs to dopamine neurons. In fact, dopamine neurons *in vitro* fire at a slow pacemaker rate but do not burst fire, indicating the critical importance of afferent inputs for burst firing[67,68]. Afferent inputs to dopamine neurons are critical for burst firing but do not act in isolation; rather, they interact with cellular conductances to generate bursts. For example, Ca^{2+} is a key component for the generation of bursts, as chelating intracellular Ca^{2+} with EGTA has been shown to prevent burst firing, whereas intracellular injection of Ca^{2+} increases burst firing[57]. It is hypothesized that Ca^{2+} conducted through excitatory NMDA receptors (NMDAR) and voltage-gated Ca^{2+} channels are key to generating bursts in VTA dopamine neurons[57,69].

Glutamatergic synaptic input to dopamine neurons is sufficient and necessary for burst firing. For example, locally applied glutamate to midbrain dopamine neurons increases burst firing[57], and central or intra-midbrain injected antagonists of fast ionotropic glutamate receptors abolishes burst firing[70,71]. Previous work indicates that the glutamatergic inputs driving burst firing primarily originate from the PFC, pedunculopontine nucleus, and the subthalamic nucleus, as stimulation of these brain regions causes burst firing in midbrain dopamine neurons[63,72-76]. Numerous pharmacological studies suggest that the ionotropic glutamate

receptor, NMDAR, is responsible for glutamate-induced burst firing. Indeed, intra-midbrain NMDAR agonists increase bursting, whereas NMDAR antagonists decrease spontaneous and evoked bursting *in vivo*[75,77-79]. In addition, genetic inactivation of NMDARs in midbrain dopamine neurons decreases burst firing[80]. In contrast, agonists and antagonists to the ionotropic glutamate AMPA receptor (AMPAR) do not affect spontaneous or evoked burst firing in midbrain dopamine neurons[75,78,79]. Nevertheless, AMPAR agonists do increase the firing rate of dopamine neurons[78], and an increase in firing rate is highly correlated with burst firing[57].

GABAergic afferents are also an important regulator of dopamine neuron firing. Midbrain dopamine neurons are innervated by GABAergic neurons from the striatum, globus pallidus, rostromedial tegmental nucleus, ventral pallidum, and NAc[19,81-85], as well as local GABAergic interneurons[21,23]. Numerous pharmacological studies examining the role of GABA in regulating dopamine neuron firing have shown the importance of GABAergic synapses and GABA receptors. GABA inhibits dopamine neurons through two receptors: fast ionotropic $GABA_A$ receptors ($GABA_AR$), and slow metabotropic $GABA_B$ receptors ($GABA_BR$) that activate G-coupled inward rectifying potassium (GIRK) channels. Midbrain application of $GABA_AR$ antagonists causes a shift from slow irregular/pacemaker firing to burst firing and increases the overall firing rate of dopamine neurons, while $GABA_BR$ antagonists shift the number of dopamine neurons firing in a slow irregular pattern to a pacemaker-firing pattern[86,87]. In addition, removing a $GABA_AR$ conductance after applying a constant NMDAR and $GABA_AR$ conductance *in vitro* drives dopamine neuron burst firing, demonstrating that removal of $GABA_AR$ input can drive burst firing[88]. In contrast, systemic and intra-midbrain injected $GABA_BR$ agonists decrease firing rate and burst firing[89-91], and $GABA_AR$ and $GABA_BR$

agonists block spontaneous tonic firing and NMDA-induced burst firing in dopamine neurons *in vitro*[88,92,93]. Lastly, GABAergic transmission may also contribute to pauses in dopamine neuron burst firing in behaving animals[69]. Pauses in firing occur in behaving animals when an expected reward is not obtained, and as dopamine neurons are abundantly innervated by GABAergic afferents, GABA is a likely candidate for mediating these pauses. Indeed, increasing $GABA_AR$ conductance causes a pause in tonic dopamine neuron firing[88]. However, a decrease in excitatory glutamate transmission may also contribute to dopamine neuron pauses[69], as removal of NMDAR conductance also causes a pause in tonic firing[88]. Thus, GABAergic afferents are an important regulator of dopamine neuron activity and burst firing.

In addition to fast ionotropic glutamate and GABA receptors, dopamine neurons express slow metabotropic receptors such as the $GABA_BR$ mentioned above and the metabotropic glutamate receptor (mGluR). These slow acting metabotropic receptors also play a role in the regulation of dopamine neuron firing. For example, along with the pauses in firing that occur in dopamine neurons in behaving animals, pauses also occur after spontaneous burst firing *in vivo* and evoked burst firing *in vitro*, and it is hypothesized that mGluRs mediate the pause following spontaneous *in vivo* and evoked *in vitro* bursts[66,69,94]. Indeed, stimulation of midbrain glutamatergic afferents using a current pulse train protocol that mimics burst firing causes a slow acting inhibitory post-synaptic current (IPSC) in dopamine neurons that is mediated by mGluRs[94]. This mGluR IPSC occurs because activation of mGluRs activates the PLC pathway, causing Ca^{2+} release from intracellular stores and subsequent activation of an inhibitory current mediated by small-conductance Ca^{2+}-dependent K^+ (sK) channels[94]. Thus, glutamate activates ionotropic and metabotropic receptors causing dopamine neurons to burst fire, followed by mGluR induced-hyperpolarization and a pause in firing[66,94]. In addition, activation of other

metabotropic receptors coupled to the PLC pathway can also cause sK channels to open through a rise in intracellular Ca^{2+} and thus may also mediate the pause following a burst[66].

Another important metabotropic receptor that regulates dopamine neuron activity is the dopamine D2 receptor (D2R). Dopamine neuron firing causes local midbrain release of dopamine from the soma and dendrites of dopamine neurons[95-99], and this somatodendritic release inhibits neighboring dopamine neurons through D2R mediated activation of GIRK channels[98,100-102]. Thus, tonic dopamine neuron firing and dopamine release in the midbrain decreases the activity of dopamine neurons, and burst firing causes an even greater decrease in dopamine neuron activity. Indeed, evoking dopamine release *in vitro* causes a D2R mediated IPSC and pause in tonic pacemaker firing in midbrain dopamine neurons[98], and systemic injection of a D2R agonist decreases evoked dopamine release in the NAc *in vivo*[103]. In contrast, systemic and midbrain injection of D2R antagonists increases the firing rate of midbrain dopamine neurons *in vivo*[104,105]. It has also been hypothesized that D2R activation terminates bursts of action potentials and is responsible for the pause following spontaneous *in vivo* and evoked *in vitro* bursts in midbrain dopamine neurons[98]. Indeed, evoking midbrain dopamine release causes a pause in dopamine neuron firing[98], and inhibition of D2Rs abolishes spontaneous pauses in dopamine neuron firing *in vitro*[106]. Thus, D2Rs are key regulators of dopamine neuron activity and dopamine release.

1.1.3 Synaptic plasticity in VTA dopamine neurons

The idea that dopamine neurons express experience-dependent plasticity was first supported by behavioral studies in animals repeatedly exposed to cocaine and amphetamine[107]. For example, animals repeatedly exposed to cocaine and amphetamine develop increased locomotor responses to these drugs known as behavioral sensitization, and intra-VTA injected

NMDAR antagonists prevent the development of cocaine induced locomotor sensitization in rats[107,108]. In addition, systemically injected cocaine causes increased glutamate levels in the VTA[109], and NMDAR antagonists block cocaine induced conditioned place preference in rats[110]. Taken together, these early studies (and numerous others not mentioned here) suggested that plasticity at excitatory synapses on VTA dopamine neurons is important for the development of behavioral sensitization to drugs.

The first study to show that VTA dopamine neurons express plasticity was by Bonci and Malenka in 1999[111]. They showed that NMDAR dependent long-term potentiation (LTP) can be induced at excitatory glutamate synapses on VTA dopamine neurons using a stimulus pairing protocol used to induce LTP in CA1 hippocampal neurons[111]. Shortly thereafter, Ungless et al. (2001) demonstrated that excitatory synapses at VTA dopamine neurons are strengthened in rodents after a single exposure to cocaine[112]. Rodents exposed to cocaine exhibit a greater ratio of AMPAR to NMDAR excitatory post-synaptic currents (EPSCs) and larger AMPAR currents in VTA dopamine neurons compared to saline treated controls[112]. This cocaine-induced plasticity is due to insertion of AMPARs lacking the GLuA2 subunit at the post-synaptic membrane[113]. Midbrain dopamine neurons also exhibit LTP of NMDAR-mediated currents, independent of AMPARs currents[114-117]. Corticotropin-releasing factor potentiates NMDAR currents[117], and applying a train of afferent stimulation that is paired with evoked post-synaptic burst firing also potentiates NMDAR currents in dopamine neurons[114]. This form of electrically evoked NMDAR LTP is dependent on enhancement of burst-induced Ca^{2+} signals[114]. Ca^{2+} signals are enhanced by activation of PLC coupled metabotropic receptors (such as mGluRs) and the subsequent release of Ca^{2+} from intracellular stores[114]. Interestingly, VTA dopamine neurons are less sensitive to NMDAR LTP than SNc dopamine neurons, but NMDAR LTP in

VTA dopamine neurons is sensitized after exposure to amphetamine or social isolation through a PKA dependent mechanism[115,116]. Thus, glutamatergic synapses on VTA dopamine neurons exhibit different forms of LTP.

Glutamatergic synapses on VTA dopamine neurons also exhibit long-term depression (LTD), and there are two known mechanisms of this glutamatergic LTD. Stimulating afferents in the VTA at 1 Hz repeatedly with small depolarization triggers Ca^{2+}-dependent LTD at excitatory synapses on dopamine neurons[118,119]. However, this form of LTD is not dependent on NMDARs or mGluRs, suggesting that Ca^{2+} most likely enters the cell through voltage-gated Ca^{2+} channels[118,119]. Applying brief bursts of afferent stimulation can also evoke LTD, but this form of LTD is dependent on activation of mGluRs[120]. mGluR dependent LTD occurs through an exchange of GluA2-lacking calcium permeable AMPARs for GluA2-containing calcium impermeable AMPARs[120]. Thus, LTD occurs at glutamatergic synapses on VTA dopamine neurons through different mechanisms.

GABAergic synapses on VTA dopamine neurons also exhibit plasticity. For example, guinea pigs chronically treated with morphine exhibit potentiation of GABAergic synaptic transmission at VTA dopamine neurons during morphine withdrawal[121], and a single exposure to ethanol has also been shown to potentiate GABAergic synaptic transmission[122]. In addition, repeated cocaine exposure depresses GABAergic synaptic transmission at VTA dopamine neurons[123]. GABAergic synaptic plasticity at VTA dopamine neurons can also be induced *in vitro.* For example, GABAergic transmission is depressed after brain slices containing the VTA are exposed to opioids[23,121], and high frequency stimulation of VTA afferents also induces LTP of GABAergic synapses through a pre-synaptic increase in GABA release[124]. Interestingly, although this form of LTP occurs through a pre-synaptic mechanism, it is dependent on post-

synaptic Ca^{2+}, NMDARs, and generation of nitric oxide, a retrograde signal[124]. This form of GABAergic LTP is blocked after *in vivo* exposure to morphine[124], and after exposure to cocaine, nicotine, or a stressful stimulus in rats[125]. Thus, GABAergic synapses on VTA dopamine neurons can undergo both LTP and LTD through different mechanisms.

D2R and $GABA_B$ IPSCs also exhibit plasticity in midbrain dopamine neurons. As mentioned above, D2R and $GABA_BR$ activation causes an IPSC by opening GIRK channels in VTA dopamine neurons. Low frequency electrical stimulation in the VTA/SNc causes LTD of stimulus evoked D2R IPSCs *in vitro*[126]. Interestingly, low frequency stimulation also causes a depression of evoked $GABA_BR$ IPSCs, but only a short-term depression as the IPSC eventually recovers[126]. LTD of D2R IPSCs is dependent on intracellular Ca^{2+} and occurs through desensitization of D2Rs[126]. The IPSCs generated by GABA acting on $GABA_BRs$ and dopamine acting on D2Rs can also be potentiated, and unlike LTD of the D2R IPSC, this form of plasticity does not affect the D2R but rather the GIRK channels activated by D2Rs and $GABA_BRs$[127]. Plasticity of GIRK channels is dependent on the firing state of VTA dopamine neurons[127]. High-frequency stimulation that mimics burst firing or depolarization in VTA dopamine neurons potentiates $GABA_BR$ and D2R currents, whereas stimulating dopamine neurons with a frequency that mimics tonic firing decreases $GABA_BR$ currents[127]. Taken together, it is apparent that dopamine neurons express plasticity at many different synapses, and many different synaptic currents exhibit LTP and LTD through both pre- and postsynaptic mechanisms.

1.2 The mesocorticolimbic dopamine system and feeding

There are many factors that control when we eat, how much we eat, and what we eat. For example, internal metabolic signals from the gut signal one's energy state so that food intake and metabolism are adjusted based on one's energy demands. Nevertheless, many other factors besides energy state trigger food intake and control the types of food eaten; stress, environmental cues, social factors, and food availability can all play a role. Life experience will tell one that this is true, but this has also been demonstrated experimentally. Satiated rats feed in response to a cue previously associated with food, demonstrating that the energy state of the animal is not the only factor influencing the initiation of feeding[128]. Satiated rats will also endure noxious cold or foot shock to eat highly palatable food such as cake, meat pâté, soda, peanut butter, or candy, even when standard chow is freely available in a neutral environment[129,130]. Thus, animals are highly motivated to obtain palatable food, and dopamine is essential for reward and reinforcement behavior. For example, food deprived rats will lever press for palatable food even when standard chow is available, but will consume standard chow rather than lever press for the more palatable food when systemically treated with a dopamine D1 receptor (D1R) antagonist, or when a D1R or D2R antagonist is injected into the NAc[131,132]. Overall, it is clear that many factors control when to eat and what is eaten, and the mesocorticolimbic dopamine system is critical for reinforcement behavior and the motivation to work for food.

There are many studies showing the importance of dopamine not only for food reinforcement behavior but also for feeding. Indeed, dopamine deficient mice are aphagic and will starve to death by 4 weeks of age if they are not treated with L-DOPA, a dopamine precursor[133], and peripheral injection of the D2R antagonist pimozide decreases free feeding and

food reward in rats[134,135]. In addition, free feeding and lever pressing for food increase dopamine release in the NAc in food-restricted rats[136-138]. Similarly, dopamine injection into the NAc increases food and water intake, while D2R antagonism blocks dopamine induced feeding[139], and injecting low doses of amphetamine into the accumbens to stimulate dopamine release increases food intake, while higher doses decrease food intake[140]. The importance of the reward system in feeding behavior has also been demonstrated in humans; for example, Parkinson's patients treated with dopamine agonists develop compulsive eating habits[141], and in human imaging studies there is an increase in activity in brain reward areas in response to pictures of food, and an even greater response to pictures of high-calorie palatable foods compared to low-calorie foods[142]. In addition, striatal D2R levels are lower in obese individuals compared to lean individuals[143], and brain reward areas of obese individuals show greater increases in activity in response to pictures of palatable food than in lean individuals[144-147]. Interestingly, there is decreased activity in brain reward areas in obese individuals in response to food consumption compared to lean individuals[144], and weight gain in women causes decreased activity in the striatum after palatable food consumption[148]. Thus, the mesocorticolimbic dopamine system is clearly an important regulator of feeding behavior, and it is essential to understand how the reward system regulates feeding, food reward, and weight gain, as the circuitry and function of the reward system appears to be altered in obese individuals.

1.2.1 Homeostatic regulators of energy balance and interactions with the mesocorticolimbic dopamine system

The homeostatic control of energy balance encompasses neural circuits, neuropeptides, and hormonal adipose and gastrointestinal signals that coordinate to balance energy intake with

energy expenditure to maintain energy equilibrium[8]. Thus, the homeostatic system increases food intake and decreases metabolic rate during an energy deficit and decreases food intake and increase metabolic rate during an energy surplus. Previous studies suggest that the homeostatic energy system interacts with the mesocorticolimbic dopamine system to regulate food intake and body weight. For example, the activity of brain reward areas and dopamine release in the NAc and VTA change with energy state. Indeed, chronically food restricted underweight rats have lower basal dopamine levels in the NAc[149,150]. Food restriction increases dopamine release in the NAc during feeding[151-153] and during a sucrose binge in rats[154]. In addition, food restriction increases the effects of drugs of abuse[155], the locomotor response to dopamine receptor agonists[156,157], and operant responding for cocaine[158] and food[159]. Acute food restriction also increases evoked somatodendritic release of dopamine in the VTA[160], and chronic food restriction increases burst firing and glutamatergic transmission in SNc dopamine neurons[161]. In human imaging studies, fasting increases the response of brain reward areas to pictures of highly palatable food and the subjective appeal of highly palatable food[162]. Thus, the reward system is affected by energy state, which may be due to homeostatic regulators of energy balance acting on the mesocorticolimbic dopamine system.

The hypothalamus is one of the primary brain areas of the homeostatic energy system that integrates peripheral satiety signals to regulate energy intake and body weight[8,163]. The hypothalamus is composed of distinct nuclei including the arcuate nucleus, ventromedial nucleus, dorsomedial nucleus, LH, and paraventricular nucleus, among others[8,163]. There is extensive evidence that the hypothalamus and mesocorticolimbic dopamine system interact to regulate food intake and food reward. Neurons of different hypothalamic nuclei project to brain areas of the reward system and vice versa[19,20,163-165]. For example, the LH is a key interface

connecting the hypothalamus with the mesocorticolimbic dopamine system, because the LH heavily innervates the mesocorticolimbic dopamine system and is connected to other hypothalamic nuclei[163,166]. The LH is also an important regulator of food intake and reward behavior[163,166]. Indeed, stimulation of the LH elicits feeding and causes an increase in dopamine release in the NAc[136], and peripheral injection of a dopamine receptor antagonist blocks LH stimulated feeding[167]. Thus, the LH may serve as a key connection between the circuits controlling homeostatic and reward feeding.

1.2.2 Hypothalamic feeding-related neuropeptides and interactions with the mesocorticolimbic dopamine system

Multiple classes of neurons in different hypothalamic regions produce orexigenic and anorexigenic neuropeptides that regulate energy balance[8,163,168]. Evidence suggests that these hypothalamic feeding-related neuropeptides can interact with the mesocorticolimbic dopamine system to regulate food intake and food reward[168]. For example, VTA dopamine neurons express receptors for many of these hypothalamic neuropeptides, and numerous studies have shown that hypothalamic neuropeptides modulate VTA dopamine neuron activity through multiple mechanisms, such as directly activating currents in dopamine neurons and/or by modulating excitatory and inhibitory inputs to dopamine neurons[168]. In addition, intra-VTA or NAc injection of several hypothalamic feeding-related neuropeptides alters food intake, food reward, and other feeding-related behaviors[168]. The interaction between hypothalamic feeding neuropeptides and the mesocorticolimbic dopamine system may be an important linkage between energy state and the motivational state of an animal. Nevertheless, how hypothalamic neuropeptides and the reward system interact to regulate feeding and food reward is not fully understood. For example, centrally injected neurotensin or neuropeptide-Y (NPY) both

increase dopamine release in the NAc, but cause opposite effects on the motivation for food[168]. In addition, injection of the hypothalamic neuropeptides neurotensin, alpha-melanocyte-stimulating hormone (α-MSH), or NPY into the VTA alters multiple aspects of feeding[169-175], but how these neuropeptides interact with the mesocorticolimbic dopamine system to alter food intake, food reward, and weight gain at both the circuit and cellular levels is not fully understood. Thus, in these studies I have tested how three distinct hypothalamic feeding-related neuropeptides, neurotensin, α-MSH, and NPY, affect VTA dopamine neuron activity to further elucidate how these peptides interact with the mesocorticolimbic dopamine system to regulate feeding and other dopamine dependent behaviors.

1.2.2.1 Neurotensin

Neurotensin is a tricapeptide that is widely expressed in both the central and peripheral nervous systems. Neurotensin regulates many different physiological processes and behaviors including analgesia, blood pressure, body temperature, locomotor behavior, drinking, feeding, and drug intake. There is substantial evidence that neurotensin interacts with the VTA to regulate dopamine-dependent behaviors. For example, neurotensin expressing neurons project to the VTA, primarily from the LH and the medial and lateral preoptic areas,[176-178], neurotensin positive fibers heavily innervate midbrain dopamine neurons[179,180], and VTA neurons express neurotensin receptors[181-187]. Intra-VTA neurotensin increases locomotor activity[188,189], induces conditioned place preference[190,191], and rats will operant respond for intra-VTA infusions of neurotensin[192]. In addition, activation of neurotensin LH neurons increases locomotor activity and dopamine release in the NAc[193].

Extensive evidence shows neurotensin regulates food intake and food reward. Central and peripheral injection of neurotensin decreases food intake in both fasted and fed rodents[194-

[196], and neurotensin antagonism or knockout of the neurotensin receptor NTS1 blocks the anorectic effects of leptin[197,198]. Intra-VTA injection of neurotensin increases latency to eat, reduces food intake, and reduces operant responding for food[169-171]. Interestingly, ablation of VTA NTS1 receptor expressing neurons in adult mice reduces body weight but increases food intake compared to control mice[187]. Previous studies suggest neurotensin signaling in the VTA modulates feeding and other dopamine dependent behaviors through an increase in dopamine neuron activity and dopamine release[188,199-207]. However, how neurotensin increases dopamine neuron activity is not fully understood.

1.2.2.2 NPY

NPY is abundantly expressed throughout the body and central nervous systems. NPY regulates many different behaviors and physiological process including anxiety, pain, stress, cardiovascular function, circadian rhythms, and feeding; in addition, NPY has also been implicated in many diseases including obesity, depression, and alcoholism[208]. NPY is expressed centrally in the hippocampus, amygdala, locus coeruleus, NAc, cerebral cortex, and hypothalamus[209,210]. Within the hypothalamus, NPY is most abundantly expressed in agouti-related protein/NPY (AgRP/NPY) neurons of the arcuate nucleus[209,210]. NPY is a strong orexigenic neuropeptide and regulator of body weight. Indeed, central administration of NPY or activation of AgRP/NPY neurons causes robust feeding in rodents[211-213]. In addition, ablation of AgRP/NPY neurons reduces food intake and body weight[214,215], and NPY knockout decreases fasting-induced feeding[216]. Substantial evidence suggests that NPY also interacts with VTA neurons to affect feeding. For example, intra-VTA NPY increases motivation for food[174]. In addition, NPY receptors are expressed in the VTA[217,218], and NPY decreases the

firing rate of VTA neurons[217]. However, how NPY decreases VTA dopamine neuron activity is unknown.

1.2.2.3 α-MSH

α-MSH is a peptide hormone that regulates multiple physiological functions throughout the body including pigmentation, inflammation, pain, and sexual arousal. α-MSH is also an anorexigenic neuropeptide and an important regulator of energy homeostasis and body weight. Within the brain, α-MSH is found in pro-opiomelanocortin (POMC) expressing neurons of the arcuate nucleus of the hypothalamus and is a derivative of the propeptide POMC. The central actions of α-MSH (as well as β- and γ-MSH) are mediated by the centrally expressed melanocortin receptors, melanocortin-3 receptor (MC3R) and melanocortin-4 receptor (MC4R)[165]. Collectively, α-, β-, and γ-MSH from POMC neurons, MC3/4Rs, and AgRP (an inverse agonist to the MC3/4Rs) from AgRP/NPY neurons make up the melanocortin system[165]. Numerous studies have shown the importance of the melanocortin system and α-MSH for the homeostatic regulation of energy balance. For example, activation of POMC neurons or injection of MC3/4R agonists suppresses food intake[211,219,220], and knockout of the MC3/4Rs is associated with obesity, increased fat mass, and reduced activity[221]. In addition, there is evidence that α-MSH interacts with VTA neurons to regulate food intake, food reward, and other dopamine-dependent behaviors. For example, POMC neurons project to the VTA[222], and MC3/4Rs are expressed in the VTA[223-226], although the literature indicates that MC3Rs are the primary melanocortin receptor expressed in the VTA with little expression of MC4Rs[223,225,226]. Intra-VTA α-MSH increases grooming, rearing, and locomotor behavior[227-230], and intra-VTA MC4R agonist decreases ethanol intake[231]. In addition, we previously showed that intra-VTA MTII (α-MSH analog) decreases food and sucrose intake and operant responding for sucrose

pellets, while intra-VTA SHU9119 (MC3/4R antagonist) increases food intake and operant responding for sucrose pellets[172,173,175]. Chronic blockage of MC3/4Rs with SHU9119 also increases body weight[172]. Intra-VTA α-MSH increases dopamine turnover in the NAc, suggesting that α-MSH modulates food intake and dopamine-dependent behaviors through an increase in dopamine neuron activity[228-230,232,233]. However, the cellular mechanism underlying the effect of intra-VTA MC3/4R agonists on food intake and reward behavior is unknown.

1.3 Summary of Introduction

Overall, the neural control of food intake and body weight is not fully understood. Determining the neural mechanisms of food intake is crucial for developing new and effective treatments for weight gain and obesity, as the global rise in obesity has largely been contributed to increased food consumption. Presented here are two important central systems that regulate food intake and body weight, the hypothalamus and the mesocorticolimbic dopamine system. The hypothalamus primarily regulates homeostatic feeding while the mesocorticolimbic dopamine system primarily regulates reward-based feeding. Thus, the hypothalamus and reward system are often studied independently of one another; yet substantial evidence suggest that they do interact to regulate food intake and food reward. Thus, I describe here our studies testing how the hypothalamic feeding-related neuropeptides neurotensin, NPY, and, α-MSH regulate VTA dopamine neuron activity to advance our understanding of how the hypothalamus and mesocorticolimbic dopamine system interact to regulate food intake and food reward.

2 OVERALL MATERIALS AND METHODS

For the below experiments I used whole-cell patch clamp and loose-cell attached electrophysiology in brain slices containing the VTA from mice[160,234-236] to test how hypothalamic neuropeptides affect dopamine neuron activity. Electrophysiological recordings were collected using an Axon multiclamp 700B microelectrode amplifier and Axograph software[160,234,236]. Putative dopamine neurons were identified by their location relative to the medial terminal nucleus of the accessory optic tract, presence of hyperpolarization-activated cation currents, spontaneous slow pacemaker firing (≤ 10 Hz), broad action potential waveform (≥ 1.2 ms), and sensitivity to dopamine[68,237-240]. Data were stored and analyzed using Axograph X (v1.3.5), LabChart (v7.3.6; ADInstruments), and Excel (v14.0; Microsoft Corporation) software. Statistics were calculated using SigmaStat (v11.0; Systat Software, Inc.).

Adapted from:
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3 NEUROTENSIN INHIBITS BOTH DOPAMINE AND GABA MEDIATED INHIBITION OF VENTRAL TEGMENTAL AREA DOPAMINE NEURONS

3.1 Abstract

Dopamine is an essential neurotransmitter that plays an important role in a number of different physiological processes and disorders. There is substantial evidence that the neuropeptide neurotensin interacts with the mesolimbic dopamine system and can regulate dopamine neuron activity. In these studies we have used whole-cell patch clamp electrophysiology in brain slices from mice to examine how neurotensin regulates dopamine neuron activity by examining the effect of neurotensin on the inhibitory post-synaptic current generated by somatodendritic dopamine release (D2R IPSC) in ventral tegmental area (VTA) dopamine neurons. Neurotensin inhibited the D2R IPSC and activated an inward current in VTA dopamine neurons that appeared to be at least partially mediated by activation of a transient receptor potential C-type channel. Neither the inward current nor the inhibition of the D2R IPSC was affected by blocking PKC or calcium release from intracellular stores, and the inhibition of the D2R IPSC was greater with neurotensin compared to activation of other Gq-coupled receptors. Interestingly, the effects of neurotensin were not specific to D2R signaling as neurotensin also inhibited $GABA_B$ inhibitory post-synaptic currents in VTA dopamine neurons. Finally, the effects of neurotensin were significantly larger when intracellular Ca^{2+} was strongly buffered, suggesting that reduced intracellular calcium facilitates these effects. Overall, these results suggest that neurotensin may inhibit the D2R and $GABA_B$ IPSCs downstream of receptor activation, potentially through regulation of G-protein coupled inwardly

rectifying potassium channels. These studies provide an important advance in our understanding of dopamine neuron activity and how it is controlled by neurotensin.

3.2 Introduction

Dopamine (DA) is an essential neurotransmitter involved in many different behaviors including motor behavior, incentive motivation, reward and reinforcement, learning, memory, drug intake, and habit formation[15], and disruptions in DA signaling have been implicated in many disorders such as drug addiction, obesity, Parkinson's disease, and schizophrenia[13,58,164,241,242]. Most DA producing neurons are found in the ventral tegmental area (VTA) and the substantia nigra *pars compacta* (SNc) of the midbrain[15]. At rest VTA/SNc DA neurons fire tonically (2-10 Hz) causing a baseline low level of DA at efferent target sites, but in response to a reward DA neurons fire in bursts causing phasic increases in DA release[58,66,69]. Phasic increases in DA release at efferent target sites are thought to be a salient signal and learning cue[58,66,69]. DA burst firing is primarily controlled by glutamatergic afferent inputs, but DA neuron activity can also be modulated by other neurotransmitters and neuropeptides acting either directly on DA neurons or indirectly through regulation of GABAergic or glutamatergic inputs to DA neurons[58,66,69]. Characterizing how DA neuron activity is regulated is important for understanding the function of DA under normal and pathological conditions.

In addition to releasing DA from their axon terminals, DA neurons release DA locally within the VTA/SNc from their soma and dendrites[95-99]. This somatodendritic DA release inhibits neighboring DA neurons through dopamine D2 receptor (D2R) mediated activation of G-coupled inward rectifying potassium (GIRK) channels[98,100-102]. D2Rs in VTA DA neurons regulate DA neuron activity and also regulate DA mediated behaviors. For example, injection

of quinpirole, a D2R agonist, directly into the VTA causes conditioned place aversion, blocks food induced conditioned place preference, decreases food intake, and decreases cocaine-induced reinstatement[243,244]. Furthermore, selective knockout of autoreceptor D2Rs within midbrain DA neurons causes increased motor activity to a novel environment, increased food self-administration, and increased responses to cocaine such as increased locomotor activity and conditioned place preference compared to wild type mice[245,246]. In addition, DA neuron burst firing is followed by a pause, and it has been proposed that D2R mediated inhibition terminates bursts of action potentials and is responsible for the pause following burst firing[98]. Thus, this autoinhibitory D2R signaling in the VTA plays an important role in the regulation of DA activity and DA mediated behaviors.

Neurotensin is a tricapeptide that was first isolated and characterized from bovine hypothalamus[247], and is widely expressed in both the central and peripheral nervous systems. The actions of neurotensin are mediated by three known neurotensin receptors: NTS1, NTS2, and NTS3 (for review see[248]). There is abundant evidence that neurotensin interacts with the DA system (for review see[184]), and dysregulation of these interactions has been proposed to be involved in pathologies such as schizophrenia, drug abuse, and Parkinson's disease[184,249-251]. Fibers containing neurotensin heavily innervate midbrain DA neurons[179,180], and DA neurons of the VTA and SNc express neurotensin receptors, primarily the NTS1 receptor[181-186]. Furthermore, D2Rs and NTS1 receptors have been shown to form heteromers in heterologous expression systems, which resulted in a decrease in D2R agonist binding and decreases in D2R signaling after treatment with neurotensin[252,253]. Previous research has shown that neurotensin modifies midbrain DA neuron activity through two NTS1 receptor dependent mechanisms: increased DA neuron firing through activation of a non-selective cation channel[199-204] and a

reduction in the inhibition of firing caused by D2R activation[201,202,204,254-256]. The majority of evidence suggests that the effects of neurotensin on DA neurons occur through activation of signaling pathways downstream of Gq-proteins, specifically through PKC, IP3, and calcium[201,203,257-259], although neurotensin has also been reported to affect DA neuron activity through a PKA dependent mechanism[256]. In these studies we sought to examine the mechanisms by which neurotensin reduces the D2R-mediated inhibition of DA neuron activity by testing the hypothesis that neurotensin inhibits the D2R-mediated inhibitory post-synaptic current (D2R IPSC)[98] that occurs in response to the local, somatodendritic release of DA within the VTA.

3.3 Materials and Methods

Animals: Male C57BL/6J male mice (5-12 weeks old) purchased from The Jackson Laboratories were used in all experiments. All protocols and procedures were approved by the Institutional Animal Care and Use Committee at Georgia State University, and conformed to the *NIH Guide for the Care and Use of Laboratory Animals*.

Slice preparation and Electrophysiology: Acute brain slices were prepared as previously described[235]. Briefly, adult male mice were anesthetized with isofluorane and decapitated. The brain was then removed and placed in carbogen (95% O_2 and 5% CO_2) saturated ice-cold artificial cerebral spinal fluid (aCSF), containing (in mM) 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 11.1 glucose, and 21.4 $NaHCO_3$. A brain block containing the VTA was made, and pseudo-horizontal sections (220 μM) were cut with a vibrating blade microtome. Slices were then incubated in aCSF (~35°C) containing 10 μM MK-801 [(+)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] for at least 30 minutes before

recording. Slices were placed in a recording chamber and perfused with carbogen-saturated aCSF at a flow rate of ~ 1-2 ml/min. Whole-cell recordings were made using an Axon multiclamp 700B microelectrode amplifier and Axograph software. Putative DA neurons were identified by their location relative to the medial terminal nucleus of the accessory optic tract, the presence of hyperpolarization-activated cation currents (H-current), the presence of spontaneous pacemaker firing, and the sensitivity to DA[237]. Although recent studies have raised questions on the utility of using these measures to identify VTA DA neurons[29], the characteristics described above have been widely used in electrophysiological studies on DA neurons to identify DA neurons within the VTA[98,235,254,258,260].

Electrodes (2.0-3.0 MΩ) were filled with a potassium gluconate (KGluconate) based internal solution containing (in mM) 128 KGluconate, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 10 BAPTA, 2 ATP, 0.3 GTP, and 10 creatine phosphate. For the experiments testing the effects of neurotensin under reduced calcium buffering conditions, low-calcium buffering potassium methyl sulfate ($KMeSO_4$) or K-Gluconate based internal solutions were used containing: (in mM) 115 $KMeSO_4$, 20 NaCl, 1 $MgCl_2$, 10 HEPES, 0.1 EGTA, 2 ATP, 0.3 GTP, and 10 creatine phosphate, or (in mM) 128 KGluconate, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 0.1 EGTA, 2 ATP, 0.3 GTP, and 10 creatine phosphate. No differences were observed between these two low calcium buffering internal solutions, so experiments using these two different internal solutions were pooled. Series resistance values were approximately ~ 3-15 MΩ. If the series resistance increased by more than 20% or if the IPSC or holding current were unstable in any of the experiments, the experiment was terminated and excluded from analysis. Neurons were voltage clamped at -60 mV for all experiments, and D2R IPSCs and $GABA_B$ IPSCs were evoked using a bipolar stimulating electrode placed 100-300 μM posterior to the recorded cell. D2R IPSCs

were evoked with 5-stimuli (0.5 ms) at 40 Hz, and $GABA_B$ IPSCs were evoked with 6-stimuli (0.3 ms) at 50 Hz. D2R IPSCs were also evoked using the iontophoretic application of DA. DA was applied iontophoretically through a ~ 70-100 MΩ glass pipette filled with 1 M DA and ejected as a cation with a single pulse (10 nA, 25 ms). Leak of DA from the pipette was prevented with a constant negative back current (2 nA). To isolate D2R IPSCs, picrotoxin (100 μM), CGP 55845 (0.5 μM), and DNQX (10 μM) were included in the perfusion solution to block $GABA_A$, $GABA_B$, and AMPA receptors, respectively. $GABA_B$ IPSCs were isolated by including sulpiride (200 nM), picrotoxin (100 μM), and DNQX (10 μM) to block D2R, $GABA_A$, and AMPA receptors, respectively. The peak amplitude of all IPSCs was measured from baseline and calculated as the mean current 30 ms before and after the peak IPSC amplitude. For all experiments cells were held for 10 minutes prior to drug application to allow for diffusion of the internal solution into the cell. For the experiments examining muscarinic acetylcholine receptor induced currents, the nicotinic receptor antagonist, mecamylamine (30 μM), was included both before and during application of acetylcholine. To determine the voltage current relationship and reversal potential of the neurotensin current, voltage ramps were applied (-120 mV to +40 mV at 160 mV s^{-1} or -120 mV to +20 mV at 140 mV s^{-1}) in the presence of TTX.

Drugs: The 8-13 active fragment of neurotensin (referred to as neurotensin) was used in all experiments. Neurotensin (8-13) was purchased from Bachem Americas Inc. (Torrance, CA, USA). CGP 55845, SKF 96365, cyclopiazonic acid, and DHPG were purchased from Tocris Biosciences (Minneapolis, MN, USA). Chelerythrine was purchased from Sigma Aldrich (St. Louis, MO, USA). Mecamylamine and acetylcholine were generous gifts from Dr. Chun Jiang's lab. All other reagents were from common commercial sources.

Data Analysis and Statistics: Data are represented as the mean +/- SEM unless otherwise noted. Data were analyzed using Axograph X (v1.3.5), LabChart (v7.3.6; ADInstruments), and Excel (v14.0; Microsoft Corporation) software. Statistics were calculated using SigmaStat (v11.0; Systat Software, Inc.). EC50 values were calculated using GraphPad Prism (v6.0f; GraphPad Software, Inc.). Pearson's correlation coefficient was used to calculate correlation. All data were initially tested for normality using the Shapiro-Wilk test and were then analyzed with Student's t-tests, Mann-Whitney U tests, ANOVAs, or a Kruskal-Wallis One Way ANOVA on ranks as appropriate with a significance level of $p<0.05$ set *a priori*.

3.4 Results

We initially examined how neurotensin affects DA neuron activity by assessing both the inward current activated by neurotensin and its ability to affect the inhibitory current generated by somatodendritic DA release in the VTA (D2R IPSC)[98]. Neurotensin dose-dependently activated an inward current in DA neurons and inhibited the D2R IPSC (Fig. 3.1A-C). Neurotensin also caused an increase in noise at all doses tested (Fig. 3.1C). The EC50 values of the effects of neurotensin on VTA DA neurons were 208.7 nM for the inward current and 4.38 nM for the inhibition of the D2R IPSC (Fig. 3.1A-B). The inhibition of the D2R IPSC positively correlated with the magnitude of the inward current ($r=0.672$, $p=0.0006$, Fig. 3.1D), suggesting that neurotensin may activate the inward current and inhibit the D2R IPSC in VTA DA neurons through a common mechanism.

The timing of the activation of the inward current and the inhibition of the D2R IPSC differed however. The inward current caused by 100 nM neurotensin reached its peak quickly, whereas the onset and peak of the inhibition of the D2R IPSC were delayed compared to the

inward current (Fig. 3.2A-C). The washout and recovery of the effects of neurotensin also differed as the neurotensin current and the increase in noise slowly reversed (in ~ 10-15 minutes), while the inhibition of the D2R IPSC never recovered during the drug washout period (Fig. 3.2A,C). Furthermore, the D2R IPSC remained inhibited 10 minutes after neurotensin application when the neurotensin current had almost completely recovered, suggesting that the inward current is not simply occluding the D2R IPSC (Fig. 3.2A-C). Thus, although the magnitude of the inward current correlated with the amount of inhibition of the D2R IPSC, there were differences in the timing of the two effects of neurotensin, suggesting that they may actually be mediated through different mechanisms.

We next sought to confirm whether the neurotensin caused inward current and inhibition of the D2R IPSC were independent by inhibiting the inward current and measuring the effect of neurotensin on the D2R ISPC. Thus, we attempted to confirm previous experiments identifying the ion channels mediating the neurotensin-induced inward current in DA neurons. The neurotensin-induced current obtained from slow voltage ramps showed a unique I-V relationship with outward rectification and an extended zero slope region around the reversal potential, which was calculated to be -36 +/- 6.6 mV (Fig. 3.3). This unique I-V curve, combined with previous reports demonstrating that neurotensin activates a slow nonselective cation conductance permeable to Na+, K+, and Cs+[199,200,261], suggests that neurotensin may be activating a member of the transient receptor potential C channel (TrpC) family[262,263]. Thus, we tested whether SKF 96365, a TrpC channel blocker that was previously shown to block the neurotensin caused increase in firing frequency in DA neurons[203], could inhibit the neurotensin-induced inward current in VTA DA neurons. SKF 96365 (100 μM) partially inhibited the neurotensin-induced inward current (Fig. 3.4C-D). Although the peak inward current caused by

neurotensin (100 nM) was only slightly decreased by SKF 96365, the sustained neurotensin-induced current rapidly decreased, and the duration of the neurotensin-induced inward current was significantly shortened (Fig. 3.4C-D) (significant main effects of treatment ($F(1, 9)=6.079$, $p<0.05$), and time ($F(20, 175)=13.888$, $p<0.001$), and a significant treatment×time interaction ($F(20,175)=2.583$, $p<0.001$). In addition, the increase in noise caused by neurotensin also partially recovered during the washout and recovery of the neurotensin-induced current. We also tested whether SKF 96365 affected the ability of neurotensin to inhibit the D2R IPSC. SKF 96365 had no effect on the ability of neurotensin to inhibit the D2R IPSC however, as the peak inhibition of the D2R IPSC caused by neurotensin was unaffected (Fig. 3.4E-F). The D2R IPSC also remained inhibited throughout the experiment even when the neurotensin current recovered (Fig. 3.4A-B), suggesting that the inward current and the inhibition of the D2R IPSC are likely independent and that the inward current caused by neurotensin does not simply occlude the D2R IPSC.

We next sought to confirm that the effects of neurotensin on the D2R IPSC were mediated post-synaptically and not through alterations in DA release, by testing whether neurotensin also inhibited the D2R-mediated current generated by the iontophoretic application of DA. As predicted, neurotensin inhibited the iontophoresis evoked D2R current (Fig. 3.5A) to the same magnitude as the electrically evoked D2R IPSC (Fig. 3.5B-C). Thus, neurotensin appears to act at the post-synaptic membrane to inhibit the D2R IPSC and not through a pre-synaptic change in DA release.

We next attempted to identify the mechanism by which neurotensin inhibits the D2R IPSC. Previous studies suggested that neurotensin inhibits D2R signaling through a Ca^{2+} and PKC dependent mechanism[201,258,259]. Therefore, we tested the role of the release of Ca^{2+} from

intracellular stores in the neurotensin caused inhibition of the D2R IPSC through the use of the reversible sarcoplasmic reticulum Ca^{2+} ATPase inhibitor, cycopiazonic acid (CPA). Pretreatment of brain slices with CPA (10 μM) for at least 15 minutes prior to neurotensin application had no effect on the neurotensin (100 nM) inhibition of the D2R IPSC (Fig. 3.6A-B). In addition, CPA had no effect on the peak inward current induced by neurotensin (Fig. 3.6C-D). We also tested whether neurotensin inhibits the D2R IPSC through a PKC-dependent process through the use of the non-specific PKC inhibitor, chelerythrine. Chelerythrine (10 μM) also had no effect on the neurotensin-induced inhibition of the D2R IPSC (Fig. 3.6A-B), and there was no significant difference between the peak inward current induced by neurotensin after chelerythrine treatment compared with neurotensin alone (Fig. 3.6C-D). Thus, in contrast to previous reports[201,258,259], the inhibition of the D2R IPSC in VTA DA neurons by neurotensin does not appear to depend on PKC activation, nor does it depend on release of Ca^{2+} from intracellular stores.

We next tested whether the inhibition of the D2R IPSC was specific to neurotensin or could be achieved by activation of other Gq-coupled receptors by examining whether activation of metabotropic glutamate receptors or muscarinic acetylcholine receptors also inhibited the D2R IPSC. To activate muscarinic receptors, acetylcholine (ACh) was added in the presence of mecamylamine (30 μM), a nicotinic acetylcholine receptor antagonist. For this experiment, we chose doses of the metabotropic glutamate receptor agonist, DHPG (10 μM), ACh (1 mM), and neurotensin (10 nM) that caused approximately the same peak inward current to allow for direct comparison of their effects on the D2R IPSC (Fig. 3.7A-D). There were no significant differences between the peak current activated by DHPG, ACh, and neurotensin ($F(2,16)=1.217$, $p=0.322$) (Fig. 3.7D), but there were significant differences in the inhibition of

the D2R IPSC, as neurotensin inhibited the D2R IPSC to a significantly greater extent than DHPG or ACh ($F(2,16)=5.181$, $p<0.05$) (Fig. 3.7E). In addition, the effects of DHPG and ACh on the D2R IPSC started to reverse upon removal of the agonist, whereas the effects of neurotensin on the D2R IPSC showed no reversal (Fig. 3.7A-C). Thus, it appears that activation of Gq-coupled receptors can inhibit the D2R IPSC, but activation of NTS1 appears to engage an additional mechanism that causes significantly greater inhibition of the D2R IPSC.

We next sought to test whether the ability of neurotensin to inhibit the D2R IPSC was specific to D2Rs or if it would also affect other inhibitory responses in VTA DA neurons. $GABA_B$ receptors and D2Rs both inhibit VTA DA neurons through the activation of G-protein coupled inward rectifying potassium channels (GIRK channels). Thus, we next tested whether neurotensin also affected the IPSC generated by activation of $GABA_B$ receptors ($GABA_B$ IPSC) (Fig. 3.8A-B). Interestingly, neurotensin inhibited the $GABA_B$ IPSC to a similar degree as the D2R IPSC (Fig. 3.8C). This was true for both the maximal dose (100 nM) of neurotensin that fully inhibited the D2R IPSC, and an intermediate dose (10 nM) closer to the EC50 value for neurotensin inhibition of the D2R IPSC (Fig. 3.8C). Thus, neurotensin does not appear to specifically inhibit the D2R IPSC in VTA DA neurons but can also inhibit the $GABA_B$ IPSC, possibly through a common mechanism.

We next sought to examine the similarity of the inhibition of the D2R IPSC and the $GABA_B$ IPSC in more detail. The D2R and $GABA_B$ IPSCs differ in their sensitivity to intracellular Ca^{2+} levels as the D2R IPSC shows increased desensitization and long-term depression when intracellular free Ca^{2+} levels are weakly buffered, whereas the $GABA_B$ IPSC is insensitive to changes in intracellular Ca^{2+}[126]. Thus, we next tested whether the effects of neurotensin on the D2R and $GABA_B$ IPSCs were affected by basal intracellular Ca^{2+} levels by

switching from a high Ca^{2+} buffering internal solution (containing 10 mM BAPTA = low levels of basal free intracellular Ca^{2+}) to a low Ca^{2+} buffering internal solution (containing 0.1 mM EGTA = higher levels of basal free intracellular Ca^{2+}). A dose of neurotensin near the EC50 value for inhibition of the D2R IPSC was used in these experiments to allow for the identification of potential increases or decreases in the effect of neurotensin on the D2R and $GABA_B$ IPSCs with the reduced calcium buffering internal solution. Neurotensin (10 nM) still caused an inward current and slightly reduced both the D2R and $GABA_B$ IPSCs when measured with a 0.1 mM EGTA internal solution (Fig. 3.9). Interestingly, the inward current induced by neurotensin was significantly reduced compared to the inward current with the internal solution containing 10 mM BAPTA (Fig. 3.9A-B) (significant main effects of treatment ($F(1, 15)=23.357$, $p<0.001$) and time ($F(19,250=33.674$, $p<0.001$), and significant treatment×time interaction ($F(19,250)=14.087$, $p<0.001$). The neurotensin-caused inhibition of both the D2R and $GABA_B$ IPSCs were also significantly reduced with the 0.1 mM EGTA internal solution compared to the 10 mM BAPTA internal solution with no differences in the magnitude of inhibition of the D2R IPSC versus the $GABA_B$ ISPC (Fig. 3.9C-F) (D2R IPSC: significant main effects of treatment ($F(1, 17)=32.102$, $p<0.001$) and time ($F(19, 288=33.157$, $p<0.001$), and a significant treatment×time interaction ($F(19,288)=11.708$, $p<0.001$); $GABA_B$ IPSC: significant main effects of treatment ($F(1,12)=17.439$, $p=0.001$) and time ($F(20, 240=18.321$, $p<0.001$), and a significant treatment×time interaction ($F(20,240)=8.165$, $p<0.001$). Thus, the effects of neurotensin on DA neurons were greater when intracellular Ca^{2+} was strongly buffered and resting levels of free intracellular Ca^{2+} were low, and were attenuated when intracellular Ca^{2+} was weakly buffered and resting levels of free intracellular Ca^{2+} were high.

3.5 Discussion

In these studies we have demonstrated that neurotensin increases DA neuron activity through multiple mechanisms. In addition to directly activating an inward current to increase DA neuron activity, neurotensin also significantly reduced the inhibition of DA neurons caused by activation of both D2R and $GABA_B$ receptors. These effects did not appear to depend on release of Ca^{2+} from intracellular stores or on PKC activation, but were sensitive to basal levels of free intracellular Ca^{2+}.

The effects of neurotensin on the inward current and the D2R and $GABA_B$ IPSCs appear to be independent and mediated by different mechanisms. The timing of neurotensin inhibition of the D2R IPSC was significantly delayed compared to the inward current caused by neurotensin, and the neurotensin-induced current recovered during the washout period after neurotensin application, whereas the inhibition of the D2R IPSC did not. The EC50 values for the two effects of neurotensin were also different, further supporting the argument that the two effects of neurotensin occur through separate mechanisms. The EC50 value for inhibition of the D2R IPSC by neurotensin was ~ 4 nM, much lower than the EC50 value for the neurotensin induced current (~ 200 nM), and lower doses of neurotensin (1-10 nM) have often been used previously to examine the effect of neurotensin on D2R signaling[201,202,204,254-256] while higher doses of neurotensin (1 nM to 5 μM) have been used to examine the neurotensin induced inward current in DA neurons[199-204]. In addition, partially inhibiting the neurotensin current with SKF 96365 did not have any effect on the neurotensin caused inhibition of the D2R IPSC. Finally, ACh, DHPG, and neurotensin caused inward currents that were similar in magnitude, but neurotensin had a much larger effect on the D2R IPSC. If the inward current caused the inhibition of the D2R IPSC, then it would be expected that neurotensin, ACh, and DHPG would

inhibit the D2R IPSC to the same magnitude when activating inward currents of the same size. In agreement with our findings, it was previously reported that neurotensin reduces quinpirole (D2R agonist) induced inhibition of DA activity even when the excitatory effect of neurotensin is blocked with heparin, an IP3 receptor antagonist[201]. Therefore, it appears that the neurotensin inward current does not block the D2R IPSC because of occlusion or net excitation, and that these are independent effects downstream of NTS1 activation.

The neurotensin-induced inward current has been characterized as a slow non-selective cation current that is equally permeable to both Na^+ and K^+, a characteristic of Trp channels[199]. The neurotensin-induced current in DA neurons was similar to that generated by activation of specific TrpC channels expressed in HEK-293 cells[262,263] suggesting that neurotensin may be activating a member of the TrpC family, potentially through the activation of phospholipase C and release of DAG[264,265]. We found that the TrpC channel blocker SKF 96365 significantly shortened the duration of the neurotensin-induced current, which is in agreement with a previous report showing that SKF 96365 blocks the neurotensin-caused increase in DA neuron firing frequency[203]. Thus, it appears that the neurotensin activated inward current is at least partially mediated by activation of TrpC channels in VTA DA neurons.

The majority of evidence suggests that the effects of neurotensin on DA neurons are mediated by signals that are downstream of PLC activation. Previously it was reported that the neurotensin-induced inward current and increase in firing frequency are dependent on Ca^{2+} and the IP3 receptor[201,203,257]. Another study found that the neurotensin-induced inward current was not dependent on Ca^{2+}, however, as the neurotensin current was not affected by buffering intracellular Ca^{2+} with 20 mM BAPTA[199]. In addition it has also been reported that neurotensin inhibits D2R signaling through a PKC and Ca^{2+} dependent mechanism[201,258,259]. In contrast to

these previous reports, we found that the neurotensin inhibition of the D2R IPSC and the neurotensin-induced current were not dependent on PKC or Ca^{2+}, and the effects of neurotensin were actually potentiated when intracellular Ca^{2+} was buffered with 10 mM BAPTA. In addition, the neurotensin inhibition of the D2R IPSC and the inward current were not dependent on Ca^{2+} release from intracellular stores. Interestingly, it has also been reported that neurotensin reduces the DA-caused inhibition of DA neuronal firing through the cAMP pathway and not through a PKC dependent mechanism[256]. Additional experiments are needed to resolve these differences, however, and to determine if the effects of neurotensin occur through dual pathways.

Although neurotensin likely inhibits the D2R IPSC via activation of Gq coupled signaling through a mechanism similar to metabotropic glutamate receptors and muscarinic acetylcholine receptors, here we have shown that there appears to be an additional mechanism activated by neurotensin to inhibit the D2R IPSC to a larger extent than other Gq coupled receptors. Interestingly, we also found that the effects of neurotensin were not specific to the D2R IPSC as neurotensin also inhibited the $GABA_B$ IPSC to the same magnitude. This suggests that the effects of neurotensin are likely not due to modulation of D2R activity by direct heterodimer interactions with NTS1 as has been observed in HEK-293 cells[252,253]. Previously it was reported that neurotensin does not block GABA caused inhibition of DA neuronal firing[202]. GABA inhibits DA neurons through the activation of both $GABA_A$ and $GABA_B$ receptors, however, so neurotensin may only block the inhibition produced by $GABA_B$ receptors and not $GABA_A$ receptors, which could explain the differences in these results. Thus the results presented here suggest that neurotensin inhibits both $GABA_B$ and D2R signaling in VTA DA neurons.

In these studies, we have shown a novel mechanism for modulation of VTA DA neuron activity by neurotensin: inhibition of $GABA_B$ IPSCs. $GABA_B$ receptors and D2Rs both activate GIRK channels to inhibit DA neurons, so it is possible that neurotensin modulates GIRK channel activity downstream of both D2R and $GABA_B$ activation to reduce D2R and $GABA_B$ caused inhibition of DA neurons (Fig. 3.10). In support of this notion, it was previously shown that neurotensin blocks D2R signaling downstream of the D2R[266]. In addition, it was recently reported that inducing high frequency bursting or depolarization in VTA DA neurons causes potentiation of GIRK currents and this potentiation was due to modulation to the GIRK channels themselves rather than regulation of the $GABA_B$ or D2 receptors[127]. Thus, neurotensin may reduce both D2R and $GABA_B$ mediated GIRK currents by causing direct modulation of GIRK channels, although further experiments will be required to test this hypothesis.

D2R IPSC desensitization and long-term depression have been reported to increase when intracellular Ca^{2+} is weakly buffered and free intracellular Ca^{2+} levels are high, whereas $GABA_B$ IPSCs do not show the same sensitivity to intracellular Ca^{2+} levels[126]. Combined with the ability of neurotensin to increase Ca^{2+} in DA neurons[201,203], these results suggest that reducing the Ca^{2+} buffering capacity of the internal solution would result in a larger effect of neurotensin on the D2R IPSC but not the $GABA_B$ IPSC. Surprisingly, the neurotensin-caused inhibition of both the $GABA_B$ and D2R IPSCs were significantly attenuated with low intracellular Ca^{2+} buffering (i.e. 0.1 mM EGTA solution). Thus, neurotensin appears to inhibit GIRK current activation downstream of D2R and $GABA_B$ receptors through a Ca^{2+} sensitive mechanism, whereby low levels of free intracellular calcium are required for the full inhibition of GIRK channel activation (Fig. 3.10). Alternatively, it is also possible that the NTS1 receptor is Ca^{2+} sensitive. We found that the neurotensin-induced inward current was also significantly

reduced with low intracellular Ca^{2+} buffering. Thus, all effects of neurotensin in DA neurons were reduced with low intracellular Ca^{2+} buffering and high intracellular levels of free Ca^{2+}. Therefore, it is possible that the NTS1 receptor may be sensitive to free intracellular Ca^{2+} levels and may desensitize or internalize with high levels of free intracellular Ca^{2+} resulting in a reduced effect of neurotensin, although future studies will be required to test this hypothesis.

In summary, we have demonstrated that neurotensin affects DA neuron activity through two seemingly independent effects: direct activation of an inward current mediated in part by TrpC channels, and inhibition of both the D2R and $GABA_B$ IPSCs. Overall these studies advance our understanding of how neurotensin regulates DA neuron activity, and further research characterizing how neurotensin affects DA neuron activity may lead to a better understanding and treatments of disorders caused by a disruption in the function of the mesolimbic DA system.

3.6 Figures

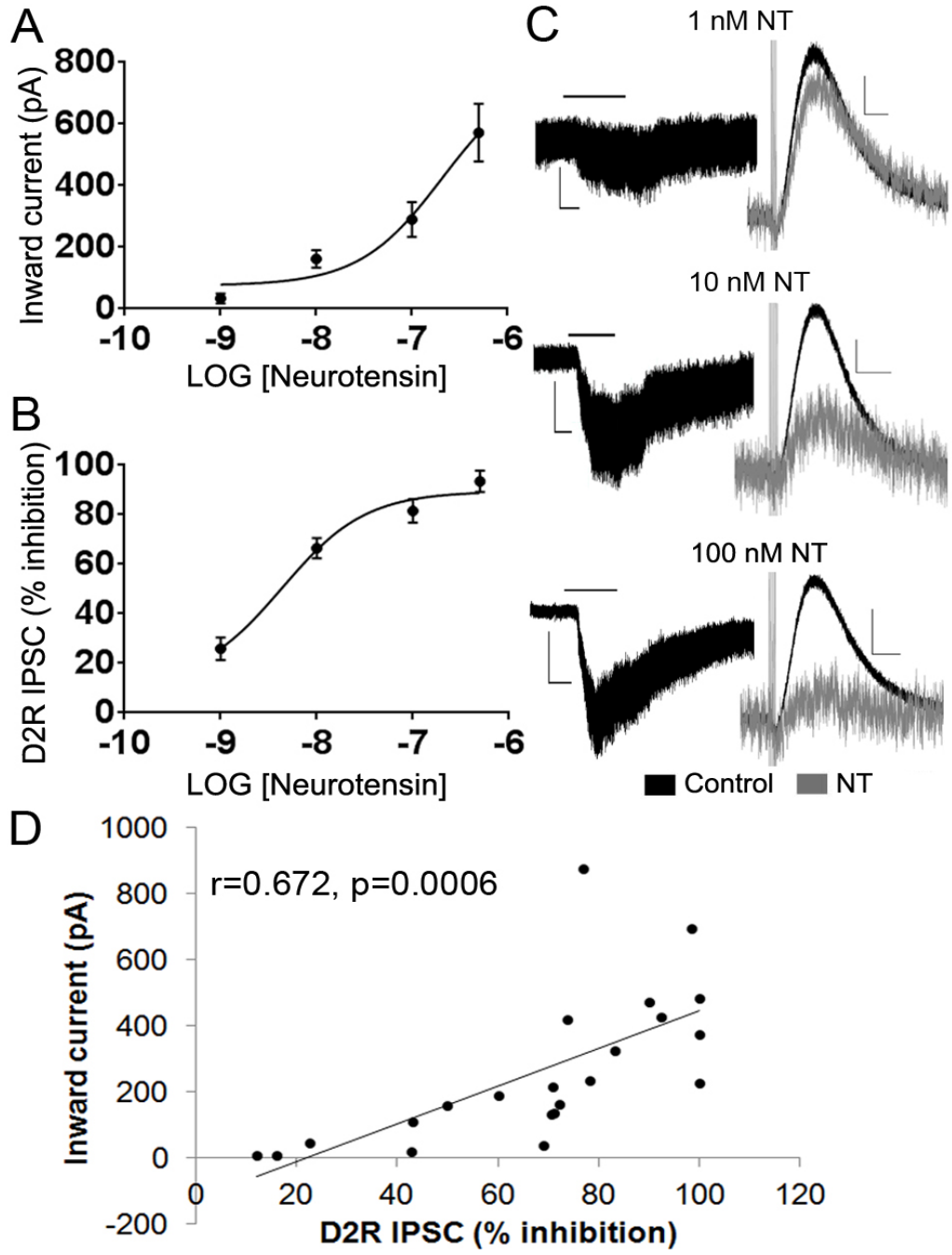


Figure 3.1: Neurotensin dose-dependently activates an inward current and inhibits the D2R IPSC in VTA DA neurons.

A. Dose-response curve of the inward current induced by neurotensin. B. Dose-response curve of the inhibitory effect of neurotensin on the D2R IPSC. C. Sample traces of the neurotensin

(NT) induced current (left) and inhibition of the D2R IPSC (right). D. The size of the inward current is positively correlated to the amount of inhibition of the D2R IPSC. Bars in C indicate time of neurotensin application. n=5-7 cells from 4-7 mice for each dose. Scale Bars: 50 pA/2 min (1 nM NT current); 100 pA/2 min (10 nM NT current); 200 pA/2 min (100 nM NT current); 20 pA/500 ms (NT inhibition of D2R IPSC).

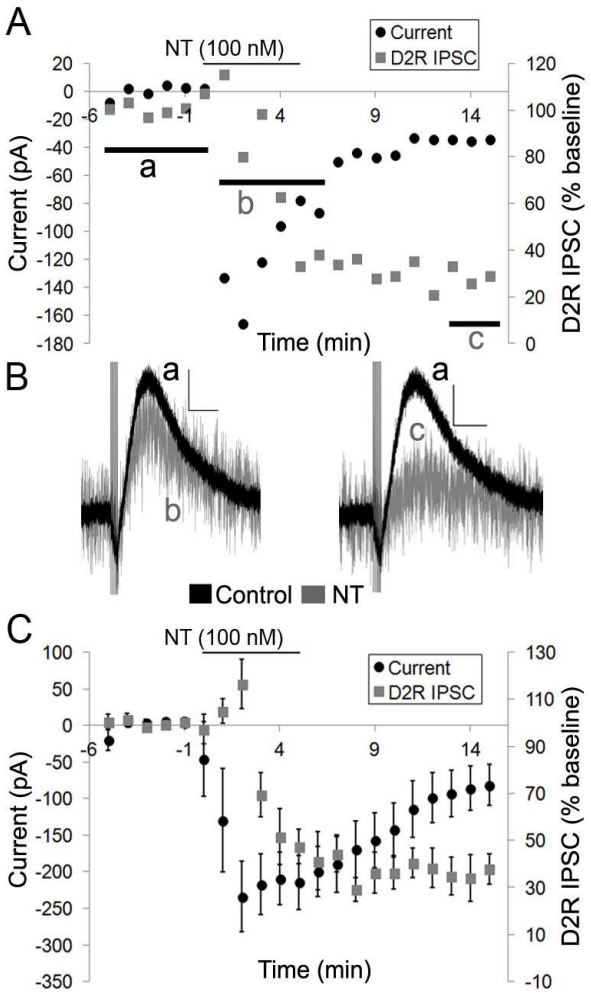


Figure 3.2: The neurotensin-induced inward current preceeds the inhibition of the D2R IPSC. A. Sample cell of the effects of neurotensin (100 nM) on the inward current and the D2R IPSC. B. Sample traces of the D2R IPSCs in A before neurotensin (a; black trace), during the peak of

the neurotensin inward current (b; grey trace), and at 8-10 minutes after neurotensin washout (c; grey trace). C. Mean responses of the effects of neurotensin (100 nM) on the inward current and the D2R IPSC. Bars in A and C indicate time of neurotensin application. n=6 cells from 6 mice. Scale Bars: 20 pA/500 ms

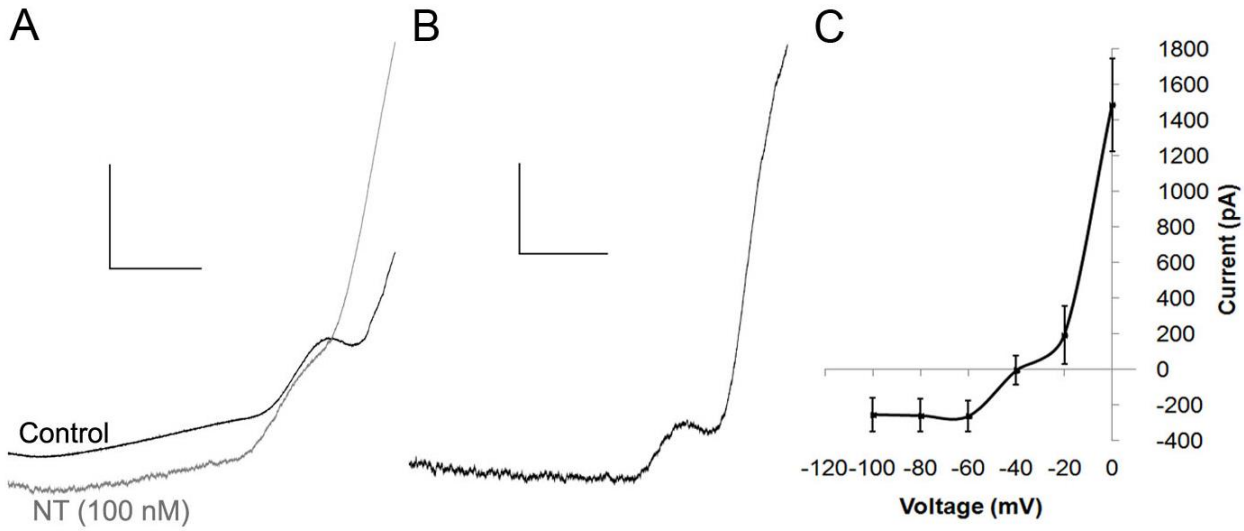


Figure 3.3: Current -Voltage relationship of the neurotensin-induced current in VTA DA neurons.

A. Sample current traces resulting from slow voltage ramps (-120 mV to +20 mV at 140 mV s^{-1}) before (black trace) and after neurotensin (100 nM; grey trace). B. Sample trace of the net neurotensin (100 nM) induced current. C. Mean current-voltage relationship of the neurotensin (100 nM) induced current. n=7 neurons from 4 mice. Scale Bars: 1 nA/200 ms

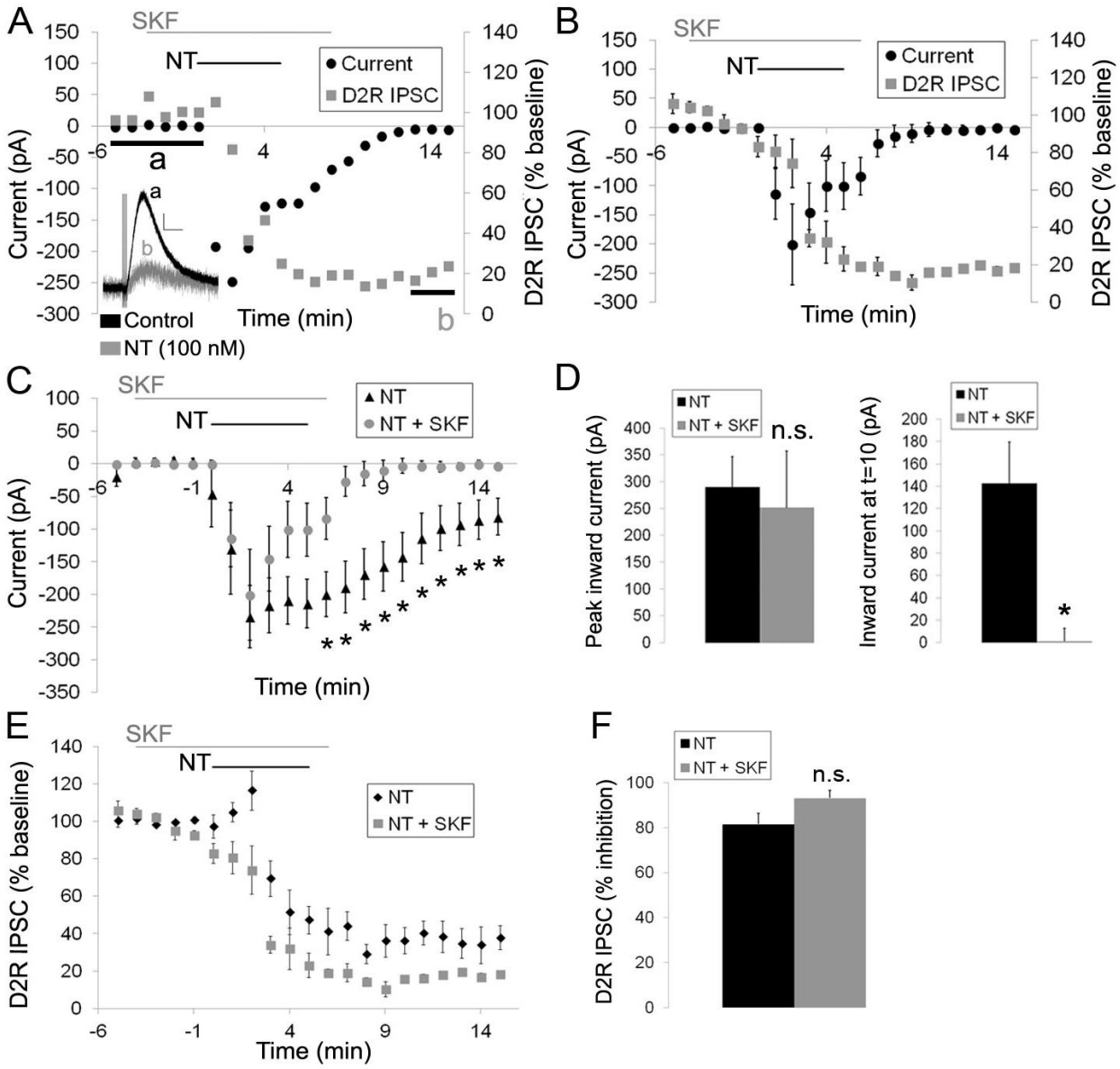


Figure 3.4: The TrpC channel inhibitor, SKF 96365, partially blocks the neurotensin-induced inward current but does not affect the inhibition of the D2R IPSC.

A. Sample cell of the effects of neurotensin (100 nM) on the inward current and the D2R IPSC in the presence of SKF 96365 (100 μM). Inset: sample trace of the D2R IPSC before neurotensin (a; black trace) and 8-10 minutes after neurotensin washout (b; grey trace) in the presence of SKF 96365. B. Mean responses of the effects of neurotensin (100 nM) on the inward current and the D2R IPSC in the presence of SKF 96365 (100 μM). C. Mean inward

current generated by neurotensin (100 nM) in the absence and presence of SKF 96365 (100 μM). D. Mean neurotensin-induced current at the peak and 5 minutes after neurotensin washout in the presence and absence of SKF 96365. E-F. Mean effect of neurotensin (100 nM) on the D2R IPSC in the absence and presence of SKF 96365 (100 μM). Bars in A-C and E indicate time of neurotensin and SKF 96365 application. n=5-6 neurons from 4-6 mice for each group. Scale bar: 20 pA/500 ms. *p<0.05 vs. neurotensin alone

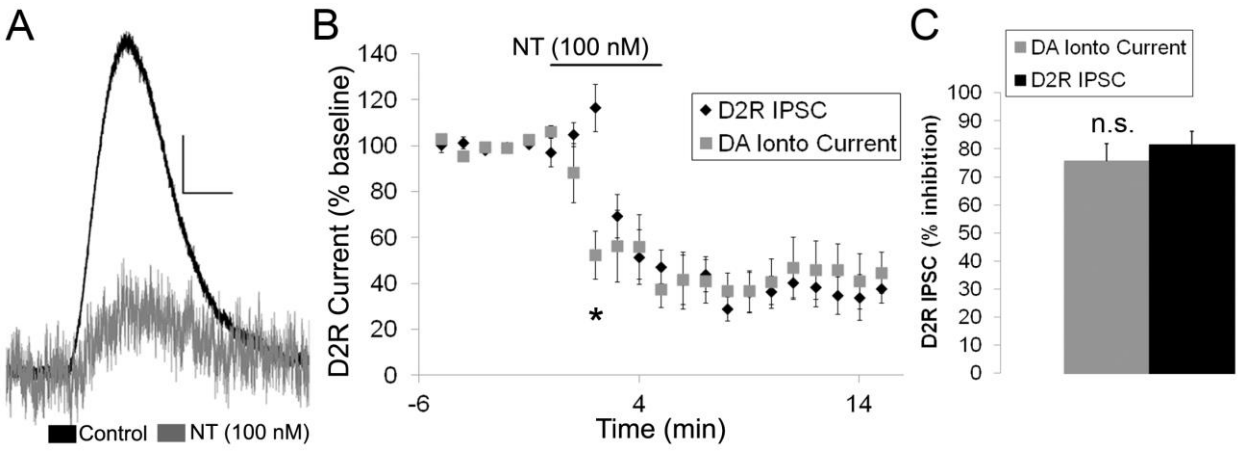


Figure 3.5: Neurotensin inhibits the D2R-mediated current produced by DA iontophoresis. A. Sample trace of the effect of neurotensin (100 nM) on the D2R-mediated current produced by DA iontophoresis. B-C. Mean effect of neurotensin (100 nM) on the D2R-mediated current produced by DA iontophoresis and on the D2R IPSC evoked with electrical stimulation. Bar in B indicates time of neurotensin application. n=6 neurons from 5-6 mice for each group. Scale Bar: 20 pA/500 ms. *p<0.05

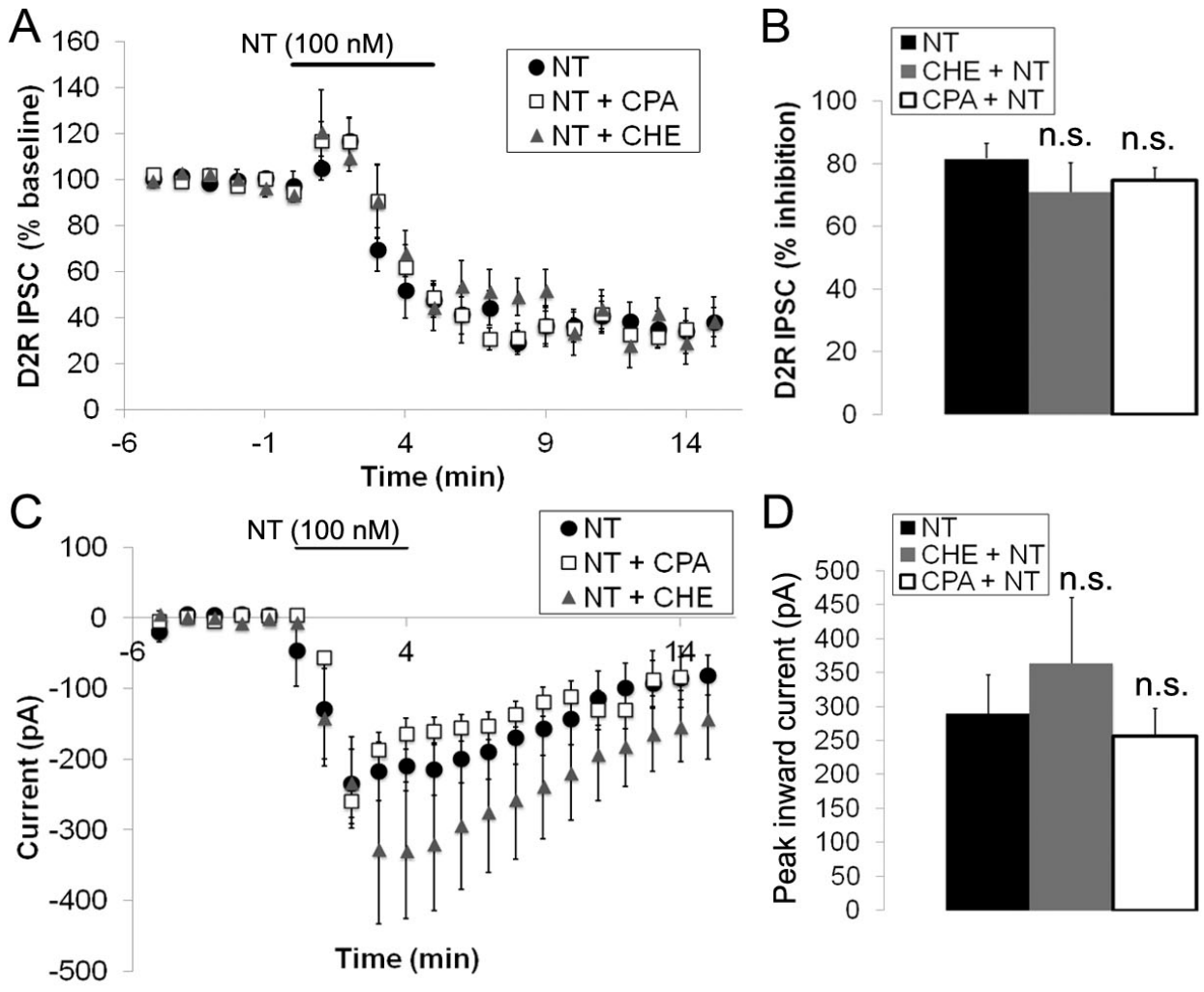


Figure 3.6: Neurotensin inhibition of the D2R IPSC and the neurotensin-induced current are not dependent on PKC activity or release of Ca^{2+} from intracellular stores.

A-B. Mean inhibition of the D2R IPSC caused by neurotensin (100 nM) in the presence and absence of CPA (10 μM) or chelerythrine (CHE) (10 μM). C-D. Mean neurotensin (100 nM) induced current in the presence and absence of CPA (10 μM) or CHE (10 μM). Bars in A and C indicate time of neurotensin application. n=5-6 cells, from 4-6 mice for each group.

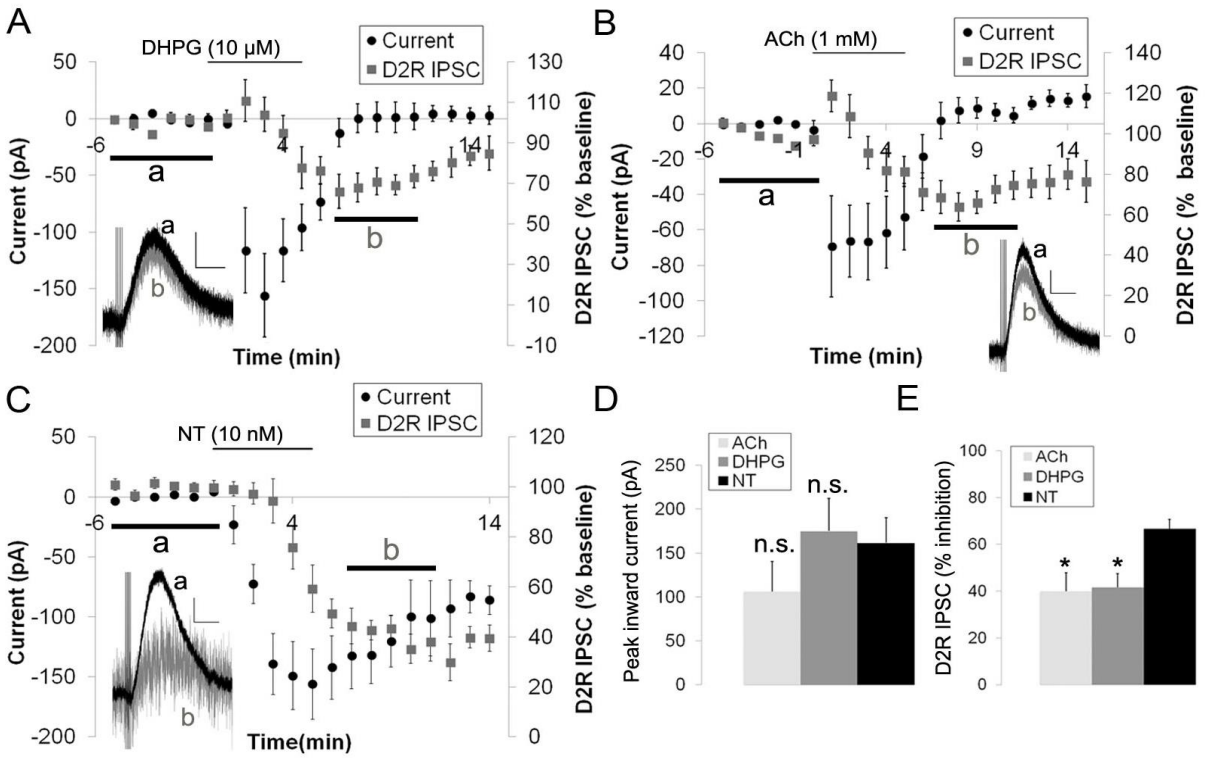


Figure 3.7: Neurotensin inhibits the D2R IPSC significantly more than DHPG or acetylcholine.

A. Mean inward current and inhibition of the D2R IPSC caused by DHPG (10 μM). Inset: Sample trace of the D2R IPSC before DHPG (a; black trace) and at 7-11 minutes after applying DHPG (b; grey trace). B. Mean inward current and inhibition of the D2R IPSC caused by ACh (1 mM) in the presence of mecamylamine (30 μM). Inset: Sample trace of the D2R IPSC before ACh (a; black trace) and at 7-11 minutes after applying ACh (b; grey trace). C. Mean inward current and inhibition of the D2R IPSC caused by neurotensin (10 nM). Inset: Sample trace of the D2R IPSC before neurotensin (a; black trace) and at 7-11 minutes after applying neurotensin (b; grey trace). D. The peak inward currents caused by DHPG (10 μM), ACh (1 mM), and neurotensin (10 nM) were not significantly different. E. Neurotensin (10 nM) inhibited the D2R IPSC significantly more than DHPG and ACh. Bars in A-C indicate time of drug application.

DHPG: n=5 cells from 4 mice; ACh: n=8 cells from 7 mice; neurotensin: n=6 cells from 5 mice. Scale bars: 30 pA/500 ms. *p<0.05 vs. neurotensin

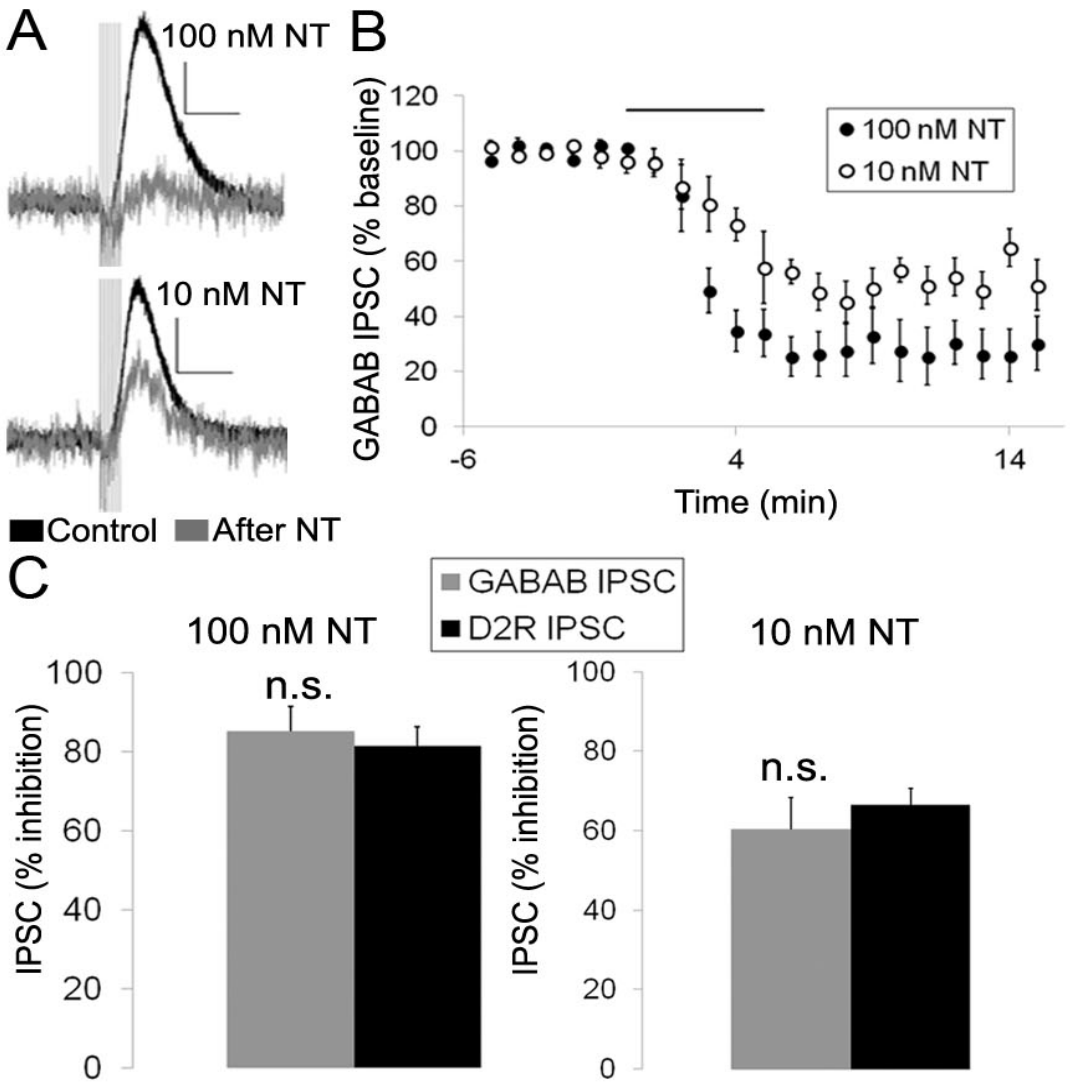


Figure 3.8: Neurotensin inhibits the $GABA_B$ IPSC in VTA DA neurons.

A-B. Sample traces (A) and mean effect (B) of neurotensin (100 nM & 10 nM; grey trace) on the $GABA_B$ IPSC. C. Neurotensin (100 nM & 10 nM) inhibited the $GABA_B$ and D2R IPSCs by the same magnitude. Bar in B indicates time of NT application. n=5-8 cells from 4-6 mice for each group. Scale Bars: 20 pA/300 ms.

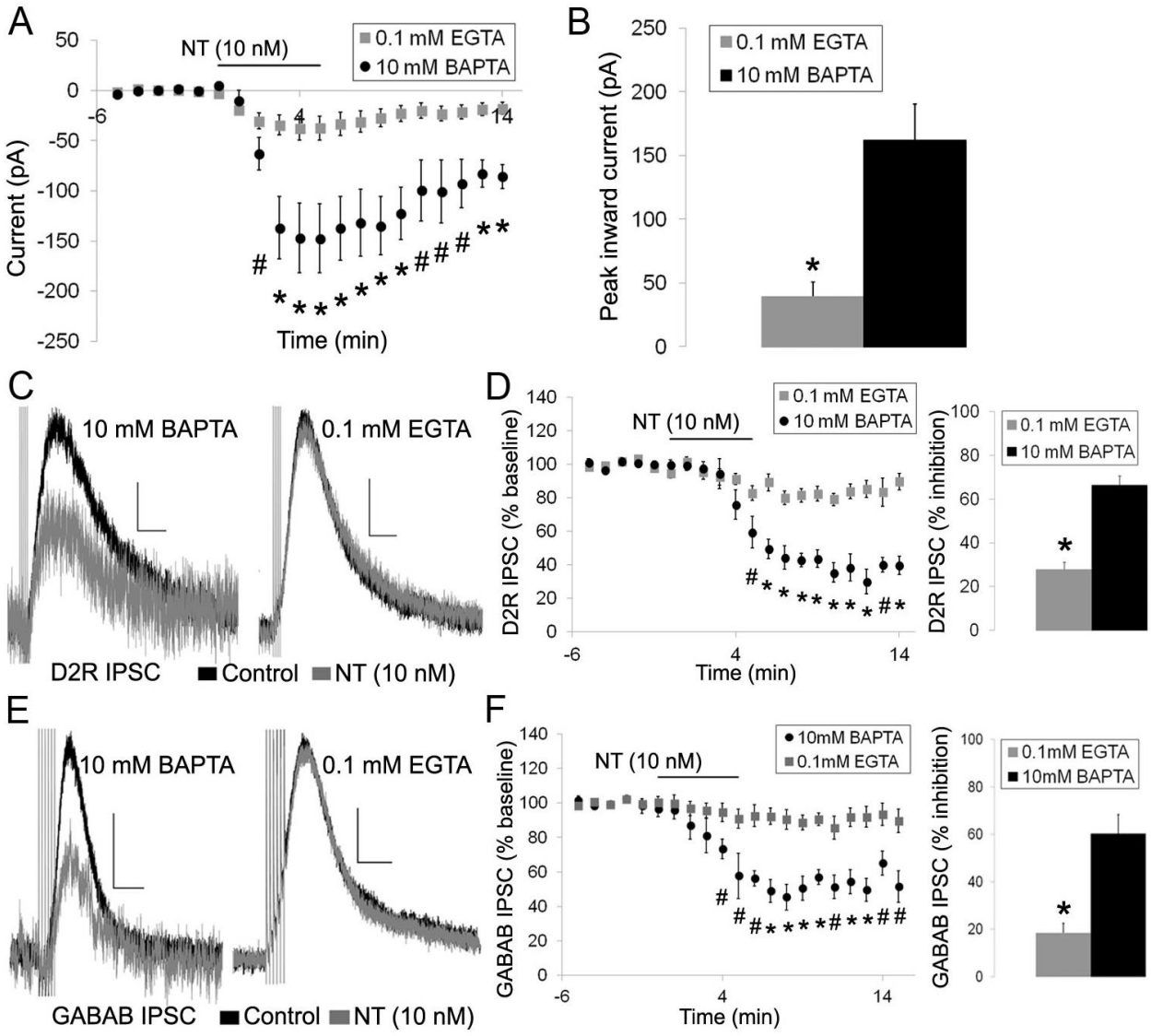


Figure 3.9: Reduced buffering of intracellular calcium attenuates the effects of neurotensin. A-B. Mean neurotensin (10 nM) induced inward current with an internal solution containing 10 mM BAPTA or 0.1 mM EGTA. C-F. Sample traces (C,E) and mean effect (D,F) of neurotensin (10 nM; grey trace) on the D2R IPSC (C-D) and $GABA_B$ IPSC (E-F) using internal solutions containing 10 mM BAPTA or 0.1 mM EGTA. Bars in A, D, & F indicate time of neurotensin application. n=5-12 cells from 4-11 mice for each group. Scale Bars: 20 pA/400 ms (C); 20 pA/200 ms (D). #$p<0.05$, *$p\leq0.001$

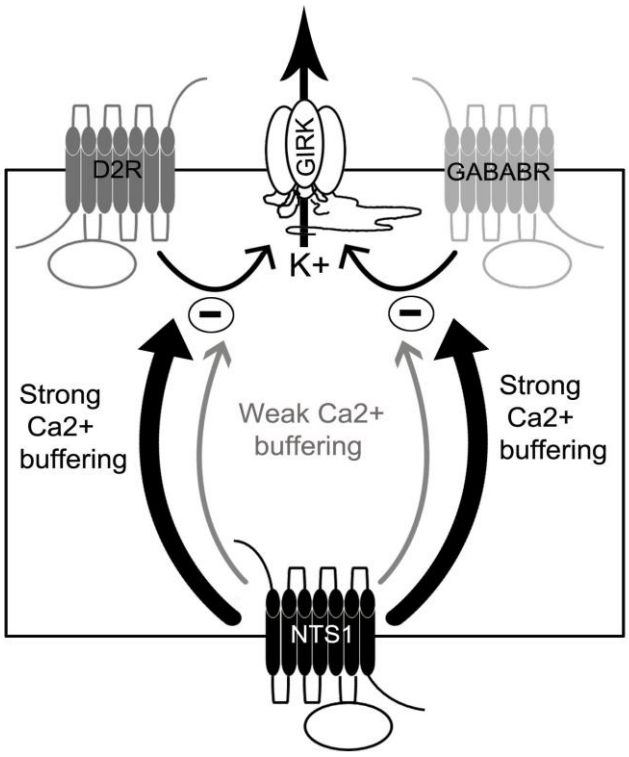


Figure 3.10: Diagram of the proposed model of neurotensin inhibition of GIRK currents activated by D2 and $GABA_B$ receptors.

Activation of NTS1 with neurotensin in VTA DA neurons causes robust inhibition of D2R and $GABA_B$ GIRK currents when the relative levels of free intracellular Ca^{2+} are low due to strong Ca^{2+} buffering, while neurotensin induced inhibition of D2R and GABAB GIRK currents is significantly attenuated when relative levels of free intracellular Ca^{2+} are higher due to weak Ca^{2+} buffering.

Adapted from:
K.S. West, A.G. Roseberry (2017) Neuropeptide-Y alters VTA dopamine neuron activity through both pre- and postsynaptic mechanisms. *Journal of Neurophysiology*, **118**(1): 625-33

4 NEUROPEPTIDE-Y ALTERS VTA DOPAMINE NEURON ACTIVITY THROUGH BOTH PRE- AND POST-SYNAPTIC MECHANISMS

4.1 Abstract

The mesocorticolimbic dopamine system, the brain's reward system, regulates many different behaviors including food intake, food reward, and feeding related behaviors, and there is increasing evidence that hypothalamic feeding-related neuropeptides alter dopamine neuron activity to affect feeding. For example, neuropeptide-Y (NPY), a strong orexigenic hypothalamic neuropeptide, increases motivation for food when injected into the ventral tegmental area (VTA). How NPY affects the activity of VTA dopamine neurons to regulate feeding behavior is unknown, however. In these studies we have used whole cell patch-clamp electrophysiology in acute brain slices from mice to examine how NPY affects VTA dopamine neuron activity. NPY activated an outward current that exhibited characteristics of a G protein-coupled inwardly rectifying potassium (GIRK) channel current in approximately sixty percent of dopamine neurons tested. In addition to its direct effects on VTA dopamine neurons, NPY also decreased the amplitude and increased paired-pulse ratios of evoked excitatory post-synaptic currents (EPSCs) in a subset of dopamine neurons, suggesting that NPY decreases glutamatergic transmission through a pre-synaptic mechanism. Interestingly, NPY also strongly inhibited evoked inhibitory post-synaptic currents (IPSCs) onto dopamine neurons by a pre-synaptic mechanism. Overall these studies demonstrate that NPY utilizes multiple mechanisms to affect VTA dopamine neuron activity, and they provide an important advancement in our understanding of how NPY acts in the VTA to control feeding behavior.

4.2 Introduction

Over one third of the U.S. adult population is obese[9,267], putting these individuals at increased risk for numerous other deleterious conditions, including diabetes, cardiovascular disease, stroke, high blood pressure, and some forms of cancer[268]. As there are currently few effective treatments available to combat obesity[269], it is essential to understand how the brain controls feeding and weight gain in order to identify new targets that can be used to develop effective treatments for obesity and weight gain.

The mesocorticolimbic dopamine system is the primary neural circuit regulating reward-related and motivational behaviors, and this system plays an important role in controlling feeding and body weight, including the appetitive and consummatory aspects of feeding[12,15,163,164,270,271]. For example, dopamine deficient mice are aphagic and will starve to death by 4 weeks of age if they are not treated with L-DOPA, a dopamine precursor[133]. Food intake, food reward, and stimuli associated with food also cause phasic increases in dopamine release[136,137,272], and blocking dopamine receptors systemically or in the nucleus accumbens decreases operant responding for food in rats[132,273,274]. Impairments in the mesocorticolimbic dopamine system have also been associated with obesity and dysregulated feeding in humans. For example, dopamine agonists cause increased compulsive eating and weight gain in Parkinson's patients[141], and obese individuals show increased activity in mesocorticolimbic areas in response to pictures of palatable food but decreased responses to food consumption compared to lean individuals[144-147,275]. Overall we have an incomplete understanding of how the mesocorticolimbic dopamine system regulates feeding, however. This includes an incomplete understanding of how other brain systems and circuits interact with dopamine circuits to regulate feeding and body weight.

Neuropeptide-Y (NPY) is a strong orexigenic neuropeptide and an important regulator of energy homeostasis[276,277]. For example, central administration of NPY robustly increases food intake[212,213], activation of NPY expressing neurons in the arcuate nucleus of the hypothalamus increases feeding[211], and ablation of NPY neurons reduces food intake and body weight[214,215]. There is also evidence that NPY interacts with the mesocorticolimbic dopamine system to regulate feeding. NPY neurons project to the VTA[278], NPY receptors are expressed in the VTA[217,279,280], and intra-VTA and intra-nucleus accumbens injection of NPY increases operant responding for food in rats[174]. There is conflicting data on exactly how NPY acts in the VTA to affect feeding, however. Intra-cerebroventricular (icv) NPY has been shown to increase dopamine efflux in the nucleus accumbens suggesting that NPY may activate dopamine neurons[281-283], but a separate study has shown that NPY decreases the firing rate of VTA dopamine neurons in *ex vivo* brain slice preparations[217]. Thus, overall, it is unknown how NPY affects VTA dopamine neurons to regulate feeding. Therefore, in these studies we have used patch-clamp electrophysiology in acute brain slice preparations to test whether NPY inhibits VTA dopamine neurons through direct action on dopamine neurons or through the pre-synaptic regulation of their synaptic inputs.

4.3 Materials and Methods

Animals: Male and female mice (5-14 weeks old) on a C57Bl/6J or a mixed C57/129 background were used in all experiments. All protocols and procedures were approved by the Institutional Animal Care and Use Committee at Georgia State University, and conformed to the *NIH Guide for the Care and Use of Laboratory Animals*.

Slice preparation and Electrophysiology: Acute brain slices were prepared as previously described[234,235]. Briefly, adult mice were anesthetized with isofluorane and decapitated. The brain was then removed and placed in carbogen (95% O_2 and 5% CO_2) saturated ice-cold artificial cerebral spinal fluid (aCSF), containing (in mM) 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 11.1 glucose, and 21.4 $NaHCO_3$. A brain block containing the VTA was made, and pseudo-horizontal sections (220 μm) were cut with a vibrating blade microtome. Slices were then incubated in aCSF (~35°C) containing 10 μM MK-801 [(+)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] for 30-60 min before recording. Slices were placed in a recording chamber and perfused with carbogen-saturated aCSF at a flow rate of approximately 1-2 ml/min. Whole-cell recordings were made using an Axon multiclamp 700B microelectrode amplifier and Axograph software. Putative dopamine neurons were identified by their location relative to the medial terminal nucleus of the accessory optic tract, the presence of hyperpolarization-activated cation currents (H-current), and the presence of spontaneous pacemaker firing[237].

Electrodes (2.0-3.0 MΩ) were filled with a potassium gluconate (KGluconate) based internal solution containing (in mM) 128 KGluconate, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 2 ATP, 0.3 GTP, 10 creatine phosphate, and 10 BAPTA or 0.1 EGTA. The internal solution contained EGTA for the experiments examining the direct effect of NPY on dopamine neuron activity under reduced calcium buffering conditions and for the experiments examining the effect of NPY on excitatory post-synaptic currents (EPSC). The internal solution contained BAPTA for all other experiments, with the exception of the measurement of inhibitory post-synaptic currents (IPSC), where a potassium methylsulfate based internal solution containing a high concentration of Cl^- was used (in mM) 57 KCl, 70 $KMeSO_4$, 20 NaCl, 1.5 $MgCl_2$, 5 HEPES, 0.1

EGTA, 2 ATP, 0.3 GTP, and 10 creatine phosphate. Series resistance values were approximately 3-15 MΩ. If the series resistance increased by more than 20% the experiment was terminated or excluded from analysis. In addition, if the holding current changed by more than 10 pA during baseline recording or during the first minute of NPY application the experiment was terminated or excluded from analysis. Neurons were voltage clamped at -60 mV for most experiments. Corrections were not made for the liquid junction potential, which was calculated to be the following for each internal: KGluconate 10 mM BAPTA, 13.9 (normal aCSF), 13.6 (high K^+ external solution); KGluconate 0.1 mM EGTA, 14.8; K methylsulfate/KCL, 6. EPSCs/IPSCs were evoked using a bipolar stimulating electrode placed 100-300 μm from the recorded cell. The electrode was placed anterior to the recorded cell to evoke EPSCs and posterior to the recorded cell to evoke IPSCs. Pairs of PSCs were evoked with a 50 ms interpulse interval every 20 s. EPSCs were isolated by including picrotoxin (100 μM) in the perfusion solution, and IPSCs were isolated by including DNQX (10 μM) in the perfusion solution. For all experiments, cells were held for at least 10 minutes prior to drug application to allow for diffusion of the internal solution into the cell. To determine the current-voltage relationship and the reversal potential of the NPY current, cells were perfused with a high K^+ external solution containing TTX: (in mM) 118.5 NaCl, 10 KCl, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 11.1 glucose, 21.4 $NaHCO_3$, and 0.001 TTX. The cells were then held at -40 mV and slow voltage ramps were applied from -100 mV to 0 mV at 100 mV s^{-1} every 30 s.

Drugs: Neuropeptide-Y and BIBP3226 were purchased from Bachem (Torrance, CA). TTX was purchased from Tocris Biosciences (Minneapolis, MN). All other reagents were from common commercial sources.

Data Analysis and Statistics: Data are represented as the mean +/- SEM unless otherwise noted. For all PSC measurements, the effect of NPY on EPSCs/IPSCs was determined by comparing the average value of the PSCs measured 5 min before the onset of NPY to the average values 5 min after the onset of NPY treatment. The pair-pulse ratio was calculated by dividing the amplitude of the second PSC by the amplitude of the first PSC. The coefficient of variation was calculated by dividing the SD by the mean of the PSC amplitude. Data were analyzed using Axograph X (v1.3.5), LabChart (v7.3.6; ADInstruments), and Excel (v14.0; Microsoft Corporation) software. Statistics were calculated using SigmaStat (v11.0; Systat Software, Inc.). Data were initially tested for normality using the Shapiro-Wilk test and were then analyzed with Student's t-tests, Wilcoxon signed-rank tests, or a two-way ANOVA with Holm-Sidek post-hoc tests as appropriate with a significance level of $p<0.05$ set *a priori*. For the experiments comparing the responses to NPY and baclofen with the BAPTA and EGTA internal solutions (Fig. 4.4), the data was log transformed to achieve normality prior to running a two-way ANOVA.

4.4 Results

There is conflicting data on whether NPY increases or decreases VTA dopamine neuron activity[217,281-283]. Thus, we used patch-clamp electrophysiology in acute brain slice preparations to test whether NPY directly regulates VTA dopamine neuron activity. NPY activated an outward current in approximately 58% of VTA dopamine neurons tested (Fig. 4.1A-C; 37 out of 64 neurons total; 10 nM= 6 of 12; 100 nM= 27 of 42; 300 nM 4 of 10). The NPY activated current was concentration-dependent (Fig. 4.1A-C) and was accompanied by a significant decrease in membrane resistance (Fig. 4.1D-E), suggesting that NPY directly activates an ionic

conductance in VTA dopamine neurons. Thus, it appears that NPY directly inhibits VTA dopamine neurons. The 100 nM and 300 nM concentrations of NPY were used in all subsequent experiments, as both appeared to be saturating concentrations.

We next sought to identify the NPY receptor mediating the NPY-induced current in VTA dopamine neurons. Previous studies have reported that the post-synaptic effects of NPY are mediated by NPY acting on Y1 and Y2 receptors[284-289]. We initially tested whether Y1 receptors (Y1R) mediated this effect using the Y1R antagonist, BIBP3226[284-289]. BIBP3226 (1 μM) reversed the NPY-induced current when it was applied at the peak of the NPY current (Fig. 4.2A-C, n=4, note the rate of reversal of the NPY-induced current with BIBP3226 compared to NPY alone in Fig. 4.1A-B). In addition, pretreatment with BIBP3226 (1 μM) completely prevented the NPY induced current in all cells tested (Fig. 4.2D-E, n=8). Thus NPY appears to directly inhibit VTA dopamine neurons by activating Y1Rs.

We next sought to determine the identity of the channel mediating the NPY activated current in VTA dopamine neurons. We tested the current-voltage relationship of the NPY current by applying slow voltage ramps (-100 mV to 0 mV 100 mV s^{-1}) in a high K^+ (10 mM) external solution containing TTX (1 μM). The current obtained from these slow voltage ramps exhibited inward rectification and had a reversal potential near that of the reversal potential for potassium ions under these conditions (Fig. 4.3A-C; E_K= -68 mV; NPY E_{rev}= -59.6 mV +/- 8.8 mV). These results indicated that NPY induced a potassium current in VTA dopamine neurons that is likely mediated by activation of GIRK channels. We then tested whether extracellular barium (1 mM) could inhibit the NPY induced current (Fig. 4.3A, D-E). Barium is a known blocker of inwardly rectifying potassium channels, including GIRK channels[290,291], and it has been shown to block NPY-induced GIRK currents in many different CNS neurons[284-289].

Extracellular barium reversed and blocked the NPY current along with a basal leak current in VTA dopamine neurons (Fig. 4.3A, D-E). Thus, it appears that NPY activated GIRK channels in VTA dopamine neurons.

We next tested whether the NPY activated current in VTA dopamine neurons was sensitive to intracellular calcium levels, because previous studies have shown that GIRK currents are smaller when intracellular calcium buffering is reduced in VTA dopamine neurons[126,260]. As a positive control, we also tested whether GIRK currents activated by the $GABA_B$ receptor agonist, baclofen (1 μM), were dependent on the strength of intracellular calcium buffering. The NPY-induced currents were significantly smaller than the baclofen-induced currents (Fig. 4.4), and, as expected, both NPY (100 nM) and baclofen (1 μM) currents were significantly smaller with reduced intracellular calcium buffering (0.1 mM EGTA) compared to strong calcium buffering (10 mM BAPTA) (Fig. 4.4; significant main effects of drug ($F(1, 23)=7.807$, $p=0.010$) and calcium buffering ($F(1, 23)=19.165$, $p<0.001$)), demonstrating that intracellular calcium regulates $GABA_B$- and NPY-induced currents in a similar manner. Thus, these results further suggest that NPY activates a GIRK channel current in VTA dopamine neurons and demonstrate that this current is sensitive to intracellular calcium levels.

In addition to directly inhibiting VTA dopamine neurons, it is possible that NPY could regulate the activity of dopamine neurons indirectly through modulation of their afferent inputs. Glutamatergic and GABAergic afferent inputs are important regulators of dopamine neuron activity[58,66,69], and NPY has been shown to affect glutamatergic and GABAergic transmission in other areas of the CNS[284,285,289,292]. Thus, we next examined whether NPY altered glutamatergic inputs to dopamine neurons. NPY decreased the amplitude of evoked EPSCs in 7 of the 10

VTA dopamine neurons tested (Fig. 4.5A-E, range of effect = 68% - 91% of baseline). In order to examine the mechanism by which NPY decreased EPSCs, we assessed whether there were changes to the paired-pulse ratio (PPR) and coefficient of variation (CV) of the EPSCs after treatment with NPY. PPR and CV are measures used to determine whether a change in synaptic strength is due to a pre-synaptic or post-synaptic modification, and PPR and CV values have been shown to significantly increase when the probability of pre-synaptic neurotransmitter release is decreased but do not change when the amplitude of PSCs are affected by a post-synaptic modification[293,294]. NPY (100 nM) significantly increased both the PPR and CV of the EPSCs inhibited by NPY (Fig. 4.5D,F,H,J; n=7 of 10) without affecting the PPR or CV of the EPSCs whose amplitude was not affected by NPY (Fig. 4.5E,G,I,K; n=3 of 10). Thus, it appears that NPY decreased glutamatergic transmission onto a subset of VTA dopamine neurons through an inhibition of presynaptic release. We next examined whether the NPY-induced current and the inhibition of EPSCs were related effects by assessing whether NPY activated GIRK currents and inhibited EPSCs in the same neurons or in distinct populations of VTA dopamine neurons. NPY inhibited EPSCs in both dopamine neurons that showed an NPY-induced outward current (n=3 of 10) and in neurons that did not directly respond to NPY (n=4 of 10), and the magnitude of the inhibition of the EPSCs was similar for both sets of neurons (Fig. 4.5L-M). These results suggest that NPY inhibited EPSCs independent of the NPY induced GIRK current, and that NPY inhibits EPSCs and activates inhibitory GIRK currents in both distinct and overlapping sets of VTA dopamine neurons.

NPY has also been reported to inhibit VTA GABA neurons[217], which provide important inhibitory input to VTA dopamine neurons[21,58,66,69]. Therefore, we next tested whether NPY also altered GABAergic inputs to VTA dopamine neurons. NPY strongly inhibited evoked

IPSCs in 4 out of 6 dopamine neurons tested (Fig. 4.6A-C; range of effect = 30% - 85% of baseline), and increased the PPR and CV of the affected IPSCs (Fig. 4.6C-F; n=4 of 6), although the CV did not reach statistical significance. NPY did not decrease evoked IPSCs in 2 out of 6 dopamine neurons tested but did activate GIRK currents in both of these neurons, suggesting that, as with NPY's effect on EPSCs, the inhibition of the IPSCs is not related to the NPY-induced GIRK current. Thus, NPY appears to also decrease GABAergic transmission onto a subset of VTA dopamine neurons through an inhibition of presynaptic release.

4.5 Discussion

In these studies we have used patch-clamp electrophysiology in acute brain slice preparations to determine how NPY alters VTA dopamine neuron activity to affect feeding. NPY inhibited a subset of dopamine neurons through two mechanisms: NPY directly inhibited dopamine neurons through Y1R mediated activation of GIRK channels, and NPY indirectly inhibited dopamine neurons by decreasing glutamatergic transmission onto dopamine neurons. Interestingly, NPY also decreased GABAergic transmission onto a subset of dopamine neurons, indicating that NPY could cause excitation of some VTA dopamine neurons.

A previous study found that NPY decreases the firing rate of a subset of VTA dopamine neurons in *ex vivo* brain slices from rats[217], but the mechanism of this NPY caused inhibition of dopamine neurons was unknown. NPY mediates its effects through five known receptors, Y1, Y2, Y4, Y5, and Y6[295,296]. All of the NPY receptors are G protein-coupled receptors that signal through $G_{i/o}$ G-proteins[295,296], and NPY causes a GIRK channel current in neurons located in different areas of the CNS[284-289]. Thus, we hypothesized that NPY inhibited dopamine neurons through a similar mechanism. Indeed, the results presented here support the hypothesis that

NPY activates GIRK channels in VTA dopamine neurons as NPY caused a concentration dependent outward current that was accompanied by a decrease in membrane resistance, reversed at the reversal potential for K^+ ions, exhibited inward rectification, and was sensitive to extracellular barium, which is similar to what has been reported in numerous other brain regions[284-289]. Thus, we can conclude that NPY activates Y1Rs that in turn release activated $G_{i/o}$-proteins to open GIRK channels.

The NPY-induced current was also sensitive to intracellular calcium levels, which is an interesting characteristic of GIRK currents in VTA dopamine neurons[126,260]. For example, GIRK currents activated by $GABA_B$ and dopamine D2 receptor agonists are reported to be smaller when intracellular calcium buffering is reduced and calcium levels are high in VTA dopamine neurons[126,260]. We found that, like the baclofen-induced currents, the NPY-induced currents were significantly smaller under reduced calcium buffering conditions. One potential caveat in these experiments is that the NPY currents could have affected the amplitude of the subsequent baclofen currents through heterologous desensitization, although this would not affect the interpretation of these results, as we would expect this to be true for both low and high calcium buffering. Thus, taken together, our findings indicate that NPY directly inhibits VTA dopamine neurons by activating a GIRK current that is sensitive to intracellular calcium levels.

In addition to the direct effects of NPY on VTA dopamine neurons, we also examined whether NPY indirectly affected dopamine neuron activity through modulation of their glutamatergic and GABAergic afferent inputs, which play an important role in controlling dopamine neuron activity[58,66,69]. Glutamatergic afferents primarily control dopamine neuron burst firing, and GABAergic afferents strongly inhibit dopamine neurons, demonstrating that

these afferent inputs are important regulators of dopamine neuron activity[58,66,69]. Surprisingly, NPY decreased both excitatory glutamatergic and inhibitory GABAergic transmission onto VTA dopamine neurons, although not to the same extent (Fig. 4.5-6). NPY decreased both glutamatergic and GABAergic transmission through a decrease in pre-synaptic release, which is similar to what has been reported in other areas of the CNS[284,285,289]. Thus, NPY modulates VTA dopamine neuron activity through two different pre-synaptic mechanisms.

The net effect of NPY on the overall activity of VTA dopamine neurons is unclear, because the responses observed here would result in both activation and inhibition of dopamine neurons. The inhibitory effects of NPY on VTA dopamine neurons were relatively small (a small (~ 50 pA) direct inhibition, and a modest ~ 18% decrease in EPSCs), whereas the excitatory effect of NPY was more robust (~ 44% decrease in IPSCs), suggesting that NPY could have a net excitatory effect on VTA dopamine neuron activity. This possibility is supported by previous studies suggesting that NPY excites VTA dopamine neurons[281-283]. For example, centrally delivered NPY increases dopamine release at VTA dopamine efferent sites[281-283] and increases dopamine associated behaviors[159,174,297-299] suggesting that NPY increases the activity of dopamine neurons to stimulate dopamine release. In contrast, Korotkova et al. have shown that NPY inhibits firing of VTA dopamine neurons in *ex vivo* slice preparations[217], indicating that NPY inhibits dopamine neurons, which is supported by our studies showing that NPY activates an outward GIRK current and inhibits EPSCs in dopamine neurons. We attempted to examine the net effect of NPY on dopamine neuron activity by testing the effect of NPY on the firing rate of VTA dopamine neurons in the cell-attached configuration in the presence and absence of inhibitors of synaptic transmission (DNQX and picrotoxin). Due to the small effects of NPY on dopamine neuron firing rate in these

experiments, we could not conclusively determine whether NPY had an excitatory, inhibitory, or no effect on the activity of all the dopamine neurons tested, however. Thus, it is still unclear whether the net effect of NPY on dopamine neuron activity *in vivo* would be excitatory or inhibitory.

One possible explanation for NPY causing both excitatory and inhibitory effects on dopamine neuron activity is that NPY could differentially modulate separate subpopulations of VTA dopamine neurons through distinct mechanisms. NPY only affected a subset of VTA dopamine neurons for each of the responses measured (direct current, EPSCs, IPSCs). Thus, NPY could excite one subpopulation of dopamine neurons and inhibit another distinct subpopulation of dopamine neurons. Historically, dopamine neurons have been thought of as a uniform population of neurons, but recent research has demonstrated that there are subpopulations of VTA dopamine neurons that project to different efferent target regions and show distinct electrophysiological and molecular properties[16,17,36,300]. In addition, aversive stimuli and rewards have also been shown to excite distinct subpopulations of dopamine neurons[16,17,36,300]. Thus, NPY could excite a specific subpopulation of dopamine neurons while inhibiting a distinct subset of neurons to differentially regulate distinct aspects of behavior (e.g. reward vs. aversion). For example, dopamine neurons encoding reward and reinforcement project to the nucleus accumbens while dopamine neurons encoding aversion project to the pre-frontal cortex[301]. Thus, it is possible that NPY could excite dopamine neurons projecting to the nucleus accumbens to promote food reward while inhibiting dopamine neurons projecting to the pre-frontal cortex to decrease aversion. This possibility is supported by the overall effects of NPY on food-motivated behavior, as injection of NPY either icv or into the VTA increases operant responding for sucrose and food pellets in rats[159,174,297], and this response is associated

with increased dopamine release in the nucleus accumbens[132,302]. Further experiments will be required to identify the net effect of NPY on overall dopamine neuron activity and dopamine output, and to determine whether NPY is activating and inhibiting distinct subpopulations of VTA dopamine neurons to promote food-seeking behaviors.

In summary, we have demonstrated that NPY modulates subsets of VTA dopamine neurons through three independent mechanisms, including both pre-synaptic and post-synaptic mechanisms. NPY directly inhibited VTA dopamine neurons through activation of a post-synaptic GIRK channel current, and indirectly inhibited VTA dopamine neurons through a pre-synaptic reduction in glutamate release. NPY also decreased GABAergic transmission onto dopamine neurons through a pre-synaptic reduction in GABA release. These results advance our understanding of how VTA dopamine neuron activity is regulated and provide further understanding of how NPY interacts with the mesocorticolimbic dopamine system to regulate feeding behavior.

4.6 Figures

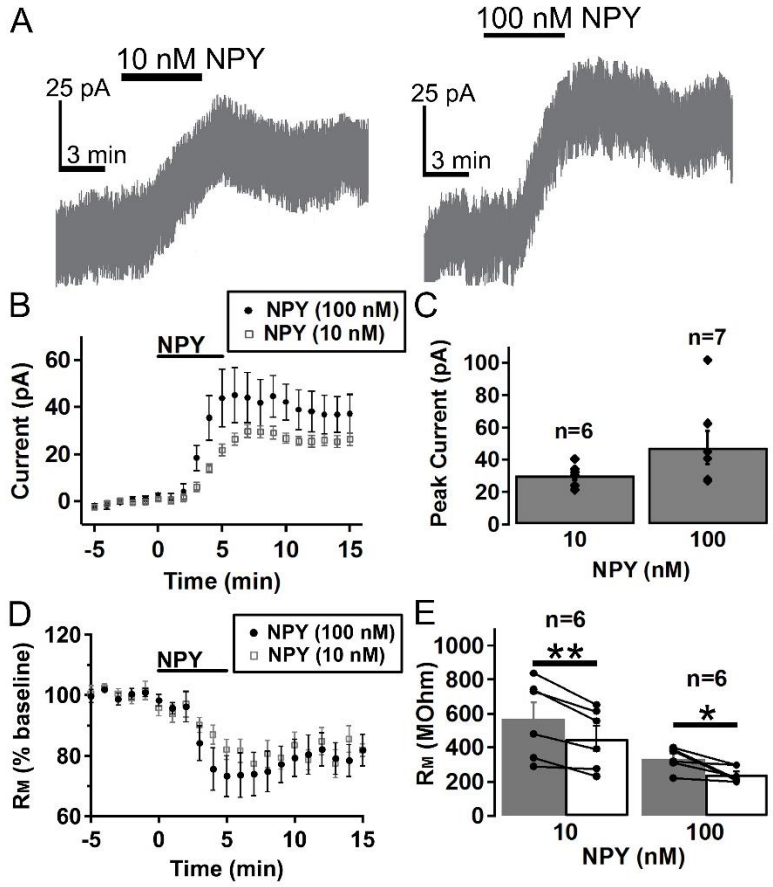


Figure 4.1: NPY concentration-dependently activated an outward current and reduced membrane resistance (R_M) in a subset of VTA dopamine neurons.

A-C. Sample traces (A), mean effect (B), and mean peak amplitude (C) of the NPY activated current at different concentrations. D-E. Mean effect of NPY on R_M (D) and mean R_M before and after NPY application (D) at different concentrations. Bars in A-B & D indicate time of NPY application. n=6-7 cells from 5-6 mice for each group. Scale Bars: 25 pA/3 min. *$p<0.05$ **$p<0.01$

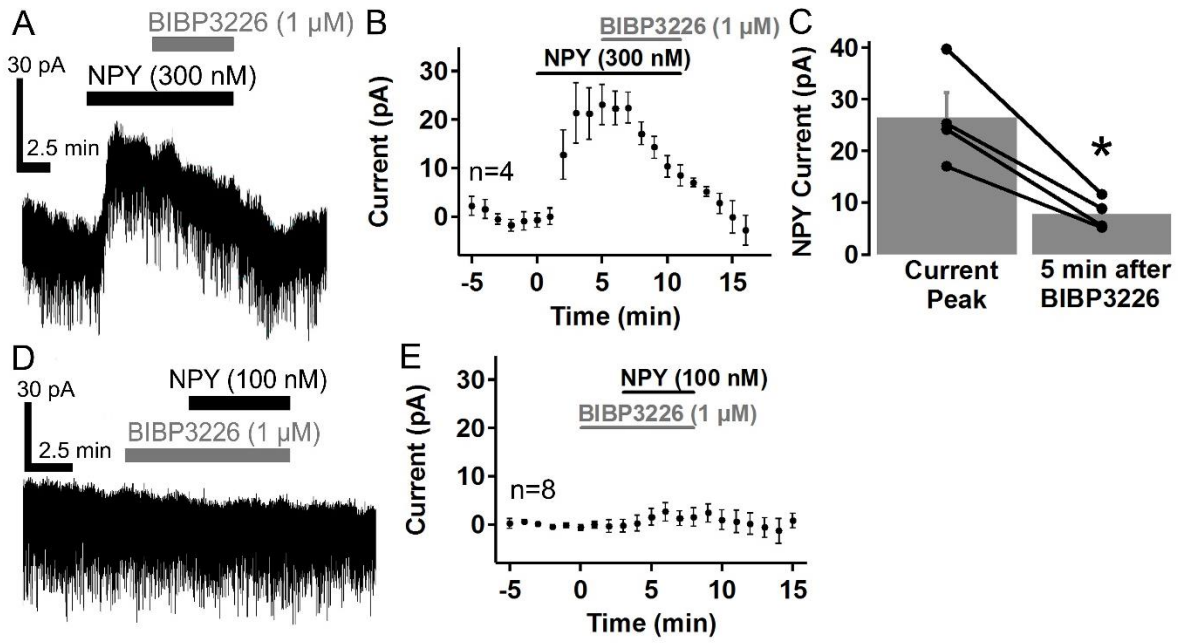


Figure 4.2: The NPY-induced current was mediated by NPY Y1Rs in VTA dopamine neurons. A-C. The Y1R antagonist, BIBP3226 (1 μM), reversed the NPY (300 nM) induced current. Sample trace (A) and mean response (B; n=4) of the NPY current before and during BIBP3326 application, and mean NPY current amplitude before and after BIBP3226 application (C; n=4). D-E. Sample trace (D) and mean response (E; n=8) of VTA dopamine neurons pretreated with BIBP3226 (1 μM) to NPY (100 nM). Bars in A-B & D-E indicate time of NPY and BIBP3226 application. Scale bars: 30 pA/2.5 min. *$p<0.05$

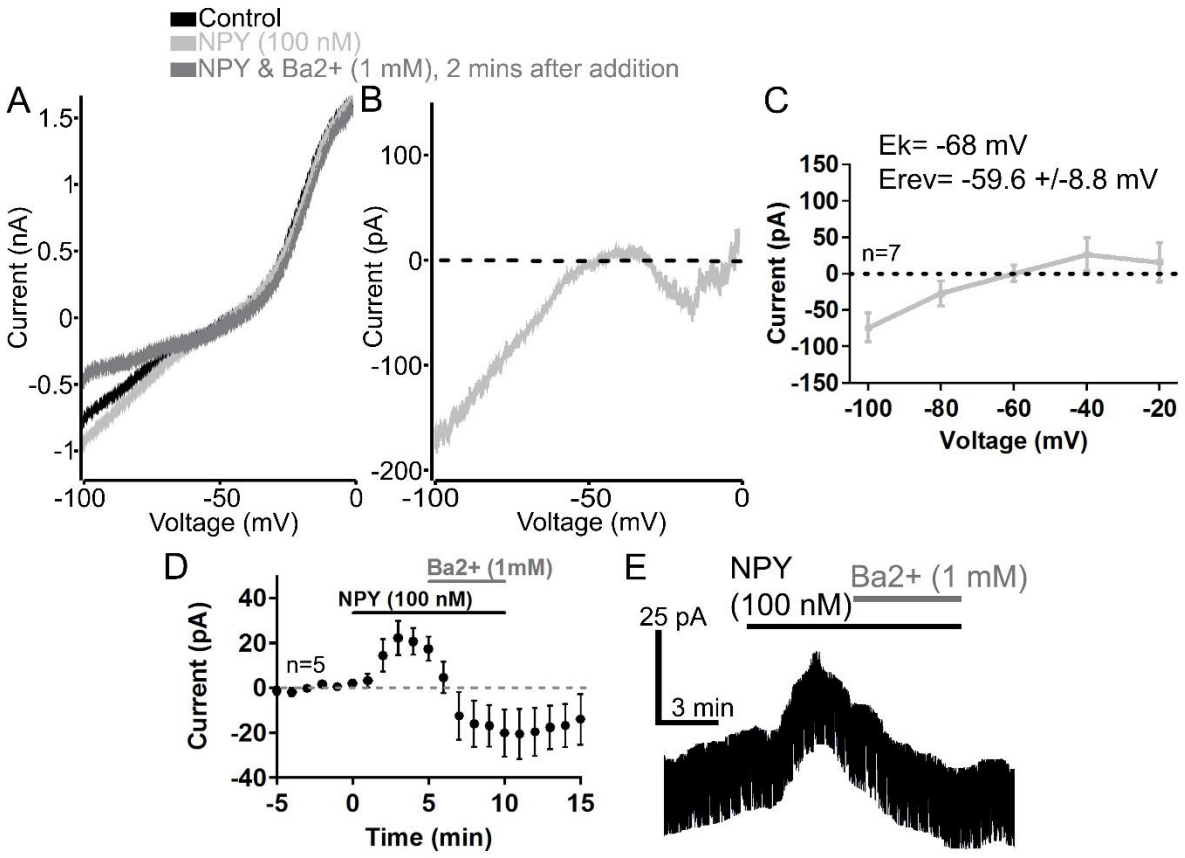


Figure 4.3: The NPY-induced current in VTA dopamine neurons exhibited characteristics of a G protein-coupled inwardly rectifying K^+ (GIRK) channel.

A. Sample current traces resulting from slow voltage ramps (-100 mV to 0 mV 100 mV s^{-1}) before (black trace) and after NPY (100 nM; light grey trace) application, and 2 min after the addition of Ba^{2+} (1 mM; NPY + Ba^{2+}, dark grey trace) using a high K^+ (10 mM) external solution containing TTX (1 μM). B. Sample trace of the net NPY (100 nM) induced current. C. Mean current-voltage relationship of the NPY (100 nM) induced current. D-E. Mean effect (D) and sample trace (E) of the NPY (100 nM) –induced current at a holding potential of -40 mV before and during Ba^{2+} (1 mM) application using a high K^+ (10 mM) external solution containing TTX (1 μM). Bars in D-E indicate time of NPY and Ba^{2+} application. n=5-7 cells from 5-7 mice for each group. Scale bars: 25 pA/3 min.

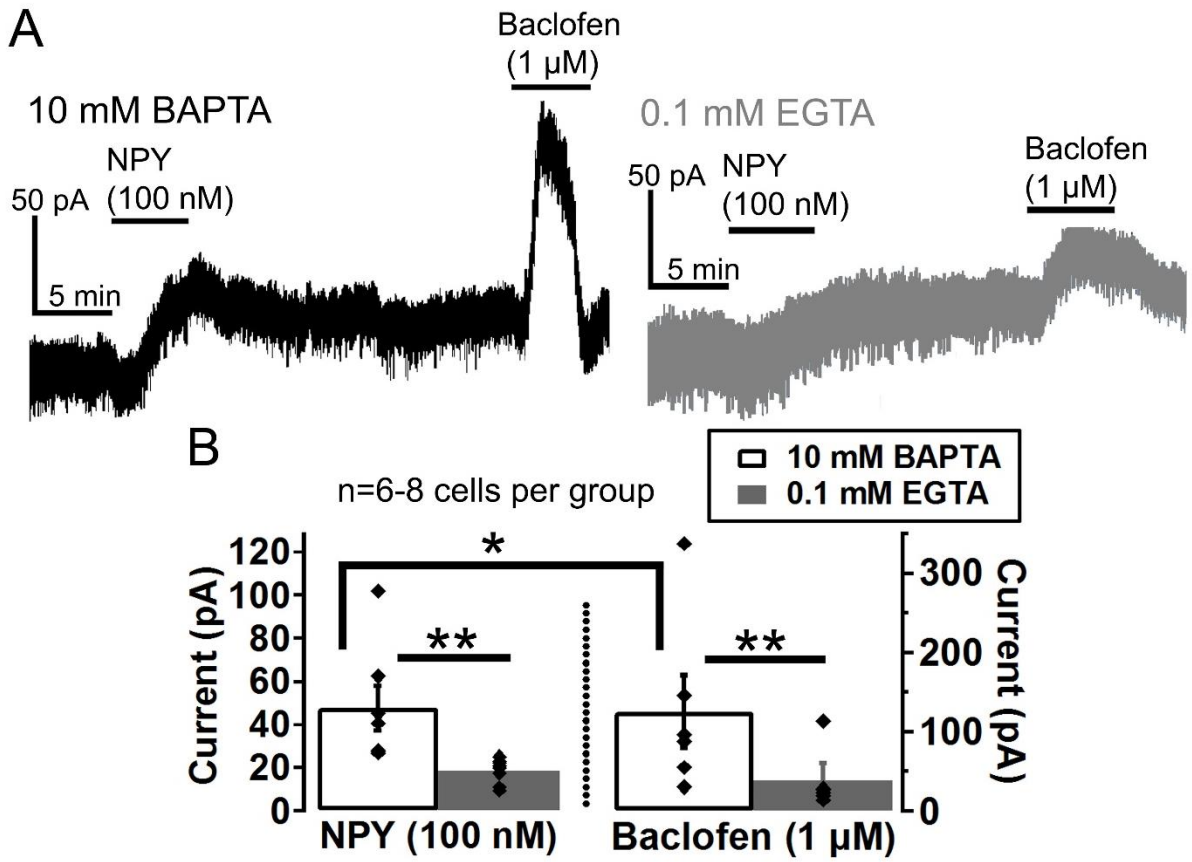
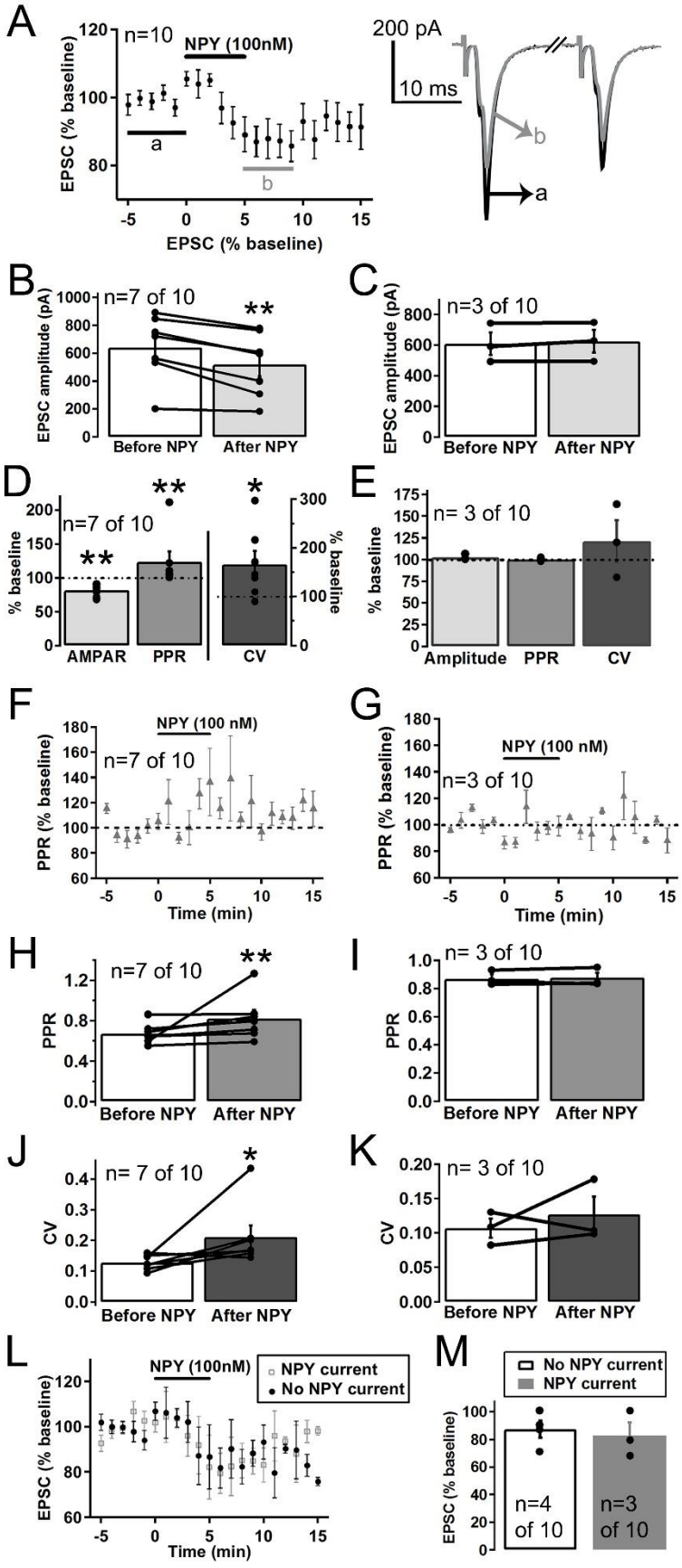


Figure 4.4: NPY and baclofen currents are affected by intracellular Ca^{2+} levels in VTA dopamine neurons.

A-B. Sample traces (A) and mean peak amplitudes (B) of the NPY (100 nM) and baclofen (1 μM) induced currents using internal solutions containing 10 mM BAPTA (black trace) or 0.1 mM EGTA (grey trace). Bars in A indicate time of NPY and baclofen application. Scale bars: 50 pA/5 min. n=6-8 cells from 5-8 mice for each group. *p≤0.05 **p≤0.01


A
n=10
NPY (100nM)
EPSC (% baseline)
a
b
200 pA
10 ms
EPSC (% baseline)
B
n=7 of 10
**
EPSC amplitude (pA)
Before NPY
After NPY
C
n=3 of 10
EPSC amplitude (pA)
Before NPY
After NPY
D
n=7 of 10
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% baseline
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n= 3 of 10
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NPY (100 nM)
n=7 of 10
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n= 3 of 10
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Before NPY
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L
NPY (100nM)
NPY current
No NPY current
EPSC (% baseline)
Time (min)
M
No NPY current
NPY current
EPSC (% baseline)
n=4 of 10
n=3 of 10

Figure 4.5: NPY decreased EPSCs in a subset of VTA dopamine neurons through a pre-synaptic decrease in glutamate release.

A. Mean effect of NPY (100 nM) on EPSCs (n=10 cells from 10 mice) and sample trace of an EPSC before (a; black trace) and after (b; grey trace) NPY. B-C. Mean EPSC amplitude before and after NPY (100 nM) application for the EPSCs inhibited by NPY (B; n=7 of 10) and for the EPSCs not affected by NPY (C; n=3 of 10). D-E. Mean effect of NPY (100 nM) on the EPSC amplitude, PPR, and CV for the EPSCs inhibited by NPY (D; n=7 of 10) and for the EPSCs not affected by NPY (E; n=3 of 10). F-G. Time course of the effect of NPY (100 nM) on EPSC PPRs for the EPSCs inhibited by NPY (F; n=7 of 10) and for the EPSCs not affected by NPY (G; n=3 of 10). H-I, J-K. Mean PPR (H-I) and mean CV (J-K) before and after NPY (100 nM) application for the EPSCs inhibited by NPY (H,J; n=7 of 10) and for the EPSCs not affected by NPY (I,K; n=3 of 7). L-M. Mean EPSC response to NPY (100 nM) (L) and mean EPSC amplitude after NPY (100 nM) application (M) in neurons in which NPY caused an outward current (n=3 of 10) compared to neurons that did not show an NPY-induced current (n=4 of 10). Bars in A, F-G, & L indicate time of NPY application. Scale Bar: 200 pA/10 ms. *$p \leq 0.05$ **$p \leq 0.01$

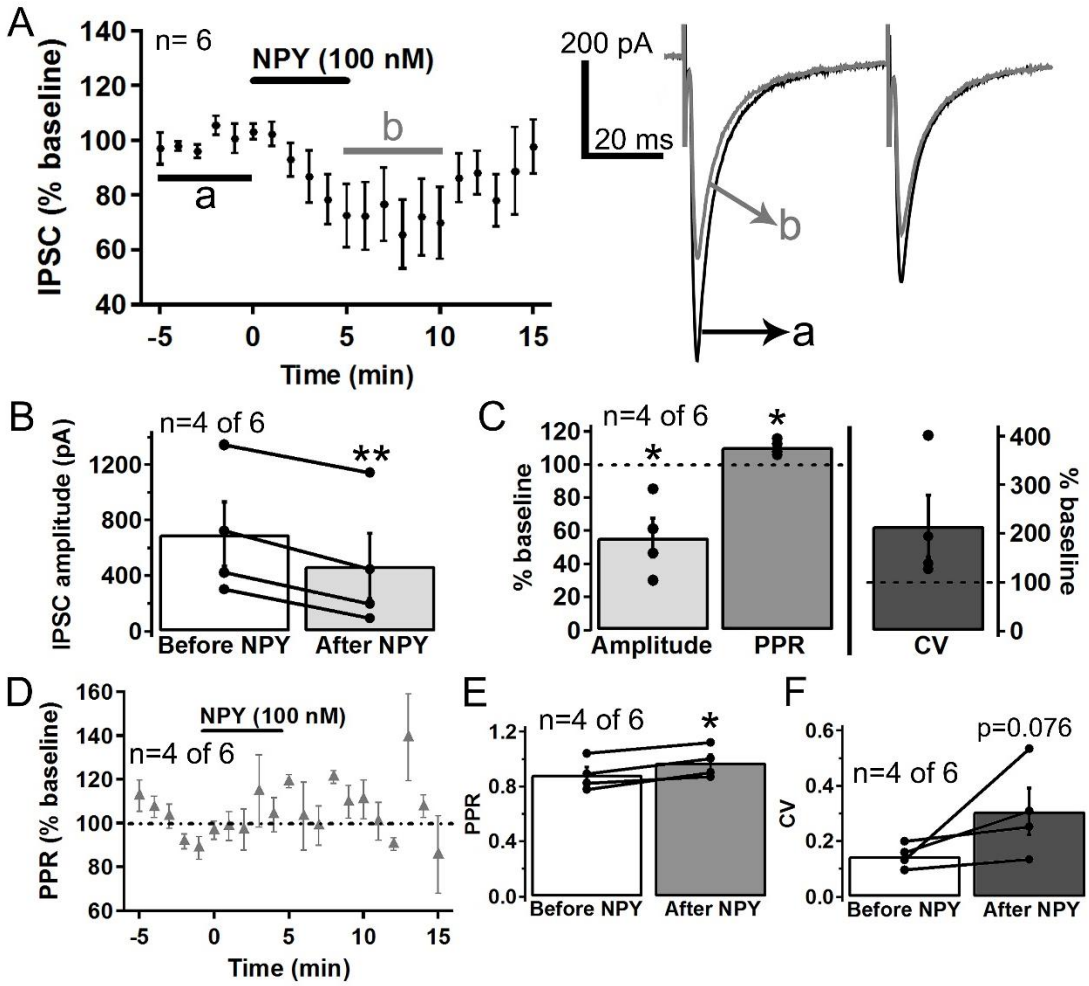

Figure 4.6: NPY inhibited IPSCs in a subset of VTA dopamine neurons through a pre-synaptic decrease in GABA release.

A. Mean effect of NPY (100 nM) on IPSCs (n=6 cells from 5 mice) and sample trace of an IPSC before (a; black trace) and after (b; grey trace) NPY. B. Mean IPSC amplitude before and after NPY (100 nM) application for the IPSCs inhibited by NPY (n=4 of 6). C. Mean effect of NPY (100 nM) on the IPSC amplitude, PPR, and CV for the IPSCs inhibited by NPY (n=4 of 6). D. Time course of the effect of NPY (100 nM) on IPSC PPRs for the IPSCs inhibited by NPY (n=4 of 6). E-F. Mean PPR (E) and mean CV (F) before and after NPY (100 nM) application for the IPSCs inhibited by NPY (n=4 of 6). Bars in A & D indicate time of NPY application. Scale Bar: 200 pA/20 ms. *$p \leq 0.05$ **$p \leq 0.01$

5 α-MSH INCREASES ACTIVITY OF MC3R-EXPRESSING NEURONS IN THE VENTRAL TEGMENTAL AREA

5.1 Abstract

The mesocorticolimbic dopamine system, the brain's reward system, regulates multiple behaviors including food intake and food reward. There is substantial evidence that the melanocortin system of the hypothalamus, an important neural circuit controlling homeostatic feeding and body weight, interacts with the mesocorticolimbic dopamine system to affect feeding, food reward, and body weight. For example, melanocortin-3 receptors (MC3Rs) are expressed in the ventral tegmental area (VTA), and our lab previously showed that intra-VTA injection of the MC3R agonist, MTII, decreases home-cage food intake and operant responding for sucrose pellets. The cellular mechanisms underlying the effects of intra-VTA α-MSH on feeding and food reward are unknown, however. To determine how α-MSH acts in the VTA to affect feeding, we performed electrophysiological recordings in acute brain slices from mice expressing EYFP in MC3R neurons to test how α-MSH affects the activity of VTA MC3R neurons. α-MSH significantly increased the firing rate of VTA MC3R neurons, but it did not increase the activity of non-MC3R expressing VTA neurons. In addition, the α-MSH-induced increase in MC3R neuron activity was independent of fast synaptic transmission and intracellular Ca^{2+} levels. Furthermore, we show that the effect of α-MSH on MC3R neuron firing rate is likely activity dependent and does not occur through a decrease in threshold potential. Overall, these studies provide an important advancement in the understanding of how α-MSH acts in the VTA to affect feeding and food reward.

5.2 Introduction

The world health organization estimates that obesity rates have nearly tripled worldwide since 1975[1]. The rapid rise in obesity rates is a major health concern as obesity increases the risk for many diseases such as heart disease, diabetes, cancers, and stroke[2]. The increase in obesity rates is largely attributed to an increase in food consumption[4-7]. Thus, understanding the mechanisms of food intake and weight gain is important for the development of new effective treatments to prevent and reverse obesity.

The melanocortin system has been widely shown to play an important role in the control of feeding and body weight. This system encompasses two neuronal populations in the arcuate nucleus of the hypothalamus, pro-opiomelanocortin (POMC) expressing neurons and agouti-related protein/neuropeptide-Y (AgRP/NPY) expressing neurons, the peptides expressed by these neurons, and the downstream receptors of these peptides[165]. POMC is a propeptide that is post-translationally processed to produce the melanocyte stimulating hormones (α-, β-, and γ-MSH). α-, β-, and γ-MSH are agonists to the centrally expressed melanocortin receptors, melanocortin-3 and melanocortin-4 receptors (MC3/4Rs), while AgRP is an inverse-agonist to the MC3/4Rs[165]. AgRP/NPY and POMC neurons respond to an animal's energy state and function in an opposing manner. For example, an energy deficit or hunger state activates AgRP/NPY neurons[303,304] while an energy surplus or satiated state activates POMC neurons[305,306]. In addition, activation of AgRP/NPY neurons or injection of MC3/4R antagonists increases feeding[211,219,307,308], while activation of POMC neurons or injection of MC3/4R agonists decrease feeding[211,219,220,309]. The melanocortin system is clearly an important regulator of food intake, and substantial evidence indicates that this system interacts with other

brain nuclei and neural systems, including the mesocorticolimbic dopamine system, to regulate food intake and body weight.

The mesocorticolimbic dopamine system is the primary circuit for reward and motivated behavior and also regulates food reward, feeding, and body weight[15,165,270,271]. The mesocorticolimbic dopamine system is comprised of dopamine neurons in the VTA and the downstream targets of dopamine neurons such as the NAc, prefrontal cortex, olfactory tubercle, and hippocampus. Numerous studies show the importance of dopamine for food intake and food reward. For example, ablation of dopamine neurons causes mice to become aphagic[133], and blocking dopamine receptors systemically or in the NAc decreases operant responding for food in rats[132,273,274]. There is also substantial evidence that intra-VTA injection of a number of feeding-related peptides alters food intake and food reward[168]. This includes injection of analogs of α-MSH and AgRP into the VTA. For example, our lab has shown that injection of the MC3R agonist, MTII, directly into the VTA decreases home-cage food intake, the intake of sucrose and saccharin intake in 2-bottle choice tests, and operant responding for sucrose pellets, whereas injection of the MC3R antagonist, SHU9119, into the VTA increases home-cage food intake and operant responding for sucrose pellets[172,173,175]. Nevertheless, how α-MSH acts in the VTA at the cellular level to regulate feeding and other reward related behaviors is unknown.

Intra-VTA α-MSH may affect food intake and food reward by regulating VTA dopamine neuron activity. For example, it has been known since the 1980s that intra-VTA injection of α-MSH increases dopamine turnover in the NAc[229,230], and intra-VTA α-MSH and MC3/4R agonists increase dopamine dependent behaviors, such as rearing, grooming, and locomotor activity[227-230]. Furthermore, additional evidence indicates that the melanocortin and mesocorticolimbic dopamine systems interact. POMC and AgRP neurons project to the

VTA[222,278], and MC3/4Rs are expressed in dopamine and non-dopamine VTA neurons[223,225,226]. The MC3R is expressed at much higher levels in the VTA than the MC4R, however[223,225,226], suggesting that the effects of α-MSH and AgRP in the VTA are likely due to actions on MC3Rs. Thus, α-MSH can clearly act in the VTA to affect food intake, food reward, and other reward behaviors, likely through activation of dopamine neurons expressing the MC3R. However, how α-MSH acts on MC3R-expressing VTA dopamine neurons to regulate food intake and reward behavior and to increase dopamine turnover in the NAc is unknown. Thus, in these studies, we tested whether α-MSH increases the activity of VTA dopamine neurons expressing MC3Rs by using electrophysiology in brain slices from transgenic mice expressing EYFP in MC3R neurons.

5.3 Materials and Methods

Animals: Male and female transgenic mice expressing EYFP in MC3R neurons (5-14 weeks old) on a mixed C57/129 background were used in all experiments. Mice were generated by crossing transgenic mice expressing Cre recombinase under the MC3R promoter (generously provided by Dr. David P. Olson, University of Michigan) with a Cre inducible EYFP transgenic mouse line (Ai3 mice from The Jackson laboratory). All protocols and procedures were approved by the Institutional Animal Care and Use Committee at Georgia State University, and conformed to the *NIH Guide for the Care and Use of Laboratory Animals*.

Slice preparation and Electrophysiology: Acute brain slices were prepared similar to what has been previously described[234-236]. Briefly, adult mice were anesthetized with isofluorane and decapitated. The brain was then removed and placed in carbogen (95% O_2 and 5% CO_2) saturated ice-cold sucrose cutting solution containing (in mM) 205 sucrose, 2.5 KCl,

0.5 $CaCl_2$, 1.25 NaH_2PO_4, 7.5 $MgCl_2$, 11.1 glucose, 21.4 $NaHCO_3$, and 0.6 kynurenic acid. A brain block containing the VTA was made, and pseudo-horizontal sections (220 μm) were cut with a vibrating blade microtome. Slices were then incubated in artificial cerebral spinal fluid (aCSF) containing (in mM) 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 11.1 glucose, 21.4 $NaHCO_3$, and 1 kynurenic acid (~ 35°C) for ~ 30 min before recording. Slices were placed in a recording chamber and perfused with carbogen-saturated aCSF at a flow rate of approximately 1-2 ml/min. Whole-cell and loose cell-attached recordings were made using an Axon multiclamp 700B microelectrode amplifier and Axograph software. MC3R-expressing neurons were identified by the presence of EYFP using a fluorescence microscope.

Cell firing was recorded in either the loose-cell attached or whole-cell configuration. Loose cell-attached recordings were obtained with electrodes (7.0-10.0 MΩ) filled with a NaHEPES based internal solution containing (in mM) 135 NaHEPES and 20 NaCl, adjusted to 290 mOsm with water. Whole-cell recordings were obtained with electrodes (2.0-3.0 MΩ) filled with a potassium gluconate (KGluconate) based internal solution containing (in mM) 128 KGluconate, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 2 ATP, 0.3 GTP, 10 creatine phosphate, and 10 BAPTA or 1 EGTA. The internal solution contained EGTA for the experiments examining the effect of α-MSH on MC3R neuron activity under reduced calcium buffering conditions. The internal solution contained BAPTA for all other whole-cell recordings. Corrections were not made for the liquid junction potential, which was calculated to be the following for each internal solution used: KGluconate 10 mM BAPTA, 13.9; KGluconate 1 mM EGTA, 14.7. Series resistance values were approximately 3-15 MΩ. If the series resistance increased by more than 20%, the experiment was terminated or excluded from analysis. Cell firing was recorded in voltage-clamp mode for loose-cell attached recordings and current-clamp mode for whole-cell

recordings. Whole-cell recordings were conducted in the presence of fast synaptic blockers (10 μM DNQX and 100 μM picrotoxin), and if the cell was not firing positive current was injected (5- 55 pA). In addition, if the cell stopped firing or did not fire for at least 1 min during baseline recordings or during the first 2 min of adding α-MSH, the experiment was terminated or excluded from analysis. Membrane potential was recorded in current-clamp mode in the presence of fast synaptic blockers and tetrodotoxin (1 μM TTX). For experiments testing the effect of α-MSH on current-step evoked action potentials, the neurons were held at ~ -70 mV and 2-sec current steps of 5 pA were applied at increasing amplitudes (5-50 pA) with a 1-sec inter-step interval. The current-step protocol was repeated every minute, and if the current steps failed to evoke action potentials or if the number of evoked action potentials decreased over time during baseline recording, the experiment was terminated. For all experiments, the change in firing rate or membrane potential was calculated as the difference between the average firing rate or membrane potential at 5 min before α-MSH treatment and the average firing rate or membrane potential at 4-6 min after α-MSH treatment. For all experiments, cells were held for at least 10 min prior to drug application to allow for diffusion of the internal solution into the cell and to ensure stability of the recording prior to drug addition.

Drugs: α-MSH was purchased from Bachem (Torrance, CA). TTX was purchased from Tocris Biosciences (Minneapolis, MN). All other reagents were from common commercial sources.

Data Analysis and Statistics: Data are represented as the mean +/- SEM unless otherwise noted. Data were stored and analyzed using Axograph X (v1.3.5), LabChart (v7.3.6; ADInstruments), and Excel (v14.0; Microsoft Corporation) software. Statistics were calculated using SigmaStat (v11.0; Systat Software, Inc.). Data were initially tested for normality using

the Shapiro-Wilk test and were then analyzed with Student's t-tests, Wilcoxon signed-rank tests, or ANOVAS with Tukey's post-hoc tests as appropriate with a significance level of $p<0.05$ set *a priori*.

5.4 Results

To determine whether α-MSH affects the activity of MC3R expressing VTA neurons ('VTA MC3R neurons'), we first tested whether α-MSH changed the spontaneous firing rate of VTA MC3R neurons in the loose-cell attached configuration. α-MSH (1 μM) significantly increased the spontaneous firing rate of VTA MC3R neurons by 0.41 +/- 0.07 Hz (Fig. 5.1; before α-MSH, 2.92 +/- 0.41 Hz; after α-MSH, 3.33 +/- 0.39 Hz). Out of the 8 MC3R neurons tested, all exhibited an action potential width ≥1.2 ms suggesting that the MC3R neurons tested were dopaminergic. A broad action potential width is a physiological characteristic of dopamine neurons that has repeatedly and reliably been used to identify dopamine neurons[68,238-240]. Thus, α-MSH increases the firing of VTA dopamine neurons expressing MC3Rs.

We next confirmed and extended these findings by testing whether α-MSH increases the firing rate of VTA MC3R neurons in the whole-cell current-clamp configuration. In addition, we included blockers of fast synaptic currents (10 μM DNQX, 100 μM picrotoxin) in the bath solution to confirm that the α-MSH induced increase in MC3R neuron firing rate was due to direct action on MC3Rs and not changes in synaptic transmission. α-MSH (1 μM) significantly increased the firing rate of MC3R neurons by 0.42 +/- 0.11 Hz in the presence of fast synaptic blockers (Fig. 5.2A-C; before α-MSH, 1.17 +/- 0.21 Hz; after α-MSH, 1.59 +/- 0.15 Hz). Intracellular Ca^{2+} plays a key role in VTA dopamine neuron excitability, firing rate, and burst firing[56,57,69], so we next tested whether reduced Ca^{2+} buffering affected the α-MSH induced

increase in MC3R neuron firing rate using an internal solution containing a low Ca^{2+} buffer (1 mM EGTA). α-MSH (1 μM) also significantly increased the firing rate of MC3R neurons using the 1 mM EGTA internal solution by 0.51 +/- 0.17 Hz (Fig. 5.2D-E; before α-MSH, 1.28 +/- 0.34 Hz; after α-MSH, 1.78 +/- 0.47 Hz), with no significant difference between the magnitudes of α-MSH induced increase in firing rate between the two groups (10 mM BAPTA, 0.42 +/- 0.11 Hz vs. 1 mM EGTA, 0.51 +/- 0.17 Hz; p=0.673). Thus, α-MSH increases the firing rate of VTA MC3R neurons through a mechanism independent of fast synaptic transmission and intracellular Ca^{2+} levels.

MC3Rs are only expressed in a subset of VTA neurons[225], so we next tested whether the α-MSH induced increase in VTA MC3R neuron firing rate was specific to VTA MC3R neurons. α-MSH did not increase the firing rate of non-MC3R expressing VTA neurons (Fig. 5.3; before α-MSH, 0.70 +/- 0.14 Hz, after α-MSH: 0.49 +/- 0.13 Hz; p=0.104). There was a trend toward a decrease in firing rate by -0.2 +/- 0.11, but this decrease was not statistically significant (Fig. 5.3C). Therefore, α-MSH induced increase in VTA neuron firing appears to be specific to MC3R-expressing VTA neurons.

α-MSH could increase the activity of VTA MC3R neurons by direct depolarization or by modifying the firing properties of the cell (e.g. threshold) independent of a direct change in membrane potential. To determine if α-MSH directly depolarizes MC3R neurons, we tested the effect of α-MSH on membrane potential in the presence of TTX (1 μM), DNQX (10 μM), and picrotoxin (100 μM). α-MSH (1 μM) slightly increased the membrane potential of MC3R neurons, but this increase was not statistically significant (Fig. 5.4; before α-MSH, -53.7 +/- 3.7 mV; after α-MSH -52.4 +/- 3.6 mV; p=0.177; Mean depolarization = 1.34 +/- 0.84 mV).

We then tested whether α-MSH altered VTA MC3R neuron firing independent of a direct depolarization by testing the effect of α-MSH on current-step evoked action potentials. The neurons were held at ~ -70 mV and a set of current steps of increasing amplitude (5-50 pA in 5 pA increments; 2-sec each; 1-sec inter-step interval) were applied every minute. α-MSH (1 μM) did not significantly affect rheobase (the minimal current required to reach threshold potential and generate an action potential) (Fig. 5.5B,E; before α-MSH, 26.9 +/- 3.5 pA; after α-MSH, 25 +/- 3 pA; p=0.35) or membrane potential (Fig. 5.5C-D; before α-MSH, -70.6 +/- 0.87 mV; after α-MSH, 68.1 +/- 1.7 mV; p=0.181). However, α-MSH (1 μM) did significantly increase the number of current-evoked action potentials at the 35, 40, 45, and 50 pA current steps in MC3R neurons (Fig. 5.5A-B; significant main effect of current step ($F(9, 63)=22.135$, $p<0.001$) and significant current-step× α-MSH interaction ($F(9,63)=3.227$, $p=0.003$)). The current-step evoked action potentials were further analyzed at the 40 pA current step, because this step consistently evoked 3-4 action potentials at baseline in 7 out of 8 neurons tested. One cell was excluded from this analysis, because the 40 pA current step failed to consistently evoke action potentials. α-MSH significantly increased the inter-spike interval at the 40 pA current step (Fig. 5.5F; before α-MSH, 119.7 +/- 20.8 ms; after α-MSH, 90.9 +/- 12.1 ms), and there was a trend towards a decrease in the latency to the first spike at the 40 pA current step (Fig. 5.5G; before α-MSH, 1.07 +/- 0.13 sec; after α-MSH, 0.85 +/- 0.07 sec; p=0.131). Thus, α-MSH facilitates MC3R neuron firing through an activity dependent mechanism that does not appear to involve direct depolarization or a change in rheobase or threshold potential.

5.5 Discussion

We have shown that α-MSH significantly increases the firing rate of VTA MC3R neurons through an activity dependent mechanism, as α-MSH increased the activity of MC3R neurons only when the neurons were firing. α-MSH did slightly increase the membrane potential of MC3R neurons, and it is possible that this slight increase in membrane potential causes a significant increase in MC3R neuron firing, however. In addition, α-MSH did not affect rheobase in MC3R neurons, suggesting that α-MSH does not increase the firing rate of MC3R neurons by lowering threshold potential. Furthermore, α-MSH increased the firing rate through a mechanism independent of fast synaptic transmission and intracellular Ca^{2+} levels. Thus, our results suggest α-MSH increases the firing rate of VTA MC3R neurons through an activity dependent mechanism that is independent of intracellular Ca^{2+} levels or altered synaptic transmission onto VTA MC3R neurons.

The effect of α-MSH on VTA MC3R neurons likely occurs through activation of MC3Rs, as MC3Rs are highly expressed in the VTA[223,225]. It is possible that α-MSH could mediate its effect on MC3R neurons by acting on MC4Rs as well, but this seems unlikely. Although MC4Rs are also expressed in the VTA, they are expressed at much lower levels compared to MC3Rs[223,225,226], and MC4Rs are most abundantly expressed in caudal regions of the VTA, while MC3Rs are expressed throughout the rostral-caudal extent of the VTA[225]. Thus, although we cannot rule out the possibility that the effects of α-MSH on VTA MC3R neuron activity are also mediated by MC4Rs in some of the MC3R neurons tested, this does not seem likely.

MC3Rs are expressed in both VTA dopamine and non-dopamine neurons (dopamine: ~ 57%; non-dopamine: ~43%)[225]. The identity of MC3R non-dopamine neurons is currently

unknown, and these neurons may be GABAergic or glutamatergic neurons, as both are found in the VTA[28,30,31]. Previous studies have shown that intra-VTA α-MSH increases dopamine release in the NAc and dopamine-dependent behaviors suggesting that α-MSH increases VTA dopamine neuron activity[227-230,232,233]. In addition, γ-MSH increases the firing rate of a subset of VTA dopamine neurons in rats[310]. In agreement, our results suggest that α-MSH increases the firing rate of VTA dopamine neurons expressing MC3Rs. All of the MC3R neurons tested in the cell-attached recordings had broad action potential widths (≥ 1.2 ms), which has been shown to reliably identify mouse VTA dopamine neurons in cell-attached recordings[238,239]. Thus, α-MSH likely increases VTA dopamine-MC3R neuron firing rate. However, it is possible that the MC3R neurons tested in the whole-cell recordings could have been both dopamine and non-dopamine neurons, as the effectiveness of previous criteria used to identify dopamine neurons in whole-cell recordings has more recently been brought into question. For example, action potential width does not reliably identify VTA dopamine neurons in whole-cell recordings in mice[239], so it could not be used to definitively identify the MC3R neurons in the whole-cell recordings here. Similarly, another electrophysiological marker for dopamine neurons, hyperpolarization-activated cation current (H-current), could not be used to identify MC3R neurons. Only a few MC3R neurons tested exhibited H-current, and electrophysiological markers of VTA dopamine neurons, such as H-current, are heterogeneous and depend on dopamine neuron projection target[16,35,311,312]. Thus, it is difficult to conclusively determine whether the VTA MC3R neurons studied here were all dopaminergic, or also contained GABA or glutamate neurons. In conclusion, we have shown that α-MSH likely increases the firing rate of VTA dopamine MC3R-expressing neurons, but α-MSH may increase the firing rate of VTA

GABA and/or glutamate MC3R-expressing neurons as well, and further studies will be required to conclusively identify the specific subtypes of VTA MC3R neurons responding to α□MSH.

We also showed that α-MSH does not significantly increase the firing rate of non-MC3R expressing VTA neurons, but there was a trend towards a decrease in firing rate in these studies, as α-MSH reduced the firing rate of 4 out of 5 of the non-MC3R neurons tested. This decrease in firing rate may be due to run down, because the firing rate slowly ran down in most of the recorded neurons. Alternatively, α-MSH may decrease the firing rate of non-MC3R expressing VTA dopamine neurons through activation of dopamine D2 receptors (D2R). VTA dopamine neurons release dopamine from their soma and dendrites[95-99], and this somatodendritic release inhibits neighboring dopamine neurons through D2R mediated activation of G-coupled inward rectifying potassium (GIRK) channels[98,100-102]. Our results suggest α-MSH likely increases the activity of VTA dopamine-MC3R neurons. Increased activity of dopamine-MC3R neurons could cause an increase in somatodendritic dopamine release and inhibition of neighboring VTA dopamine neurons, but this possibility remains to be tested.

MC3Rs are G protein-coupled receptors that signal through G_s and thus activate adenylyl cyclase and subsequently cAMP and PKA[223]. However, additional experiments have demonstrated that the MC3R is coupled to other G-proteins and can activate other signaling pathways. For example, activating MC3Rs in HEK293 cells activates MAP kinase through a Gi protein-PI3K signaling pathway[313]. It is possible that α-MSH increases MC3R neuron activity by activating one of these kinases and subsequently increasing or decreasing the conductance of an ion channel through phosphorylation of that ion channel, but the signaling cascade activated by α-MSH acting on MC3Rs in VTA neurons is currently unknown. Thus, further experiments

are needed to determine how α-MSH increases VTA MC3R neuron firing rate, and the intracellular signaling pathway activated by MC3Rs in VTA neurons.

Previous studies have shown that activation of MC3Rs increases intracellular Ca^{2+} in neurons[314-316] and one study showed α-MSH increases intracellular Ca^{2+} in MC3R-expressing HEK293 cells[317]. Intracellular Ca^{2+} is an important regulator of dopamine neuron excitability[56,57,69] and increasing intracellular Ca^{2+} increases dopamine neuron burst firing[57]. Thus, α-MSH could alter VTA MC3R neuron activity through the alteration of intracellular calcium levels, but our results suggest that the effect of α-MSH on MC3R neuron firing rate is independent of intracellular Ca^{2+} levels. α-MSH increased the firing rate of MC3R neurons under both increased (10 mM BAPTA) and reduced (1 mM EGTA) intracellular Ca^{2+} buffering conditions, and there was no significant difference in the magnitude of the effect of α-MSH with the two conditions. Thus, α-MSH induced increase in VTA MC3R neuron firing is not Ca^{2+} dependent.

In summary, we have shown that α-MSH increases the firing rate of MC3R expressing VTA neurons through an activity dependent mechanism. These results advance our understanding of how intra-VTA α-MSH regulates food intake, food reward, and other dopamine dependent behaviors, and how intra-VTA α-MSH increases dopamine turnover in the NAc.

5.6 Figures

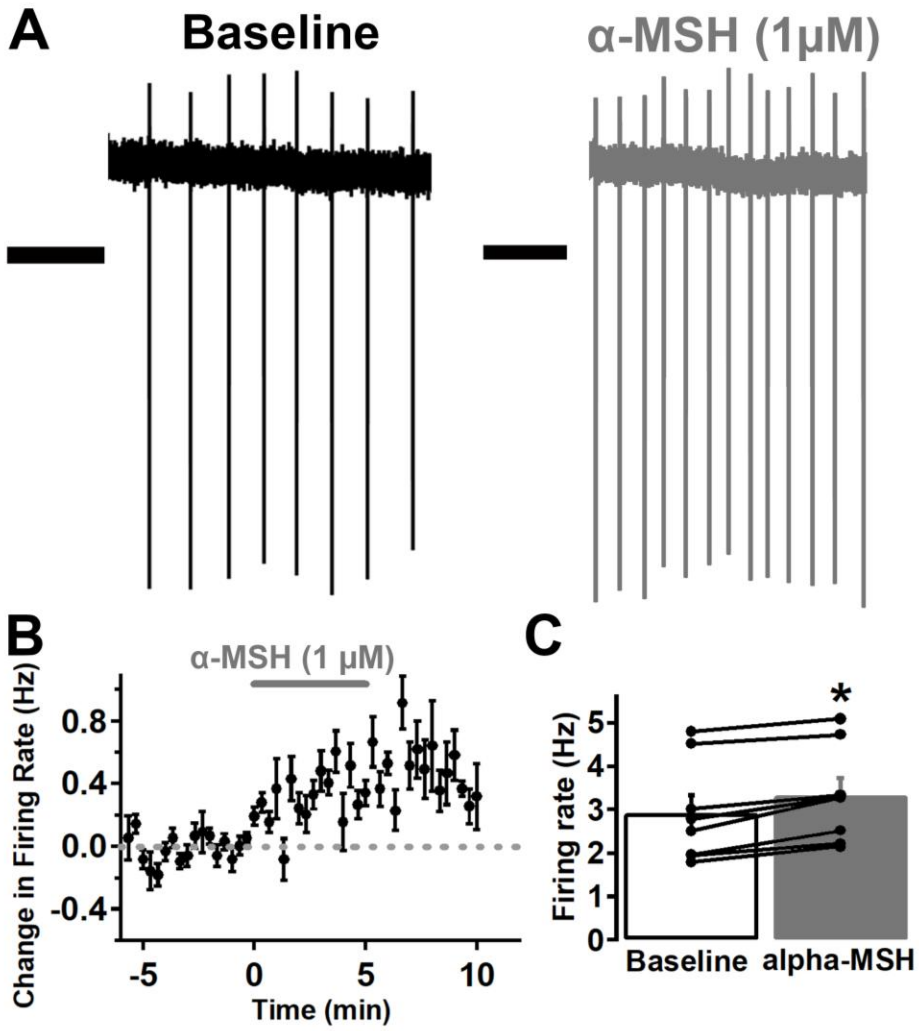

Figure 5.1: α-MSH increased the spontaneous firing rate of VTA MC3R neurons in loose cell-attached recordings.

A. Sample traces of a MC3R neuron before (black trace) and after (grey trace) α-MSH (1 μM).

B. Mean effect of α-MSH on the firing rate of MC3R neurons. C. Mean firing rate of MC3R neurons before and after α-MSH. n= 8 neurons from 7 mice. Scale Bars: 1 sec. *p<0.001

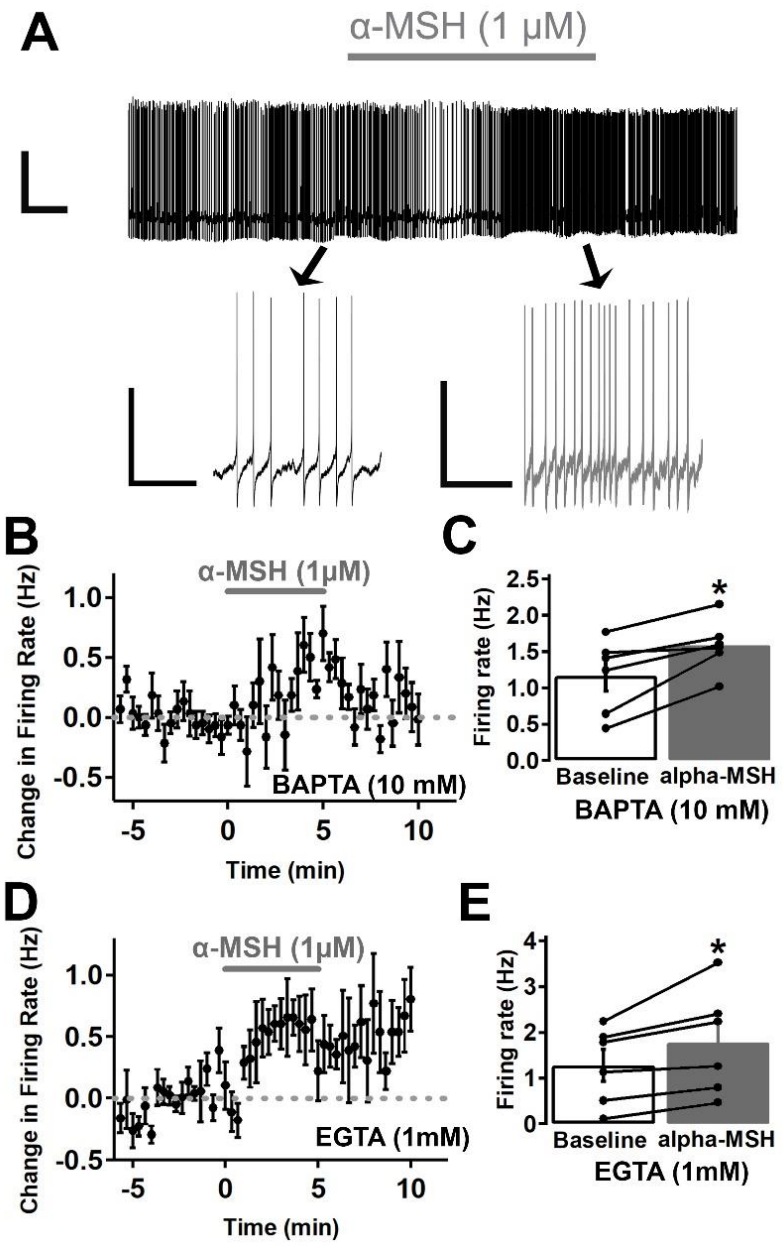

Figure 5.2: α-MSH increased the firing rate of VTA MC3R neurons in whole-cell current clamp recordings in the presence of inhibitors of fast synaptic transmission (DNQX: 10 μM; picrotoxin: 100 μM).

A. Sample traces of the firing rate of a MC3R neuron before (black trace), during, and after (grey trace) α-MSH (1 μM) application. B,D. Mean effect of α-MSH on the firing rate of MC3R neurons using an internal solution containing 10 mM BAPTA (B) or 1 mM EGTA (D). C,E. Mean firing rate of MC3R neurons before and after α-MSH using an internal solution

containing 10 mM BAPTA (C) or 1mM EGTA (E). n= 6 neurons from 6 mice for each group. Scale Bars: top trace, 40 mV/1 min; bottom trace, 40 mV/4 sec. *p<0.05

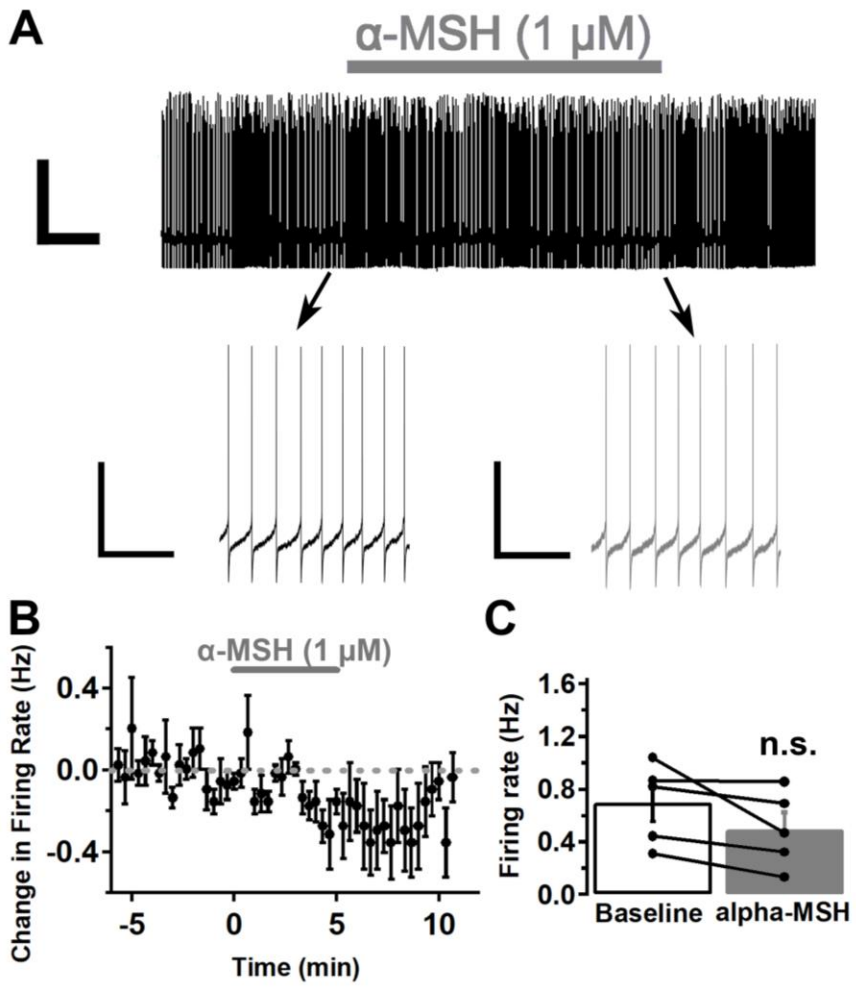

Figure 5.3: α-MSH did not increase the firing rate of non-MC3R expressing VTA neurons in whole-cell current clamp recordings in the presence of inhibitors of fast synaptic transmission (DNQX: 10 μM; picrotoxin: 100 μM).

A. Sample traces of the firing rate of a non-MC3R neuron before (black trace), during, and after (grey trace) α-MSH (1 μM) application. B. Mean effect of α-MSH on the firing rate of non-MC3R neurons. C. Mean firing rate of non-MC3R neurons before and after α-MSH. n= 5 neurons from 5 mice. Scale Bars: top trace, 50 mV/1 min; bottom trace, 40 mV/4 sec.

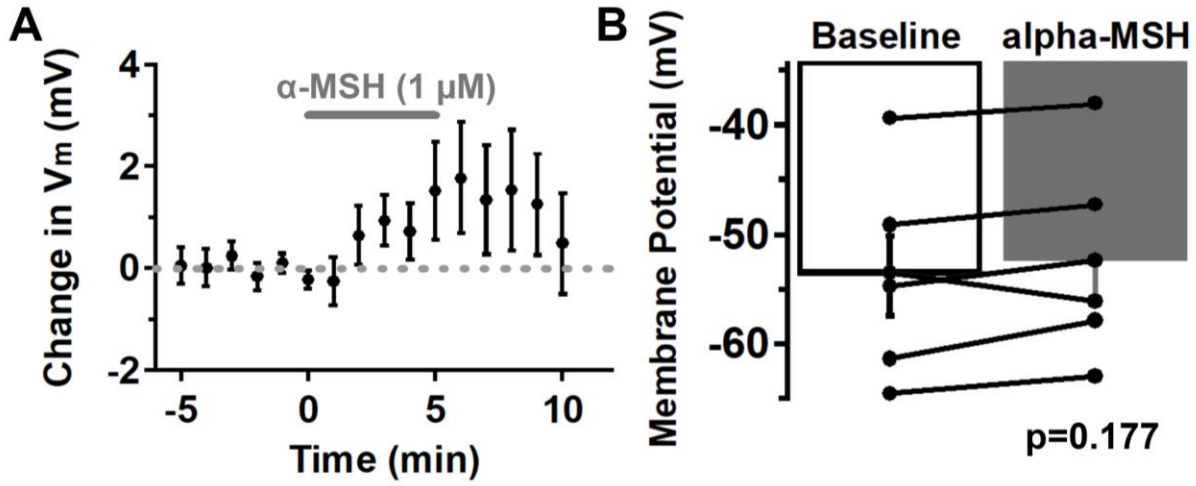

Figure 5.4: α-MSH did not significantly affect the membrane potential of VTA MC3R neurons in the presence of TTX (1 μM).

A. Mean effect of α-MSH (1 μM) on the membrane potential of MC3R neurons. B. Mean membrane potential of MC3R neurons before and after α-MSH. n= 6 neurons from 6 mice.

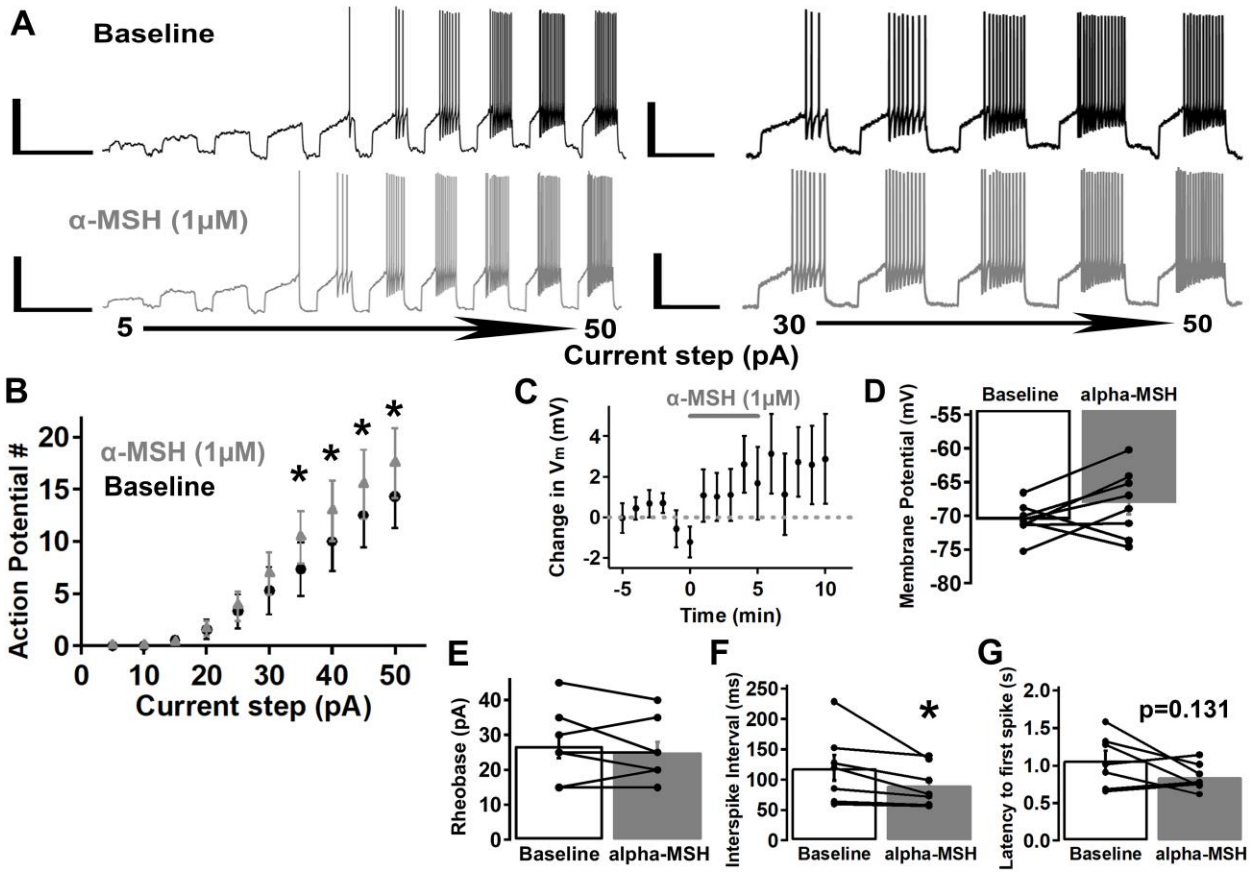

Figure 5.5: α-MSH increased the number of current-step evoked action potentials but did not affect rheobase in VTA MC3R neurons.

Current steps (2 sec) of increasing amplitude (5-50 pA) were applied every minute before, during, and after α-MSH (1 μM) application. A. Sample traces of a MC3R neuron before (black trace) and after (grey trace) α-MSH. B. Mean effect of α-MSH on the number of action potentials evoked by incrementing 5 pA current-steps (5-50 pA). C-D. Mean effect of α-MSH on membrane potential (C) and mean membrane potential before and after α-MSH (D) for the MC3R neurons in B. E-F. Mean rheobase (the minimum current step required to initiate an action potential; E), mean inter-spike interval at the 40 pA step (F), and mean latency to the first spike at the 40 pA step (G) before and after α-MSH. n= 7-8 neurons from 6-7 mice for each group. Scale Bars: left traces, 40 mV/5 sec; right traces, 40 mV/2 sec. *p<0.05

6 CONCLUSIONS

In summary, I have shown here that overall neurotensin appears to increase VTA dopamine neuron activity by directly activating a slow non-selective cationic current and by decreasing D2R and $GABA_B$ IPSCs[234]. In contrast, NPY appears to decrease the activity of a subset of VTA dopamine neurons by directly activating an inhibitory GIRK current and by decreasing glutamatergic transmission onto VTA dopamine neurons, although NPY likely increases the activity of a subset of VTA dopamine neurons through dis-inhibition as NPY also decreases GABAergic transmission onto a subset of VTA dopamine neurons[236]. Lastly, I have shown that α-MSH increases the activity of VTA MC3R-expressing neurons. Neurotensin and α-MSH are both anorexigenic peptides that decrease feeding and food reward when injected into the VTA[169-173,175] whereas NPY is an orexigenic peptide that increases operant responding for food when injected into the VTA[174]. If only the results presented here are considered, then the data suggest that anorexigenic peptides increase dopamine neuron activity to decrease feeding and food reward while orexigenic peptides decrease dopamine neuron activity to increase food reward, but a review of the literature reveals the opposite to be true[168]. For example, insulin and leptin (anorexigenic) decrease dopamine neuron activity[318-320] while ghrelin (orexigenic) increases dopamine neuron activity[321]. Orexigenic peptides usually increase dopamine neuron activity and dopamine release while anorexigenic peptides have been shown to both increase and decrease dopamine neuron activity and dopamine release[168]. Nonetheless, the theory that increased dopamine neuron activity and dopamine release lead to increased food intake is too simple to adequately describe how the mesocorticolimbic dopamine system regulates food intake. In light of this, it is unclear how anorexigenic and orexigenic peptides interact with the mesocorticolimbic dopamine system to regulate food intake, food

reward, and weight gain. Three possible models are described below as to how neuropeptides act in the VTA to regulate food intake and food reward: 1- neuropeptides regulate tonic and burst firing through different mechanisms (Fig. 6.1), 2- neuropeptides affect specific subpopulation of dopamine neurons to control distinct behaviors (Fig. 6.2), 3- neuropeptides regulate VTA glutamate and GABA neuron activity (Fig. 6.3). In addition, how the effects of neurotensin, NPY, and α-MSH on VTA dopamine neuron activity contribute to these models is described below. Finally, I describe how VTA dopamine neurons may integrate peptide signals of energy state to regulate feeding along with the future direction of the projects presented here.

6.1 Model 1- Neuropeptides regulate tonic and burst firing through different mechanisms

Neuropeptides may not increase or decrease overall dopamine neuron activity to modulate food intake and food reward. Rather, it is possible that neuropeptides affect food intake and food reward by changing only tonic or only burst dopamine neuron firing or by decreasing tonic firing but increasing burst firing and vice versa (Fig. 6.1). For example, testing the effect of evoking tonic or burst firing in VTA dopamine neurons on behavior has revealed that evoking tonic vs. burst firing affects reward consumption and reward-behavior differently. Previous studies suggest that tonic firing affects reward consumption whereas burst firing affects reward behavior. Indeed, evoking tonic firing in VTA dopamine neurons using optogenetics decreases sucrose and ethanol consumption in a two bottle-choice test[322,323] while evoking burst firing has no effect on ethanol consumption[322]. In contrast, evoking burst firing in VTA dopamine neurons using optogenetics causes conditioned place preference whereas evoking tonic firing does not[324]. In addition, reducing burst firing by knocking out NMDARs in

VTA dopamine neurons has no effect on food consumption, but does impair conditioned place preference for food and cue-dependent learning tasks[80]; thus, burst firing is important for reward behavior and cue-induced learning. Numerous studies also show that evoking burst firing in VTA dopamine neurons enhances reward reinforcement learning. For example, optogenetically evoking burst firing in dopamine neurons reinforces lever pressing[325], enhances lever pressing for food reward, and reactivates food-seeking behavior after extinction training in mice[302]. Thus, feeding neuropeptides may affect tonic and burst firing differently to regulate food intake and food reward.

The different effects of tonic and burst firing on food intake and reward behavior may explain why both anorexigenic and orexigenic neuropeptides have seemingly excitatory effects on VTA dopamine neuron activity but opposite effects on feeding behavior. Furthermore, these different effects of tonic and burst firing on behavior may explain why some peptides have both excitatory and inhibitory effects on VTA dopamine neuron activity. I have shown here that NPY has both excitatory and inhibitory effects on dopamine neuron activity[236], and it was previously shown that intra-VTA NPY increases operant responding for sucrose pellets but has no effect on free feeding[174]. Thus, taken from what is known about the effects of tonic and burst firing on behavior, NPY may increase burst firing to enhance food reinforcement behavior. Indeed, central NPY increases operant responding for food to equivalent levels as food restriction[159], and food restriction enhances dopamine neuron burst firing[161] and dopamine responses to sucrose, food intake, and operant responding for food[151-154,326]. Furthermore, centrally injected ghrelin, an orexigenic peptide like NPY, enhances the phasic dopamine response in the NAc to a reward-predicting cue and to pellet retrieval[153,327]. NPY may increase dopamine neuron burst firing through its excitatory effect of reducing GABAergic transmission,

as $GABA_A$ receptor antagonism also increases dopamine neuron burst firing[86,87]. Conversely, the inhibitory effects of NPY on VTA dopamine neuron activity may decrease tonic firing of dopamine neurons. Indeed, NPY levels increase during fasting[328,329] and fasted animals do have lower basal levels of dopamine in the striatum[149,150,330], which is set by the tonic release of dopamine. Thus, NPY may reduce tonic firing and basal dopamine levels through inhibitory actions but enhance burst firing and phasic dopamine release through excitatory actions. These opposing effects on tonic and burst firing would increase the change in dopamine from baseline during phasic dopamine release and may explain how intra-VTA NPY increases reward behavior. If the hypothesis described here holds true, then changes in the amount of dopamine from baseline may regulate food intake and reward behavior rather than the absolute amount of dopamine. Thus, higher basal levels of dopamine may occlude phasic dopamine signals and reduce the change in dopamine from baseline during phasic release and decrease food intake and reward behavior. In addition, increases in tonic dopamine release may further decrease phasic dopamine release through an auto-inhibitory feedback mechanism[323]. For example, D2Rs are located on dopamine neuron afferent terminals and tonic dopamine release may inhibit phasic dopamine release through dopamine mediated D2R activation. In conclusion, orexigenic peptides, such as NPY, may enhance burst firing and decrease tonic firing to increase food intake and food reward (Fig. 6.1).

As described above, anorexigenic and orexigenic neuropeptides have seemingly excitatory effects on VTA dopamine neuron activity. I have shown that the anorexigenic peptide neurotensin increases the activity of dopamine neurons[234] and that the anorexigenic neuropeptide α-MSH increases VTA MC3R neuron activity. Previous studies have shown that α-MSH and neurotensin both decrease food intake and reward behavior when injected into the

VTA[169-173,175]. Therefore, in agreement with the above hypothesis, α-MSH and neurotensin may increase dopamine neuron tonic firing and increase basal dopamine release to decrease food intake (Fig. 6.1). Neurotensin has also been shown to have inhibitory effects on dopamine neurons as neurotensin increases evoked $GABA_A$ currents in SNc dopamine neurons[331] and decreases evoked EPSCs in VTA dopamine neurons[332,333]. These effects of neurotensin may decrease bursting in dopamine neurons, because antagonizing glutamate receptors abolishes burst firing[331,332] and $GABA_A$ receptor agonist block NMDA induced burst firing[92,93]. Thus overall, α-MSH and neurotensin may increase dopamine neuron tonic firing while neurotensin may also decrease dopamine neuron burst firing through its inhibitory effects to decrease food intake and food reward (Fig. 6.1). Similarly, insulin (anorexigenic hormone) increases the firing rate of a subpopulation of VTA dopamine neurons[334], but also causes LTD of glutamatergic transmission[320]. Thus intra-VTA insulin may increase dopamine neuron tonic firing but decrease burst firing through a reduction in glutamate transmission to decrease feeding. In addition, drugs of abuse such as cocaine and amphetamine also increase dopamine levels in the NAc but decrease food intake. Nevertheless, drugs of abuse do increase other dopamine dependent behaviors, such as locomotor behavior, and interestingly both neurotensin and α-MSH have also been shown to increase locomotor behavior[188,189,228,229]. Thus, increases in basal dopamine levels seem to reduce food intake and food reward but may decrease feeding behavior because of increased activity levels.

Another component of dopamine neuron activity is the pauses in burst firing. Pauses occur in behaving animals when an unexpected reward is omitted or is less than what is expected and after spontaneous bursts and *in vitro* stimulated bursts; thus, pauses are an important component of the reward prediction error. Interestingly, high-frequency stimulation

that mimics burst firing potentiates $GABA_BR$ and D2R mediated GIRK currents[127] while low frequency stimulation that mimics tonic firing decreases $GABA_BR$ and D2R mediated GIRK currents[126,127]. Thus if $GABA_BR$ and D2R mediated GIRK currents mediate pauses in burst firing as has been hypothesized[69,98], then it is fitting that they would be potentiated by burst firing and attenuated by tonic firing. I have shown that neurotensin inhibits GIRK currents, while NPY activates a GIRK current[234,236]. If NPY does promote burst firing to increase food reward, then the NPY caused GIRK current may contribute to pauses in burst firing. Likewise, if neurotensin disrupts burst firing and increases basal dopamine levels, then neurotensin-induced inhibition of GIRK currents may disrupt the pauses in burst firing and increase tonic dopamine neuron firing.

It has been argued here that neuropeptides may affect food intake and food reward by modulating either tonic or burst dopamine neuron firing or by modulating tonic and burst firing through different mechanisms, but this does not exclude the possibility that neuropeptides regulate feeding by changing the overall activity of dopamine neurons. Orexigenic and anorexigenic neuropeptides both increase dopamine neuron activity, and it may be that increases in dopamine neuron activity and dopamine release both increase and decrease food intake. For example, it could be that the amount of dopamine that causes a feeding response follows an inverted-u curve such that high and low levels of dopamine decrease feeding, whereas a median level of dopamine increases feeding. Indeed, increasing dopamine release in the NAc using a low dose of amphetamine increases food intake while higher doses decrease food intake in rats[140]. In addition, completely depleting dopamine inhibits feeding in mice[133]. Thus, neurotensin and α-MSH may increase dopamine release to a level that attenuates feeding behavior, while NPY increases dopamine release to a level that potentiates feeding behavior.

Overall, how feeding neuropeptides alter dopamine neuron activity to regulate food intake and food reward and reinforcement is fairly complex, and neuropeptides may modulate dopamine neuron tonic and bust firing differently or change the overall activity of dopamine neurons and dopamine release to affect feeding behavior.

6.2 Model 2- Neuropeptides affect specific subpopulations of dopamine neurons to control distinct behaviors

It is possible that feeding-related neuropeptides regulate specific subpopulations of dopamine neurons to regulate specific behaviors such as food reward or aversion (Fig. 6.2). Our data, and others, demonstrate that the same neuropeptide can have both excitatory and inhibitory effects on VTA dopamine neurons; therefore, it is also possible that neuropeptides simultaneously activate one subpopulation of dopamine neurons while inhibiting another subpopulation to regulate different behaviors (Fig. 6.2). Subpopulations of dopamine neurons projecting to distinct target regions have been shown to regulate different behaviors. For example, rewards cause an increase in dopamine neuron activity, but subpopulations of dopamine neurons also increase their activity in response to aversive or stressful stimuli[335-338], and aversive or stressful stimuli cause an increase in dopamine release in the PFC, NAc, and amygdala[338,339]. Dopamine neurons encoding aversion seem to project to the PFC, while dopamine neurons encoding reward project to the NAc. Indeed, a single exposure to cocaine causes LTP of glutamatergic synapses on lateral shell NAc projecting dopamine neurons, but does not affect glutamatergic synapses on VTA dopamine neurons projecting to the medial PFC[312]. Interestingly, exposure to an aversive stimulus does cause LTP of glutamatergic synapses on medial PFC projecting dopamine neurons but not on NAc projecting dopamine

neurons, suggesting PFC projecting neurons encode aversion and NAc projecting neurons encode reward[312]. In agreement, excitation of laterodorsal tegmentum inputs to the VTA causes conditioned place preference while excitation of lateral habenula (LHb) inputs to the VTA causes conditioned place aversion, and laterodorsal tegmentum inputs excite lateral shell NAc projecting dopamine neurons, while LHb inputs excite medial PFC projecting dopamine neurons[301]. Thus, neuropeptides may modulate the activity of these distinct neuron populations through different mechanisms or modulate one specific subpopulation of dopamine neurons to regulate a specific behavior. For example, a neuropeptide that increases food intake and reward behavior may activate NAc projecting dopamine neurons while inhibiting PFC projecting dopamine neurons or only activate NAc projecting dopamine neurons to increase feeding and reward behavior (Fig. 6.2).

The different effects of neurotensin on dopamine neuron activity reported here are all excitatory and occurred in all dopamine neurons tested. Nevertheless, a recent study has also shown that neurotensin has inhibitory actions on SNc dopamine neurons. Neurotensin increases GABAergic transmission on SNc dopamine neurons by increasing $GABA_A$ currents through an increase in GABA release while simultaneously decreasing $GABA_B$ currents in the same neuron[331]. However, it is unknown whether neurotensin has similar inhibitory effects in the VTA. In addition, neurotensin has also been shown to attenuate evoked EPSCs in VTA dopamine neurons[333], but one study suggests neurotensin potentiates EPSCs[340], while another suggests neurotensin specifically potentiates NMDAR EPSCs and attenuates AMPAR EPSCs[332]. It is unknown if the inhibitory and excitatory effects of neurotensin occur in different populations of dopamine neurons, or if neurotensin modulates one distinct population of dopamine neurons to regulate behavior. NTS1 is the primary receptor expressed in VTA

dopamine neurons, and these NTS1 neurons primarily project to the ventral striatum and adjacent regions[187,341]. NTS1 neurons do not project to the PFC or hippocampus and only send minor projections to the amygdala[187,341]. Therefore, it is very unlikely that neurotensin significantly affects the dopamine neurons projecting to these brain regions, although neurotensin may affect these dopamine neurons through an indirect presynaptic mechanism. Overall, it seems that intra-VTA neurotensin primarily affects food intake and reward behavior by activating dopamine neurons projecting to mesolimbic regions.

I have reported that NPY has both excitatory and inhibitory effects on dopamine neurons. All three effects of NPY occurred in only a subset of dopamine neurons tested, so it is possible that NPY affects distinct subpopulations of dopamine neurons through different mechanisms. For example, NPY may excite NAc projecting dopamine neurons and inhibit PFC projecting dopamine neurons to increase food reward and suppress a stress response. Indeed, rats will endure aversive stimuli in order to obtain palatable high-calorie foods[129,130], and hunger attenuates pain through NPY signaling in the lateral parabrachial nucleus[342]. Thus, neuropeptides that increase food intake, such as NPY, may suppress the activity of brain regions encoding aversion and stress while activating feeding circuits to increase food intake when the food is highly rewarding or during an energy deficit. The target sites of dopamine neurons expressing NPY receptors are unknown, so additional experiments are needed to determine whether NPY affects subpopulations of dopamine neurons through distinct mechanisms to regulate different behaviors.

In contrast to NPY and neurotensin, only an excitatory effect of α-MSH on VTA dopamine neuron activity has been reported. It is possible that α-MSH also has inhibitory effects on VTA dopamine neurons. For example, α-MSH-induced increases in dopamine

neuron activity may increase the somatodendritic release of dopamine in the VTA and inhibit neighboring dopamine neurons through dopamine mediated D2R IPSCs. MC3Rs are only expressed in a subset of VTA dopamine neurons[225]. Therefore, α-MSH may regulate a distinct subpopulation of dopamine neurons. The projection targets of MC3R-expressing VTA dopamine neurons are currently unknown, but our preliminary data suggest that MC3R VTA neurons project to the NAc but not to the PFC. Thus, it seems that α-MSH likely decreases food intake and reward behavior primarily by activating MC3R neurons projecting to the NAc, but α-MSH may also regulate VTA MC3R neurons projecting to other brain regions through an indirect presynaptic mechanism.

6.3 Model 3- Neuropeptides regulate VTA glutamate and GABA neuron activity

It is possible that feeding neuropeptides regulate food intake and food reward by changing the activity of VTA GABA and glutamate neurons in addition to dopamine neurons, as the VTA contains GABA and glutamate neurons, and dopamine neurons that co-release either GABA or glutamate (Fig. 6.3). Activating VTA GABA or glutamate neurons has been shown to affect food intake, reward behavior, and aversion. For example, activation of VTA glutamate neurons is reinforcing[33,343], and causes a real time place preference[343]. However, the effect of VTA glutamate activation on behavior depends on the specific population of VTA glutamate neurons activated. Indeed, in contrast to the above studies, specifically activating LHb projecting or NAc projecting VTA glutamate neurons causes real-time place avoidance[344-346] (Fig. 6.3A). Activating local VTA glutamate interneurons increases glutamatergic transmission onto VTA dopamine neurons and activates dopamine neurons (Fig. 6.3B); thus, it has been hypothesized that activating local VTA glutamate interneurons increases reward

behavior through activation of dopamine neurons, whereas activating LHb or NAc projecting VTA glutamate neurons causes aversion[33,343,346] (Fig. 6.3).

Activating VTA GABA neurons also affects reward and aversion behavior. Unsurprisingly, the effect of VTA GABA neuron activation on behavior is opposite to VTA glutamate neuron activation. Indeed, activating VTA GABA neurons causes conditioned place aversion[347] and decreases reward consumption[348]. In addition, inhibiting VTA GABA neurons increases reward behavior[349]. Activating local VTA GABA interneurons increases GABAergic transmission onto VTA dopamine neurons and inhibits dopamine neurons[348]. Therefore, activating local VTA GABA interneurons may decrease reward behavior and reward consumption by inhibiting dopamine neurons (Fig. 6.3B). Conversely, specifically activating LHb projecting VTA GABA neurons increases reward behavior (Fig. 6.3A). For example, activation of LHb projecting VTA GABA neurons is reinforcing and causes real time place preference[345,350]. Overall, activation or inhibition of different populations of VTA GABA and glutamate neurons affects reward consumption, reward behavior, and aversion, and one way neuropeptides may interact with the mesocorticolimbic dopamine system to regulate behavior and food intake is through modulation of VTA GABA and glutamate neuron activity (Fig. 6.3).

It is unknown whether NPY, neurotensin, or α-MSH modulates the activity of VTA glutamate and GABA neurons, but there is some evidence suggesting that these neuropeptides do regulate their activity. For example, the neurotensin receptor NTS1 is expressed in LHb projecting VTA neurons[341], and VTA GABA and glutamate neurons project to the LHb but dopamine neurons do not[32-34,350]. Intra-VTA neurotensin may increase the activity of these LHb projecting GABA or glutamate neurons through NTS1, as I have shown that neurotensin induces an excitatory current in VTA dopamine neurons[234] which is mediated by the NTS1

receptor[201-204]. It is possible that neurotensin decreases food intake by activating LHb projecting glutamate neurons, as stimulation of LHb projecting glutamate neurons is aversive[344,345]. However, the effect of activating LHb projecting glutamate or GABA VTA neurons on food intake has not been tested. In addition, neurotensin modulates glutamatergic transmission in the VTA[332,333,340] and GABAergic transmission in the SNc[331]. Neurotensin may modulate glutamate and GABAergic transmission by directly affecting the activity of local VTA GABA and glutamate neurons. Thus, it is possible that neurotensin regulates VTA GABA and glutamate neuron activity to control feeding and food reward, but further experiments are needed to test this hypothesis.

NPY likely regulates the activity of GABA and glutamate neurons in the VTA. NPY has been shown to decrease the firing rate of putative VTA GABA neurons[217]. Thus, intra-VTA NPY may increase food reward through inhibition of VTA GABA neurons, as inhibiting VTA GABA neurons does increase reward behavior[349]. In addition, I have shown here that NPY decreases glutamatergic and GABAergic transmission onto a subset of VTA dopamine neurons through a presynaptic mechanism[236]. It is possible that NPY directly inhibits local VTA glutamate and GABA interneurons to decrease presynaptic glutamate and GABA release. Thus, intra-VTA NPY may modulate food reward by modulating VTA glutamate and/or GABA neurons, but further experiments are needed to test this hypothesis.

α-MSH may also modulate GABA and glutamate neuron activity to regulate feeding. I have shown that α-MSH increases the activity of MC3R-expressing neurons, and MC3Rs are expressed in both dopamine and non-dopamine neurons[225]. However, the identity of the non-dopamine neurons is unknown. If MC3Rs are expressed in VTA glutamate and/or GABA neurons, then intra-VTA α-MSH may regulate the activity of VTA glutamate and GABA

neurons to decrease food intake and food reward. For example, our preliminary results suggest that MC3R neurons project to the LHb; therefore, α-MSH may decrease food intake and reward by activating LHb projecting glutamate neurons. Further experiments are needed to determine the identity of non-dopamine MC3R-expressing neurons.

6.4 Integration of anorexigenic and orexigenic peptide signals in VTA dopamine neurons

Numerous feeding related peptides have been shown to interact with the mesocorticolimbic dopamine system to regulate food intake and food reward[168]. It is unknown how VTA dopamine neurons integrate all of these anorexigenic and orexigenic peptide signals to regulate feeding, or whether one subpopulation of VTA dopamine neurons responds to all feeding peptides. It is very unlikely that one subpopulation of dopamine neurons responds to all of these peptides, and different feeding peptides have been shown to affect non-overlapping populations of dopamine neurons[311]. Nevertheless, it would be interesting to test if NPY and α-MSH have opposite effects on the activity of VTA MC3R neurons, because AgRP/NPY neurons and POMC neurons (α-MSH containing) have opposite effects on food intake and metabolism. In fact, AgRP/NPY neurons inhibit POMC neurons through NPY and GABA[286,351,352]. It would also be interesting to test whether NPY or α-MSH affect the activity of NTS1 expressing dopamine neurons, because NTS1 is only expressed in a distinct subset of mesolimbic VTA dopamine neurons and these NTS1 neurons are hypothesized to be essential for regulating energy balance[187,341]. For example, ablation of VTA NTS1 neurons increases food intake but decreases body weight[187]. It is likely that α-MSH affects the activity of at least

some of the NTS1 expressing dopamine neurons, because our preliminary results suggest that the projection pattern of VTA MC3R neurons is similar to that of NTS1 VTA neurons.

It is expected that the effects of anorexigenic and orexigenic neuropeptides on VTA dopamine neuron activity would be in opposition. Indeed, the effects of neurotensin and NPY on VTA dopamine neuron activity may be in opposition. Intra-VTA neurotensin and NPY have opposite effects on food reinforcement[170,174], and I have shown that NPY activates a GIRK current while neurotensin inhibits dopamine and GABA mediated GIRK currents[234,236]. Hence, neurotensin may inhibit the NPY-mediated GIRK current. In contrast, the effects of α-MSH and neurotensin on VTA dopamine neuron activity may be complementary as both peptides have seemingly excitatory effects on VTA dopamine neurons and both peptides decrease food intake and food reward when injected into the VTA[169-173,175]. Thus, overall it is unclear how dopamine neurons integrate all of these feeding-related neuropeptide signals, and additional experiments are needed to determine how VTA dopamine neurons integrate feeding signals.

6.5 Future directions

6.5.1 Neurotensin

I have shown that neurotensin inhibits GIRK currents mediated by D2Rs and $GABA_B$ receptors[234] and this has been verified by additional studies[331,353]. Future experiments are needed to test whether neurotensin also inhibits NPY mediated GIRK currents. If neurotensin specifically inhibits GIRK currents, then it is expected that neurotensin would inhibit the NPY current. The mechanism of inhibition of neurotensin-induced GIRK currents is still unknown, and additional experiments are also needed to determine how neurotensin inhibits GIRK currents. I have discussed possible explanations of how neurotensin inhibits GIRK currents

above, so I will not go into great detail here. I have shown that neurotensin does not inhibit GIRK currents through the PKC pathway or through Ca^{2+} release from intracellular stores, but have shown that increased intracellular Ca^{2+} levels do reduce neurotensin-induced inhibition of GIRK currents[234]. In addition, Tschumi and Beckstead have shown that neurotensin does not inhibit $GABA_B$ mediated GIRK currents in SNc dopamine neurons through the PKA pathway or through general kinases and phosphatases as nonspecific kinase and phosphatase blockers do not affect neurotensin inhibition of $GABA_B$ GIRK currents[331]. Thus, additional experiments are needed to determine how neurotensin inhibits GIRK currents in VTA dopamine neurons in order to better elucidate how neurotensin regulates VTA dopamine neuron activity.

6.5.2 NPY

NPY affects VTA dopamine neuron activity through both pre- and postsynaptic mechanisms, and through mechanisms that both inhibit and excite VTA dopamine neurons. All of the effects of NPY occurred in only a subset of dopamine neurons tested. Therefore, NPY may modulate distinct subpopulations of dopamine neurons through different mechanisms. Future experiments are needed to test this hypothesis, and future experiments should also determine which specific NPY receptors are expressed in VTA neurons, as well as the projection targets of these neurons. In addition, the MC3R is only expressed in a subset of VTA dopamine neurons, and it is unknown if NPY specifically modulates that activity of this distinct population of dopamine neurons. Thus, further experiments are also needed to test the effect of NPY on MC3R VTA neurons. Finally, the brain regions from which VTA NPY afferent inputs may arise from are currently unknown. AgRP fibers do innervate the VTA[278] suggesting that AgRP/NPY neurons project to the VTA. However, additional NPY neuron populations may also innervate the VTA, and future experiments should determine if NPY neurons from other

brain regions project to the VTA. These experiments would lead to a better understanding of how NPY interacts with the mesocorticolimbic dopamine system to control food reward.

6.5.3 α-MSH

I have shown that α-MSH increases the firing rate of VTA MC3R-expressing neurons, but how α-MSH increases MC3R neuron activity is unknown. MC3Rs are G protein-coupled receptors that signal through G_s-proteins and thus activate the cAMP pathway[223], but there is also evidence suggesting that MC3Rs are coupled to Gi-proteins and activate MAP kinase through PI3K signaling[313]. Future experiments testing the effect of blocking different intracellular signaling pathways on α-MSH-induced increases in MC3R neuron activity are needed to determine how α-MSH increases VTA MC3R neuron activity.

Previous studies have shown that activation of MC3Rs increases intracellular Ca^{2+} in neurons and HEK293 cells[314-317]. Ca^{2+} is an important regulator of dopamine neuron excitability, burst firing, and glutamatergic and GABAergic synaptic plasticity[56,57,69,114,118,119,124]. Ca^{2+} also activates sK currents in VTA dopamine neurons, which are also an important regulator of dopamine neuron firing and burst firing[66,69,354]. sK currents are activated by action potential induced Ca^{2+} influxes and release of Ca^{2+} from intracellular stores[355,356]. CRF and forskolin have been shown to potentiate sK currents and enhance Ca^{2+} release from intracellular stores through a PKA dependent mechanism in VTA dopamine neurons[116,357,358]. As α-MSH uses a similar signaling pathway, α-MSH may enhance intracellular Ca^{2+} release and sK currents through a similar mechanism. Thus, future experiments should be conducted to determine if α-MSH increases sK currents, and experiments using Ca^{2+} imaging should be conducted to determine whether α-MSH increases intracellular Ca^{2+} levels in VTA MC3R neurons.

α-MSH may affect VTA MC3R neuron activity through additional mechanisms not tested here, such as changing the strength of afferent inputs to VTA MC3R neurons. For example, previous studies have shown that α-MSH and MTII (α-MSH analog) change glutamatergic and GABAergic transmission in different areas of the CNS[359-363]. α-MSH may also affect glutamatergic and GABAergic transmission on VTA MC3R neurons. Future experiments testing the effect of α-MSH on glutamatergic and GABAergic transmission in VTA MC3R neurons are needed to determine the effect of α-MSH on VTA afferent inputs.

Finally, our results suggest that α-MSH likely increases the activity of VTA MC3R-expressing dopamine neurons, but α-MSH may also increase the activity of glutamate and/or GABA MC3R-expressing VTA neurons. The identity of non-dopamine MC3R neurons is unknown; thus, future experiments should determine whether GABA and/or glutamate VTA neurons express MC3Rs, and whether α-MSH modulates the activity of dopamine and non-dopamine MC3R-expressing VTA neurons through different mechanisms. The additional experiments described here would further elucidate how α-MSH acts at the cellular level in the VTA to regulate food intake and food reward.

6.6 Summary to Conclusion

I have shown that neurotensin, NPY, and α-MSH affect VTA dopamine neuron activity through multiple mechanisms and have described three possible models as to how these peptides may alter the activity of VTA neurons to control food intake and food reward. Feeding neuropeptides acting in the VTA most likely do not regulate food intake and food reward through one mechanism or through only one of the methods described above. For example, one feeding peptide may regulate the activity of a distinct subpopulation of dopamine neurons (Fig.

6.2), affect tonic and burst dopamine neuron firing differently (Fig. 6.1), and affect VTA glutamate and/or GABA neuron activity to control food intake and reward behavior (Fig. 6.3). In addition, the different effects of neuropeptides on the mesocorticolimbic dopamine system may affect different feeding and reward behaviors, so one neuropeptide acting in the VTA may not affect both food intake and food reward but only affect one or the other. Thus, how feeding-related neuropeptides affect VTA dopamine neuron activity to control food intake and reward is complex, but the experiments presented here have added to the existing body of knowledge and advanced the collective understanding of how hypothalamic feeding-related neuropeptides interact with the mesocorticolimbic dopamine system to regulate food intake and food reward.

6.7 Figures

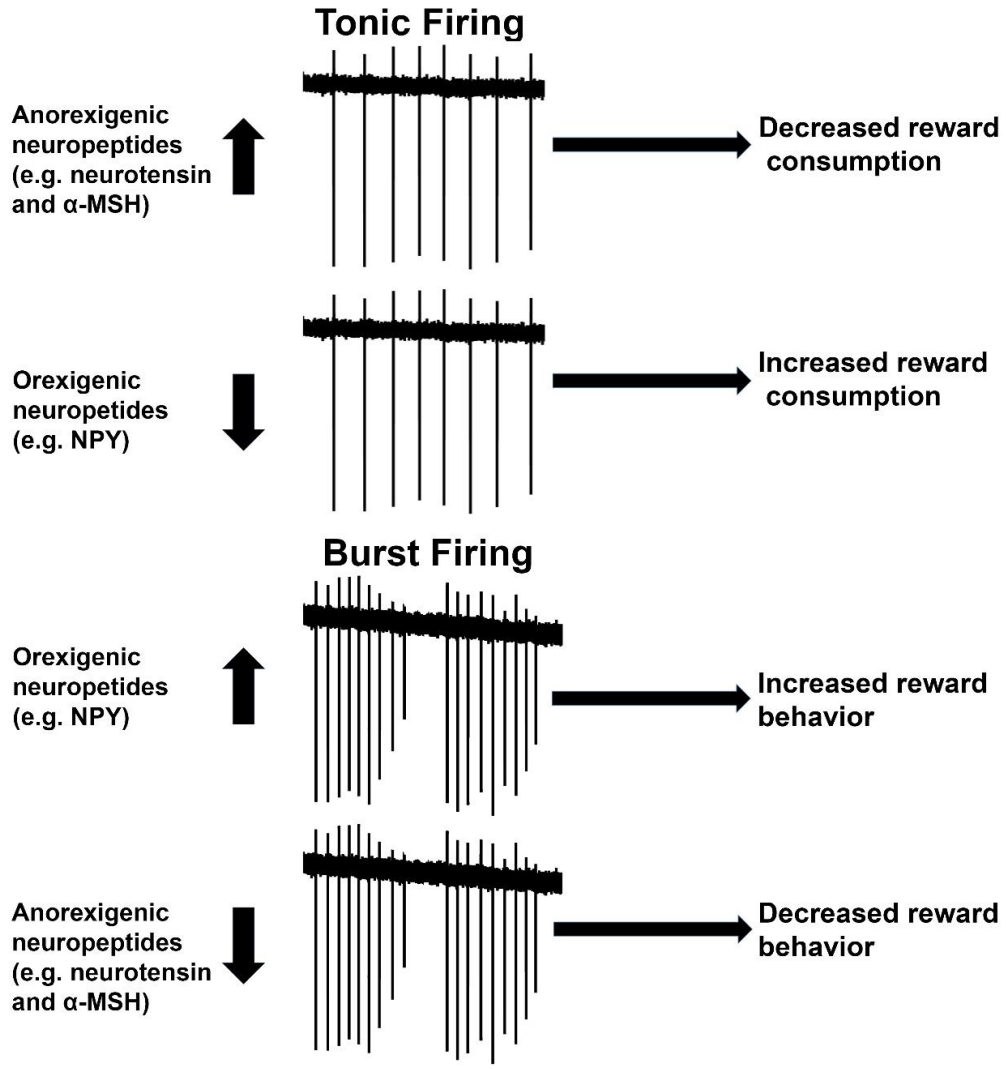

Figure 6.1: Model 1- Neuropeptides regulate tonic and burst firing through different mechanisms.

Feeding-related neuropeptides may regulate food intake and food reward by specifically affecting either tonic or burst firing in VTA dopamine neurons or by decreasing tonic firing but increasing burst firing and vice versa. Evoking tonic firing decreases reward consumption whereas evoking burst firing increases reward behavior in VTA dopamine neurons. Anorexigenic neuropeptides such as neurotensin and α-MSH may decrease food intake and reward behavior by increasing tonic firing and decreasing burst firing. Orexigenic

neuropeptides such as NPY may increase food intake and reward behavior by decreasing tonic firing and increasing burst firing in VTA dopamine neurons.

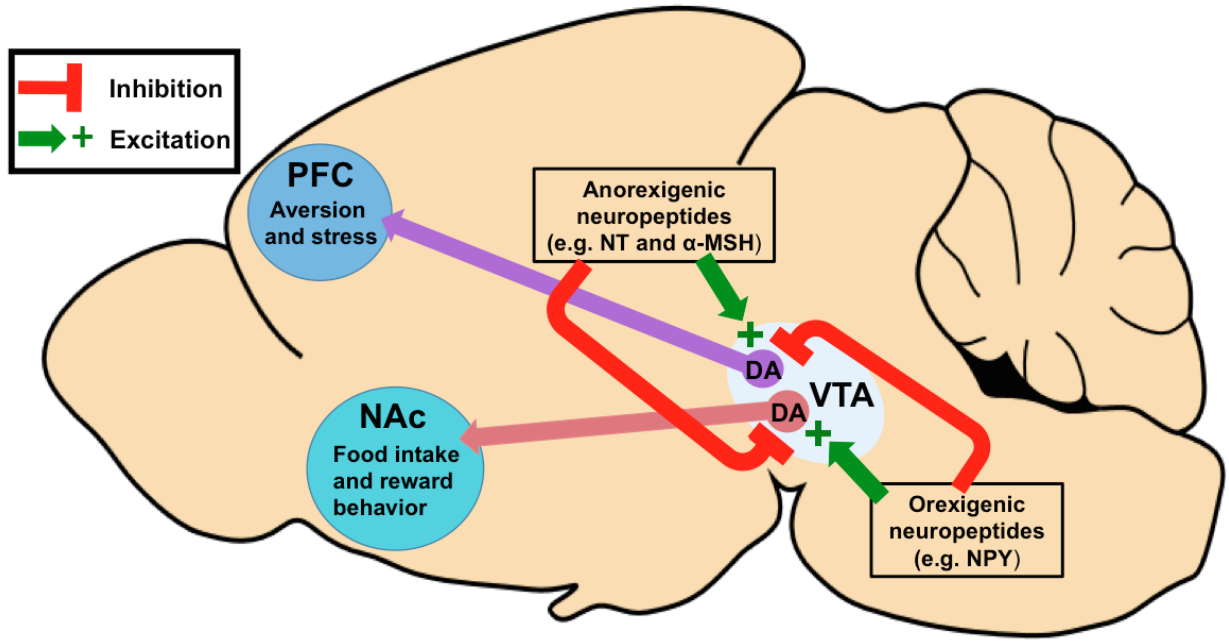

Figure 6.2: Model 2- Neuropeptides affect specific subpopulations of dopamine neurons to control distinct behaviors.

Feeding-related neuropeptides may regulate food intake and food reward by regulating the activity of specific subpopulations of dopamine (DA) neurons to regulate specific behaviors. NAc projecting dopamine neurons encode reward behavior while PFC projecting dopamine neurons encode aversion and stress. Anorexigenic neuropeptides such as neurotensin (NT) and α-MSH may decrease food intake and reward behavior by activating PFC projecting dopamine neurons and inhibiting NAc projecting dopamine neurons. Orexigenic neuropeptides such as NPY may increase food intake and reward behavior by inhibiting PFC projecting dopamine neurons and activating NAc projecting dopamine neurons.

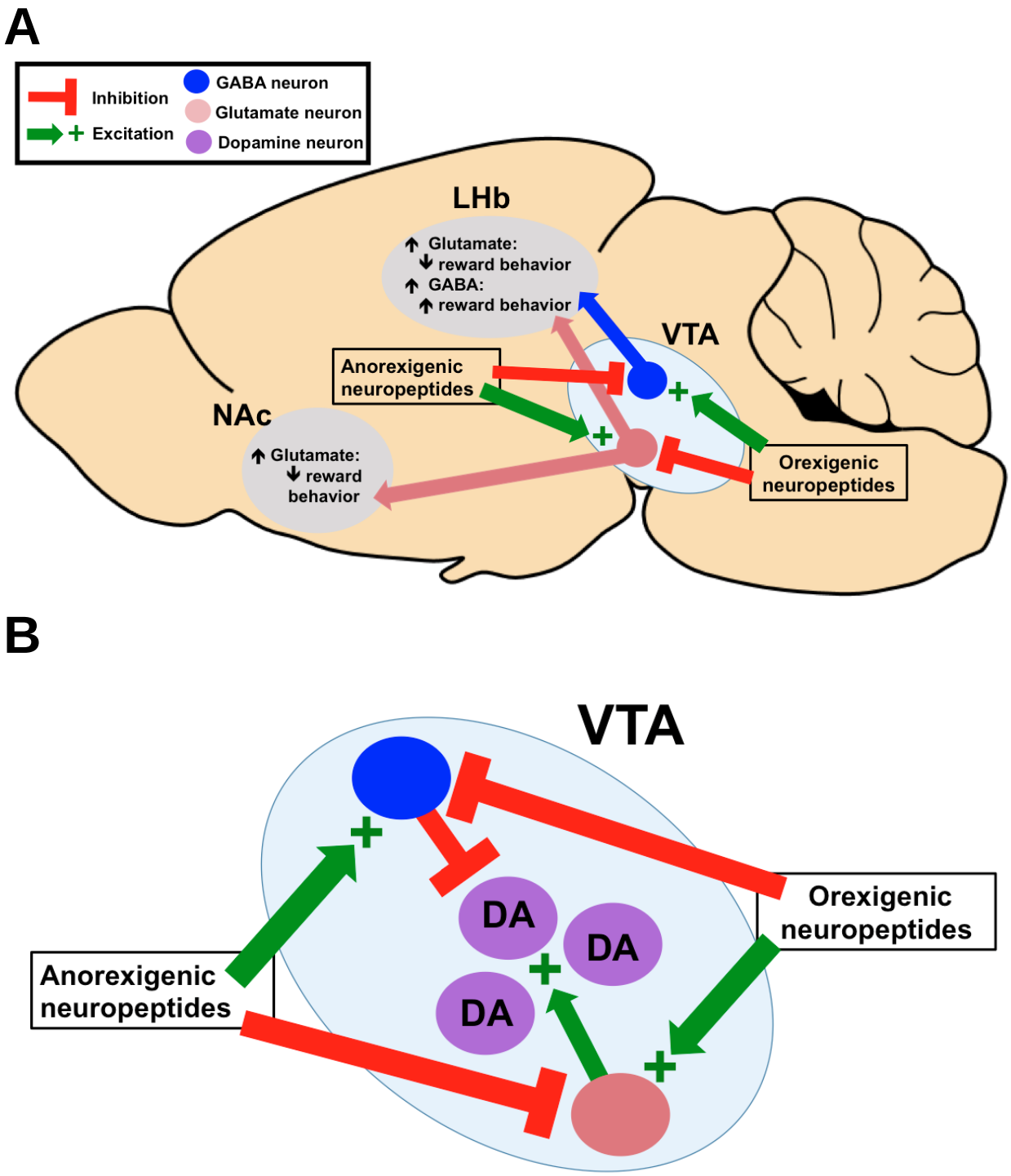

Figure 6.3: Model 3- Neuropeptides regulate VTA glutamate and GABA neuron activity. Feeding-related neuropeptides may regulate food intake and food reward by changing the activity of VTA GABA and glutamate neurons in addition to dopamine (DA) neurons. A. Activation of NAc and LHb projecting VTA glutamate neurons causes aversion whereas

activation of LHb projecting VTA GABA neurons increases reward behavior. Anorexigenic neuropeptides (such as neurotensin (NT) and α-MSH) may decrease food intake and reward behavior by activating LHb and NAc projecting glutamate neurons and inhibiting LHb projecting GABA neurons. Orexigenic neuropeptides (such as NPY) may increase food intake and reward behavior by inhibiting NAc and LHb projecting glutamate neurons and activating LHb projecting GABA neurons. B. Activation of locally projecting VTA glutamate neurons increases reward behavior through excitation of VTA dopamine neurons whereas activation of locally projecting VTA GABA neurons causes aversion and decreases reward consumption through inhibition of VTA dopamine neurons. Anorexigenic neuropeptides (such as NT and α-MSH) may excite VTA GABA neurons and inhibit VTA glutamate neurons to decrease food intake and reward behavior. Orexigenic neuropeptides (such as NPY) may inhibit VTA GABA neurons and excite VTA glutamate neurons to increase food intake and reward behavior.

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