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Hypothalamic neurotensinergic projections promote reward by enhancing glutamate transmission in midbrain

by

Kimberly A. Kempadoo

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by
Kimberly A. Kempadoo

Acknowledgements

This dissertation was completed with the guidance and support of a number of individuals to whom I am extremely grateful. I would like to express my appreciation for the role these individuals have played in enriching my experience and shaping my scientific development. Given the great number of people who have done so, this acknowledgement also includes those who are not mentioned by name.

I would first like to thank my dedicated thesis committee: Patricia H. Janak, Roger A. Nicoll, Luis de Lecea, Howard L. Fields, and Antonello Bonci. The scientific advice that my committee provided has been invaluable to the execution of this dissertation and I am very appreciative of their commitment to providing insightful guidance throughout my graduate career.

I am grateful to have had Patricia H. Janak as thesis committee chair. In addition to leading with a quiet, but firm hand, Tricia is able to provide the calm guidance while simultaneously providing clear perspective and renewed sense of purpose. Roger A. Nicoll has provided keen insight throughout the project and I am honored to have had the opportunity to interact with and learn from Roger. Luis de Lecea has been an excellent mentor from the start of my time in the Bonci Lab. Not only did Luis welcome me into his lab to collaborate on an optogenetics project, but his doors were always open to me, an invaluable resource.

Howard L. Fields has been my co-advisor, and I am truly in awe of Howard's approach to science. He has taught me what a scientist could and should be. His perspective on how responsible investigators should design and execute their experiments is refreshing and motivates those around him to reach and surpass the highest of

standards. Howard's mentorship has been the steady force that guided me in the right direction and I am truly appreciative to have had the honor of working with one of the most inspiring and enlightening minds in neuroscience.

I would also like to express my gratitude to Antonello Bonci for all of the opportunities that he has afforded me. Working in Anto's lab has been a memorable experience that has helped me grow as a person and a scientist. Anto has keen insight and intuition that enables him to help guide a person towards their personal best. I am thankful for this opportunity and everything that I have gained from the experience.

In addition to the support of the thesis committee, I would also like to acknowledge the people who make the Gallo Center such a unique and wonderful research center. The Animal Facilities at the Gallo are stellar and I would like to thank Nourredine Azouaou and the animal facilities staff including Sergio Pedroza, David Hampton and Danielle Fernandez. The IT Department has rescued me from many technical mishaps, particularly Brandon Abram and Calvin Hom. I would also like to thank Administration, Marcus Cavness and Julie Anderson.

For my training, I would like to thank Stephanie Borgland, M. Scott Bowers, Viktor Kharazia, Emanuela Argilli, Steven Lieske, Woody Hopf, and the collaborators at Stanford University, Luis de Lecea, Antoine Adamantidis, Matt Carter, Karl Deisseroth and Feng Zhang. Thank you to the technicians who have helped things run smoothly including Saemi Cho, Francesco Magnani, and Shao-Ju Chang.

At the University of California, San Francisco, I appreciate the leadership of Louis Reichardt, the Neuroscience Program director, as well as Pat Veitch and Carrie Huckaba, the program administrators. I would like to send a special thank you to the National Institute of General Medical Sciences (NIGMS) for providing my fellowship throughout graduate school, and particularly the directors who transformed a funding source into a community and support system: Christine DesJarlais and Donald Woodson. Thank you to the Minority Graduate Student Organization for providing a strong network of support and for working so diligently to increase the diversity of the UCSF student population. I would also like to thank the mentors who have guided my early scientific experiences prior to my studies at UCSF, Phyllis Citrin, Donald S. Faber, Matthew Walker III, William Martin and Chris I. Moore.

I must send a warm message of appreciation to the friends who have made this journey enjoyable and exciting, and who have help me maintain a balanced life. M. Scott Bowers and Taban Seif, previous Bonci Lab members; my close friends and UCSF colleagues, Ashley Robinson, Lauren Goins, Georgette Charles and Olusegun Williams; dear friends from MIT collectively known as M'05; and my good friends Nicholas de Priest Bishop and Jennifer Weekes.

Last but not least, I would like to extend my deepest sentiment of gratitude to my family for their unconditional love and support that has enabled me to complete this graduate program. To my father, Nelson Kempadoo, who has mastered his field of hematology. His passion for tirelessly helping patients inspires me to continue to approach my work with the same conviction for helping others. My mother, Oreann Kempadoo, has also dedicated her career to saving and improving lives as a registered

nurse and nurse manager. She has demonstrated the kindness, gentle patience and wisdom that only a mother could. I aspire to be like both of my parents and cannot express how appreciative I am to have been raised by such intelligent, giving and loving parents. I would like to share a special thank you with my sister, Latasha N. Kempadoo-Riddle, who is a beacon of hope, an endless source of strength and courage, and one of the most graceful people I know. My sister is truly a phenomenal person and I have always strived to hold myself to the same standards of excellence that I have seen her reach and surpass as a young girl. I would like to thank my brother-in-law, Raymond L. Riddle, for his support and for being the big brother that I never had. Finally, I would like to dedicate this dissertation to my nephew, Tyler V. Kempadoo Riddle. My nephew is a shining star in every sense, and I cannot wait for the world to see his brilliance.

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Abstract

The lateral hypothalamus (LH) is a brain region that provides one of the largest sources of glutamatergic and peptidergic input to the ventral tegmental area (VTA). Electrical stimulation in this area produces reward, however, the medial forebrain bundle courses through the LH. Therefore, understanding the role of the LH to VTA projection in mediating reward-related behavior and pathological states of reward-seeking such as drug abuse can be informed by separating the role of LH neurons from other descending forebrain inputs to the VTA. In the present study, we focus on the action of neurotensin (NT), one of the most abundant peptides in the LH to VTA projection, on excitatory synaptic transmission in the VTA and its relevance in goal-directed behavior. Whole-cell patch clamp techniques in midbrain slices of C57Bl/6 mice were used to demonstrate that NT potentiates NMDA-mediated excitatory postsynaptic currents (EPSCs) via the NT 1 receptor (Nts1). NT release at optogenetically-isolated LH-VTA terminals indicates synapse-specific effects *ex vivo*. Using *in vivo* optogenetic techniques, we demonstrate that animals display robust intracranial self-stimulation of lateral hypothalamic terminals in the VTA. This behavior is significantly attenuated by blockade of either Nts1 or NDMA receptors in the VTA. The striking behavioral and electrophysiological effects of lateral hypothalamic NT in the VTA highlight this pathway as an important component in mediating reward-related behavior.

Chapter 1

Introduction

A major challenge in understanding motivated behavior is to isolate the function of specific neural pathways and characterize the contribution they make to processing salient signals that drive goal-oriented behavior. The overarching goals of this subfield are to advance the knowledge of the neural systems that produce reinforced behavior in nature, define the mechanisms by which these innate processes become pathological, and develop strategies for treating pathological motivational dysfunctions such as drug addiction. In the present work, we use electrophysiological, behavioral, and optogenetic techniques to identify the mechanism of action of a specific pathway underlying reward-related behavior, the glutamatergic and peptidergic projections from the lateral hypothalamus (LH) to the ventral tegmental area (VTA).

History of Intracranial Self-Stimulation

In the early 1900s, Edward Thorndike described a form of associative learning in which animals consistently repeat actions that produce a reward, a phenomenon called operant conditioning (Squire and Kandel, 1999). In order to quantify this behavior, B.F. Skinner created an apparatus in which animals learned to press a lever for food or other rewards (Kandel et al., 2000). In the 1950s, investigators demonstrated that in addition to food, rodents will lever-press to receive electrical stimulation of the septal nucleus (Olds and Milner, 1954). Subsequent studies extended this finding by systematically mapping brain

sites where rats would lever press to receive electrical stimulation (intracranial self-stimulation or ICSS). Effective sites included the lateral hypothalamus, medial forebrain bundle, VTA, locus coeruleus, regions of the frontal cortex, hippocampus, amygdala, and nucleus accumbens (for review see Wise, 1996). Given that rodents returned to areas of a cage previously associated with brain stimulation, it was proposed that ICSS activates reinforcing brain regions directly involved in processing natural reward (Olds and Milner, 1954). Indeed, electrical activation of specific brain regions promotes consummatory or appetitive behaviors such as feeding and drinking (Wyrwicka and Doty, 1966). Of particular interest are two brain regions that produce robust ICSS, the lateral hypothalamus and the ventral tegmental area (Olds and Olds, 1963; Miliaressis and Cardo, 1973).

A substantial body of literature has established that stimulation of the medial forebrain bundle (MFB) at the level of the lateral hypothalamus is a “brain reward stimulation” site that exerts its reinforcing properties via efferents to the VTA (Koob et al., 1978; Shizgal et al., 1980; You et al., 2001). In this dissertation reward will refer to the ability of a stimulus to promote appetitive behavior. These classic studies have laid the foundation for studying animal models of reward, and now neuroscience is entering a new phase of research in which more precise isolation of distinct cell populations and neurotransmitter systems are required to understand the microarchitecture underlying neural substrates of behavior.

VTA and Reward

VTA dopamine neurons (Dalhstrom and Fuxe, 1964) are critical for various behaviors from pursuing natural reward experiences to pathological states of reward seeking such as drug addiction (Bunney et al., 1973; Roberts and Koob, 1982; White and Kalivas, 1998; Kauer, 2004; Wise, 2004; Marinelli et al., 2006; Fields et al., 2007). Focus has been placed on the mesoaccumbens pathway, a primarily dopaminergic projection from the VTA to the nucleus accumbens (NAc) (Mogenson et al., 1980; You et al., 2001, Margolis et al., 2006). Activation of the VTA promotes locomotor activity, induces exploration, reinforces specific actions, and promotes the formation of the association between a stimulus and its outcome (Stinus et al., 1980; Shumake et al., 2010; Flagel et al., 2011). Intracranial self-stimulation studies of the VTA demonstrate that dopamine metabolites are significantly increased in the nucleus accumbens, striatum and olfactory tubercle (Fibiger et al., 1987). Dopamine antagonists cause a rightward shift in the response rate-stimulus frequency curve, but do not affect the maximal response rate for self-stimulation, suggesting reduced stimulus reinforcement (Wise et al., 1985, Gallistel et al., 1987). It was therefore hypothesized that dopamine is intimately involved in reward processing (Wise et al., 1985; Gallistel et al., 1987).

It has been proposed that the locomotor behaviors induced by dopamine release are critical components of seeking drugs of abuse as well as natural rewards (Wise, 2004; Willuhn et al., 2010). In fact, drug addiction can be described as a state of over active reward-seeking. It is marked by users who will: (1) work increasingly hard to obtain the drug, (2) seek drugs despite negative consequences, (3) usually develop tolerance to the substance, and (4) experience withdrawal symptoms in the absence of administration

(According to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition). Aspects of human drug addiction can be simulated in animal models.

Psychostimulants such as cocaine and amphetamine promote dopamine release into the nucleus accumbens (Willuhn et al., 2010). In an ICSS paradigm, amphetamine produce a leftward shift in the rate-frequency curve, the opposite direct as dopamine antagonists, suggesting that psychostimulants, most likely via dopamine signaling, share a common neural mechanism will brain reward stimulation (Carboni et al., 1989; Gallistel and Karras, 1984). The involvement of dopamine neurons in reinforced behaviors is also supported by other behavioral models of drug addiction.

Behavioral sensitization is an enhanced locomotor response to administration of a substance after repeated exposure to that compound. Infusion of amphetamine and cocaine into the VTA produces locomotor sensitization by promoting dopamine release into the nucleus accumbens (Kalivas and Weber, 1988; Kalivas and Duffy, 1993a and b). Antagonists or other pharmacological agents that reduce or prevent behavioral sensitization may serve as therapeutic targets for reducing the physiological changes that occur during withdrawal periods, potentially reducing susceptibility to drug-seeking relapse.

Also integral for drug-induced behavior is the formation of the association between a specific stimulus or context and the drug experience. Investigators utilize conditioned place preference (CPP) to explore this behavior in rodents. CPP is a paradigm in which a distinct experimental chamber is paired with drug exposure, while another place is not. If the drug is inherently rewarding, or provides relief from a stressful state, the animal will choose to spend more time in the paired chamber when given the option in absence of the

stimulus (Spyraki et al., 1982). Psychostimulants produce robust CPP, an effect attenuated by neuroleptics, or agents with antagonist action at dopamine receptors (Mackey at al., 1985, Kosten et al., 1994, Veenaman, 2010). Taken together, these behavioral models of drug addiction and reward clearly demonstrate the importance of dopamine signaling in the VTA for motivated behaviors.

LTP in the VTA

The neural mechanisms underlying VTA dopamine neuron involvement in drug addiction have been extensively studied (Jones and Bonci, 2005; Kauer and Malenka, 2007). In order for an animal to develop a pattern of sustained reward or drug seeking, it must learn the association between stimuli or actions and the outcome (drug effect). Synaptic plasticity, or long-term potentiation (LTP), is an important neural substrate underlying learning and memory (Malenka and Nicoll, 1997; Whitlock et al., 2006). Multiple mechanisms of LTP induction have been extensively studied in the hippocampus (Bliss and Lomo, 1973; Kauer et al., 1988; Zalutsky and Nicoll, 1991), and LTP in the VTA requires N-methyl-D-aspartate (NMDA) receptors (Bonci and Malenka, 1999). Since NMDA-dependent LTP typically augments α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor signaling, a common tool used to identify the relative contribution of AMPA and NMDA receptors to the glutamate response is the AMPA/NMDA ratio (Bonci and Malenka, 1999; Hsia et al., 1998).

LTP induction in the VTA is thought to be critical for the expression of specific responses to drugs of abuse (Robinson and Berridge, 1993; White and Kalivas, 1998; Overton and Clark, 1997). Cocaine promotes synaptic strengthening of glutamatergic

synapses onto VTA dopamine neurons after one exposure to the drug (Ungless et al, 2001; Saal et al., 2003). In particular, NMDA receptor signaling is enhanced via a PKC-dependent pathway activated by dopamine D5-like receptors (Schilstrom et al., 2006). This produces an insertion of NR2B subunits as indicated by a prolonged NMDA-mediated current decay (Schilstrom et al., 2006). This NMDA-dependent form of LTP is characterized by an increase in GluR2-lacking, calcium-permeable AMPA receptors, that enhance AMPA-mediated current (Bellone and Luscher, 2006).

Interestingly, this augmentation of AMPA receptor activity does not solely explain the effects of cocaine. In mice lacking GluR1, the AMPA subunit translocated to the synapse after LTP induction in the VTA (Bellone and Luscher, 2006; Argilli et al., 2008), cocaine-induced plasticity in the VTA was absent, however behavioral sensitization remained intact (Dong et al., 2004). In mice lacking a functional NMDA receptor, compensatory mechanisms enhanced AMPA-mediated currents, and although initial cocaine sensitization was normal, the sensitization that occurs after long periods of withdrawal was absent (Zweifel et al., 2008). Further analysis reveals that single or repeated injections of cocaine induce LTP for up 5 days (Borgland et al., 2004). Interestingly, cocaine self-administration produces an increase in the AMPA/NMDA for up to 3 months, while animals receiving passive injections of cocaine no longer display LTP 21 days after exposure (Chen et al., 2008). This suggests that instrumental actions to obtain drug are critical for long-lasting synaptic strengthening in the VTA, and suggests that pharmacological actions on synapses can be specific to a behavioral context.

Dopamine Hypotheses

It is widely accepted that VTA dopamine neuron activation promotes goal-directed behavior, however competing theories debate whether dopamine encodes hedonic value, incentive-salience or reward learning. The three theories will be discussed.

Hedonic Value

Wise and others have pioneered the hypothesis that dopamine neurons directly process the hedonic value of stimuli. As discussed above, intracranial self-stimulation is thought to activate rewarding brain regions and is attenuated by dopamine antagonists (Wise, 1996). Demonstrations that the behavioral effects of drugs of abuse and other rewards are attenuated by neuroleptics led to the notion that dopamine is essential for hedonic value, or for "liking" a particular stimulus (Bailey et al., 1986; Fibiger et al., 1976). Others suggest that the pleasurable effects of drugs diminish with repeated use and the subsequent increase in drug seeking is an attempt to regain a positive state, or "hedonic homeostasis" (Koob and Le Moal, 1997). While this popular theory has become ingrained in dopamine literature, the fact that dopamine is often released in anticipation of a reward suggests that dopamine mediated actions are more complex than simply "liking" the stimulus (Schultz, 1998). Brischoux and colleagues have demonstrated that dopamine neurons in the dorsal VTA respond not only to positive signals, but also to noxious stimulation such as footshock (2009). Additionally, dopamine lesions created by 6-OHDA do not impair the ability of a rat to switch between hedonic and aversive learning rules (Berridge and Robinson, 1998). It is now widely accepted that the hedonic

aspects of dopamine signaling are central, though not sufficient to fully describe the role of dopamine in reinforced behaviors (Wise, 2008).

Reward Learning or Prediction

Classic studies by Wolfram Schulz and colleagues have demonstrated that putative dopamine neurons increase firing rate in response to an unconditioned stimulus such as unexpected sucrose delivery. After repeated pairing of the unconditioned stimulus with a conditioned stimulus such as a tone, peak dopamine neuron activity temporally shifts from the reward to the predictive cue (Schultz, 1998). If dopamine truly indicates reward prediction, then neurons should be inhibited by aversive stimuli or cues predicting a negative consequence. Reduced activity of dopamine neurons to both cues predicting the absence of a reward and to aversive stimuli has been observed (Schultz and Romo, 1987; Ungless et al., 2004). Evidence challenging this hypothesis is substantial. *In vivo* recordings from monkeys performing a Pavlovian test in which distinct cues predict either positive or aversive outcomes demonstrate that while some dopamine neurons respond to the reward-predicting cue, a population of midbrain dopamine neurons respond to both cues, and a distinct group of neurons respond directly to aversive cues (Matsumoto and Hikosaka, 2009). This suggests that discrete populations of midbrain dopamine neurons encode associations that extend beyond reward learning. Animals lacking dopamine or dopamine signaling via 6-OHDA lesions typically become severely aphagic, however they will consume food if it is administered to them (Zhou and Palmiter, 1995). Additionally, facial expressions associated with “liking” food remain intact in these animals (Berridge et al., 1989). This suggests that animals have maintained

the association between the pellet and its metabolic reward, and still "like" consuming their chow, but have lost the drive or motivation to actively seek the food pellet.

Incentive Salience

Robinson and Berridge (1998) have proposed that dopamine signaling indicates which stimuli warrant directed action, or what is "wanted." The main distinction between incentive salience and associative learning is that the latter assigns predictive value to cues or stimuli that have been previously paired with positive hedonic experiences, whereas the former involves an assessment of which stimuli or actions are "wanted" whether novel or previously experienced. The concept of "wanting" is a component of motivation that converts a neutral stimulus into one that promotes action by attributing degrees of incentive to distinct goals. The predictive ability of dopamine neuron activity proposed in the prediction theory does not exclude incentive salience. It is possible that predictive cues gain incentive salience of their own (Berridge and Robinson, 1998). In fact this theory essentially incorporates components of the two other theories to explain dopamine function. There are three steps in the process of developing a new reward according to this theory. The first is the hedonic experience with a novel stimulus. Then, associative learning must take place in order to link the conditioned stimulus to the unconditioned stimulus in the formation of a memory. And lastly, the incentive salience induced by the conditioned stimulus incorporates how much the animal "wants" the reward with the homeostatic needs in the context of a given association (Berridge and Robinson, 1998). Reward learning, as Schultz describes it (Schultz, 1998), and incentive salience are not necessarily mutually exclusive hypotheses, depending on the definition

of reward learning or expectation of reward. The reward learning theories of Schultz and others conflict with the incentive salience because reward learning hypothesizes that dopamine is critical for the stimulus-outcome associations of outcomes with positive hedonic value, whereas incentive salience posits that the indication of motivational value occurs independent of hedonic value (Berridge and Robinson, 1998). If reward learning theories define expectation of reward not as a cognitive experience, but as an enhanced level of motivation or incentive to attend to or execute an action to obtain a goal independent of hedonic value experience, then both theories can coexist (Berridge and Robinson, 1998).

LH to VTA Pathway

The lateral hypothalamus (LH), a major source of glutamatergic and peptidergic input to the VTA (Geisler and Zahm, 2006; Geisler et al., 2007), strongly activates reward circuitry (Lorens, 1966). The LH is known for its involvement in motivated behaviors driven by homeostatic state (Boutrel et al., 2010). Such behaviors include feeding, drinking, arousal, and attack (Margules and Olds, 1962; Hrabovsky et al., 2005). Classic electrical ICSS studies revealed that the medial forebrain bundle (MFB) at the LH is one of the most activating regions in the brain (Margules and Olds, 1962). Given that electrical stimulation of the LH could not be isolated from that of the MFB, a number of studies were performed to identify which regions of the MFB were required to produce reward responses. Gallistel and colleagues used autoradiography techniques to identify which brain regions show metabolic changes in response to LH/MFB stimulation (1985). Stimulation in the posterior region of the MFB produced metabolic changes in the

anterior region of the MFB, the diagonal band of Broca, and to a lesser extent, the bed nucleus of the stria terminalis and the medial preoptic area. The VTA showed strong activation posterior to the stimulation site, suggesting that forebrain structure innervation of the VTA contributes to LH/MFB ICSS (Gallistel et al., 1985).

Thus, the robust operant behavior induced by LH/MFB stimulation was attributed to the activation of fibers descending from forebrain structures to the VTA (Wise, 1984). Interestingly, lesions of the MFB do not eliminate the anatomical or functional connectivity between septal or preoptic regions and the VTA (Simmons et al., 1998). This suggests that these regions are interconnected not solely via the MFB, but by a branched network of interconnected fibers. If forebrain projections to the VTA remain functional after lateral hypothalamic lesions, then it is likely that neurons within the lateral hypothalamus are responsible for the loss of motivated behaviors after LH inactivation.

Lesion studies and fiber-cut experiments revealed that ablation of the prefrontal cortex, bed nucleus of the stria terminalis and other forebrain regions did not attenuate LH/MFB stimulation (Arvanitogiannis et al., 1999). Murray and colleagues performed a fiber collision test in which they stimulated fibers in the anterior lateral hypothalamus and the VTA at varied time intervals (1999). If action potentials collided between the two sites they were likely along the same fiber, and in fact, reward-producing signals were transmitted along single fibers between the anterior LH and the VTA (Shizgal, 1989; Murray and Shizgal, 1999). These studies highlight the anatomical and functional connection of the LH to VTA and suggest that this pathway produces the motivating effects of LH/MFB stimulation (Mogenson and Huang, 1973; Maeda and Mogenson,

1981). Interestingly, LH/MFB ICSS was then treated as a tool to simulate natural reward, leaving unanswered questions of whether, and by which mechanisms, LH neurons directly drive neural reward circuitry.

Lateral Hypothalamic Peptides and Reward

The LH is a heterogeneous brain region that produces a number of peptides such as neurotensin (NT), hypocretin (or orexin), melanin-concentrating hormone (MCH), cocaine-and-amphetamine-regulated transcript (CART), neuropeptide Y, neuropeptide S, and corticotrophin releasing factor (CRF), among others. There are distinct as well as overlapping populations (Elias et al., 2001; Cvetkovic et al., 2004; Kerman et al., 2007). NT neurons are concentrated in the anterior region of the LH and send a dense project to the VTA (Geisler et al., 2007). A small portion of NT neurons colocalize with CRF, and CRF neurons are distinct from two other isolated cell populations, hypocretin and MCH (Kerman et al., 2007).

In 1998, hypocretin was identified by Luis de Lecea as a LH peptide with excitatory action (de Lecea et al., 1998). This group went on to describe the critical role this peptide plays in mediating transitions from sleep to wake states (Adamantidis and de Lecea, 2008). Hypocretin is orexigenic along with MCH and is found in cell bodies in the perifornical region of the posterior LH (Nambu et al., 1999).

Until recently, much of the work describing actions of LH neurons has focused on feeding, arousal and homeostasis maintenance (de Lecea et al., 2006). Gary Aston-Jones and colleagues placed new light on the role of LH peptides in reward. His group demonstrated that hypocretin neurons are activated proportionally with conditioned

place-preference for morphine, cocaine and food (Harris et al., 2005). Hypocretin antagonism attenuates morphine CPP (Harris et al., 2005) and the expression of cocaine reinstatement in a self-administration paradigm (Smith et al., 2009). Not only does this highlight the importance of lateral hypothalamic input to the VTA, but it also calls into question which neuropeptides are involved in reward-related behaviors.

NT Overview

NT (NT) is a tridecapeptide found in approximately 30% of the lateral hypothalamic neurons that project to the VTA (Carraway and Leeman, 1973; Binder et al., 2001). Up to 66% of VTA neurons have NT-containing terminals within 5 μm of their cell bodies or processes, and 50% of NT boutons in the VTA originate in the lateral preoptic area-rostral LH (Beaudet and Woulfe, 1992; Geisler and Zahm, 2006). Many previous studies focus on NT release from the VTA into the NAc, however, NT colocalizes with dopamine neurons in the rat VTA, not the VTA of mice or humans (Bayer et al., 1991; Berger et al., 1992; Szigethy and Beaudet, 1989). Therefore, the current study utilizes mice to model human NT action on dopamine neurons.

NT actions are primarily mediated via the high-affinity NT 1 receptor (Nts1), though signaling also occurs through NT receptors 2 (Nts2) and 3 (Nts3) (Vincent et al., 1999; Binder et al., 2001). NT increases the firing rate of dopamine neurons by increasing the conductance of nonselective cation channels (Farkas et al., 1996). This effect is mediated via a calcium-dependent, IP3-dependent mechanism and can be blocked with a non-selective NT receptor antagonist (Farkas et al., 1996). NT increases tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis in the VTA, and promotes dopamine

release by acting at both the nerve terminal and at the somatodendritic level (Sotty et al., 1998; Binder et al., 2001). The peptide decreases the affinity of the dopamine D2 autoreceptor for dopamine and other agonists (Jomphe et al., 2006). Also, activation of NT receptors depolarizes dopamine neurons via two mechanisms. The fast component of depolarization is induced by an increased conductance of non-specific cation channels via activation the second messenger, IP3. The slow phase is modulated by a decrease in the I_h current, or inwardly rectifying K+ channel and is protein kinase C-dependent (Wu et al., 1995; Binder et al., 2001). Nts1 interacts with $G_{q/11}$ via the third intracellular loop of the receptor, and with G_s and $G_{i/o}$ at the C-terminus tail (Pelaprat, 2006).

Behaviorally, NT produces psychostimulant-like actions. Intra-VTA NT alone increases locomotor activity, supports conditioned place preference, induces behavioral sensitization, and is actively self-administered (Kalivas and Duffy, 1990; Glimcher et al., 1984; Elliott and Nemeroff, 1986; Rompre et al., 2006; Glimcher et al., 1987). Not only does this clearly highlight the psychostimulant-like action of NT, but it also suggests that NT receptor antagonists may be effective at blunting effects of drugs of abuse. Indeed, systemic administration of a NT antagonist attenuates the acute and repeated cocaine-induced increases in locomotor activity and rearings. Pre-exposure to NT antagonists delays the induction of behavioral sensitization to cocaine. Also, endogenous NT peptide release in the VTA is necessary for the induction of amphetamine behavioral sensitization.

NT and Glutmate

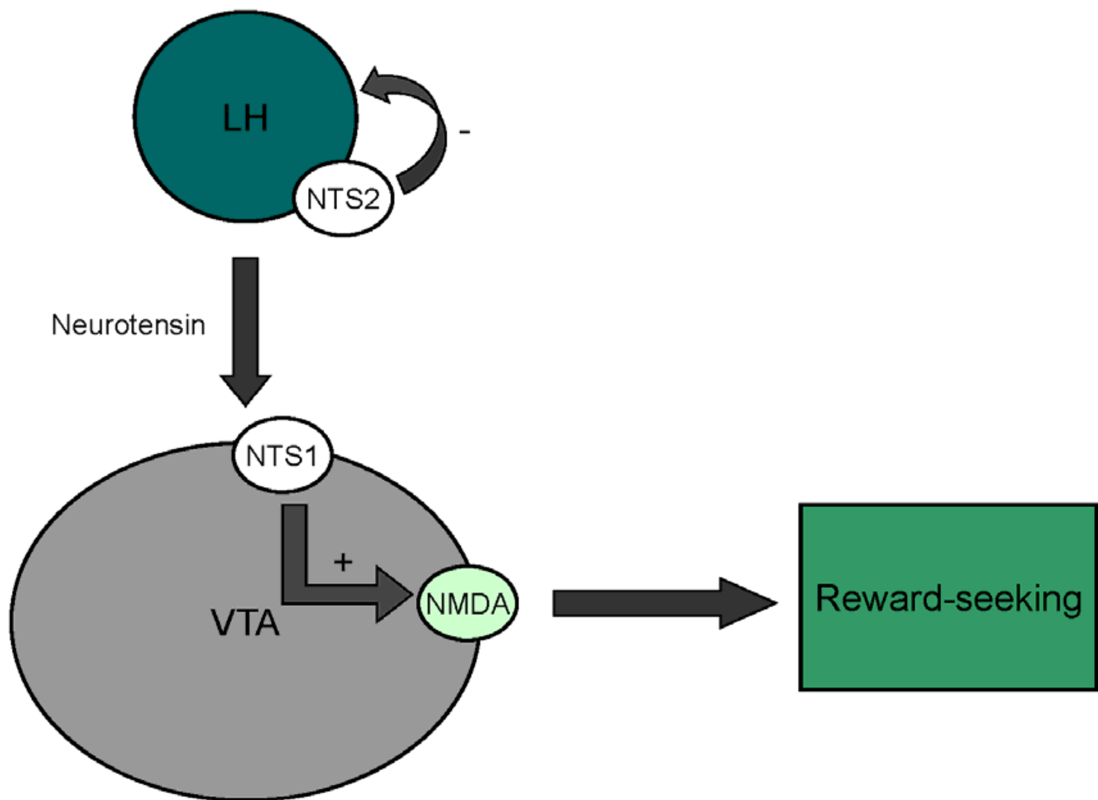
LTP at excitatory synapses is a neural substrate of learning and memory (Whitlock et al., 2006), and given the actions produced by NT signaling, it is appropriate to investigate the ability of NT to modulate glutamate signaling. NT enhances glutamate levels in primary cultures of rat cortical neurons (Antonelli et al., 2004). Co-application of ineffective concentrations of NT and NMDA to cultured neurons promotes glutamate release, suggesting a shared mechanism of these receptors (Antonelli et al., 2004). Microdialysis studies in the rat cortex and striatum demonstrate that NT enhances NMDA-induced glutamate release (Ferraro et al., 2007). This evidence suggests that NT can modulate glutamate responses in the VTA.

Rationale for Dissertation

The mesolimbic dopamine system is a critical component of the neural circuitry underlying motivated behavior. Dopamine release from the VTA into the NAc and long-term alterations in this pathway can contribute to the reinforcing properties of natural rewards and drugs of abuse. The lateral hypothalamus (LH), a significant source of glutamatergic and peptidergic input to the VTA (Geisler and Zahm, 2006; Geisler et al., 2007), responds to modulators released from the periphery (Sahu, 2004), and strongly activates reward circuitry (Lorens, 1966). NT is abundant in the LH to VTA projection, and is anatomically positioned to influence VTA neurons. Given that NT promotes glutamate release (Ferraro et al., 2007) and exerts psychostimulant-like action in the midbrain, we propose that modulation of glutamate transmission in the VTA by NT release from neurons arising in the LH drives reward-related behaviors. The inability to

isolate the actions in the VTA of lateral hypothalamic neurons has prevented close examination of the mechanisms by which LH neurons and associated peptides mediate reward processing in the VTA. Therefore, the current study uses a novel approach to selectively activate lateral hypothalamic afferents within the VTA and identify the role of NT in modulating this pathway. We demonstrate that NT modulates glutamate activity in the VTA to promote reward-related behavior in mice.

Working Hypothesis



Chapter 2

NT promotes glutamate signaling in the VTA

INTRODUCTION

Mesolimbic dopamine neurons are part of the neural circuitry underlying the reinforcing properties of natural and drug reward (Wise, 2004). Drugs of abuse induce long-term potentiation at excitatory synapses in the VTA, rendering dopamine neurons more sensitive to glutamate input (Bonci and Malenka, 1999; Ungless et al., 2001). This suggests that neuromodulators that increase VTA neuron excitability or contribute to LTP induction may also affect reward-seeking.

Understanding LTP induction in VTA dopamine neurons could be significantly expanded by more precisely defining the afferents and physiologically relevant signals that drive dopamine neuron activity. The heterogeneity of VTA neurons is well established (Margolis et al., 2006), making dissection of distinct afferents in the VTA critical for a more complete understanding of mesolimbic neural circuitry.

The LH is a major source of glutamatergic and peptidergic input to the VTA and strongly activates reward circuitry (Lorens, 1966; Sahu, 2004; Geisler and Zahm, 2006; Geisler et al., 2007). NT is one of the most abundant peptides in the projection from the LH to the VTA, is closely anatomically connected with VTA dopamine neurons and is thought to exert excitatory action in the midbrain (Binder et al., 2001). Behaviorally, NT functions quite similarly to psychostimulants calling into question whether the peptide shares similar molecular mechanism as drugs of abuse (Glimcher et al., 1987; Rompre et

al., 1997). Given that NT promotes glutamate release in cortical regions (Ferraro et al., 2000), it is possible that the peptide modulates glutamate signaling in the VTA.

One limitation of slice electrophysiology recordings of VTA neurons is that electrical stimulation activates terminals nonspecifically. Afferents from various brain regions cannot be selectively stimulated, hindering the ability to dissect neural circuits and describe input-selective modulation of VTA neurons. Using optogenetic techniques, we isolate the synapses formed by excitatory lateral hypothalamic neurons and VTA dopamine neurons. To identify the role and locus of NT action in the VTA, we measure the effect of NT on glutamate currents at specific synapses onto dopamine neurons the VTA.

METHODS

Animals and Pharmacological Agents

All procedures conformed to animal care standards set forth by the National Institute of Health and the Ernest Gallo Clinic and Research Center (EGCRC). Male C57Bl/6 mice, age P21-adult, were obtained from Jackson Laboratories or Charles River Laboratories and maintained in accordance with EGCRC IACUC guidelines. NT-cre animals were generated and generously shared by Martin Myers at the University of Michigan.

NT (8-13) was purchased from Sigma-Aldrich; AP5 (50 μM) and DNQX (10 μM) were obtained from Tocris Inc.; SR48692 (100 nM and 500 nM) was ordered through the National Institutes of Mental Health Chemical Synthesis and Drug Supply Program.

Electrophysiology

Horizontal brain slices containing the ventral tegmental area were prepared from C57Bl/6 male mice as previously described (Argilli et al., 2008). The brain was rapidly dissected, and horizontal slices (200 μm thick) containing the VTA were prepared using a Leica vibratome. Slices were allowed to recover for at least 45 min in artificial CSF [aCSF; containing the following (in mM): 126 NaCl, 1.6 KCl, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 18 $NaHCO_3$, and 11 glucose, saturated with 95% O_2 and 5% CO_2] before being transferred individually to the recording chamber and superfused with continuous flow (2 ml/min) of aCSF at 32°C containing picrotoxin (100 μM) to block $GABA_A$ receptor-mediated synaptic currents.

Cells were visualized using an upright microscope with infrared illumination. Whole-cell voltage-clamp recordings were made using an Axopatch 1D amplifier (Molecular Devices) with 3–6 M glass electrodes containing the following (in mM): 120 $CsCH_3SO_3$, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 $N(CH_2CH_3)4Cl$, 2.5 Mg-ATP, and 0.25 Mg-GTP, pH 7.3. Putative dopamine cells were identified by the presence of a large hyperpolarization-activated potassium current, I_h (Lacey et al., 1990; Johnson and North, 1992) or by green fluorescence in TH-GFP mice (n = 9). According to previous studies in mice, tyrosine hydroxylase (TH) is present in approximately 98% of neurons identified by presence of the I_h current (Wanat et al., 2008), and there is general agreement that I_h(-) neurons are not dopaminergic (Margolis et al., 2006). To confirm dopamine neuron identity, cells were filled with biocytin, fixed overnight with paraformaldehyde (4%) and post-hoc labeled with tyrosine hydroxylase. In the present study, 92.3% of the I_h(+) biocytin-filled cells were positive for TH (12 out of 13). Given that experimental procedures and

recording area (lateral VTA) were consistent across experiments, studies utilizing either method of dopamine neuron identification were included. We are therefore confident that experiments were conducted on VTA dopamine neurons.

A bipolar stimulating electrode was placed rostrally at a distance of 100–300 µm from the recording electrode for electrical experiments, and a fiber optic was aimed at the region of interest at a distance of 100–200 µm for optical experiments. Peak evoked AMPA-mediated excitatory postsynaptic currents (EPSCs) were recorded from neurons voltage clamped at -70 mV. Evoked NMDA-mediated EPSCs were measured 25 ms after the stimulus artifact in neurons voltage clamped at +40 mV. Recordings were conducted using 3–5 MΩ electrodes filled with a cesium methanesulfonate solution containing the following (in mM): 120 cesium methanesulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 MgATP, and 0.25 NaGTP (pH 7.2–7.3, 270–285 mOsm). Series resistance (10–30 MΩ) and input resistance were monitored online with a 4 mV depolarizing step (50 ms) given just after every afferent stimulus. Afferents were stimulated at 0.1 Hz, and the evoked EPSCs were filtered at 2 kHz, digitized at 5–10 kHz, and recorded using Igor Pro software (WaveMetrics). Statistics were computed between minutes 16-22 for the 30 minute electrophysiology experiments and minutes 32-40 for 50 minute experiments. Student t-tests were used to determine significance of drug treatment compared to baseline, and one-way ANOVA tests with Tukey's post-hoc analysis to compare differences between three or more groups.

Optogenetics

Animals P21 to adult were anesthetized with ketamine/xylazine prior to the surgical procedure. An adeno-associated virus (AAV) coding for the light-sensitive cation channel, channelrhodopsin-2 (ChR2) and enhanced yellow fluorescent protein (EYFP) under control of the CaMKII promoter were used (courtesy of Karl Deisseroth at Stanford University). In a subset of experiments, NT-cre mice were injected with a double-floxed virus, enabling specific expression of ChR2 in NT-containing neurons. The AAV was injected bilaterally (0.3 µl over 3 minutes) in the anterior region of the lateral hypothalamus (AP: -0.4, ML: ±1.0, DV: -4.9). Electrophysiology experiments were conducted at least three weeks after surgery. Brain slices were prepared as described above and stimulated with a fiber optic coupled to a laser (Laserglow Ltd) aimed at the recording site. Pulses of blue light (wavelength 430-473 nm) were delivered to the slice to evoke EPSCs at 0.1 Hz.

Immunohistochemistry

For immunohistochemical staining, animals were perfused with phosphate-buffered saline (PBS) for 5 min, followed by 4% paraformaldehyde. Brains were dissected, refrigerated at 4˚C in fixative overnight and then transferred to 30% sucrose solution until saturated. 50µm sections were prepared on a Leica cryostat (CM3050). Slices were washed with PBS and refrigerated until pre-blocking the tissue with PBT (0.1% triton in PBS) and 10% normal donkey serum at 25°C for 30 min on a shaker. Slices incubated at 4°C for 16 hours with 1:100 rabbit anti-tyrosine hydroylase (Chemicon, Temecula, CA, USA). Slices were then washed and blocked with 2% normal donkey serum prior to

incubating in Alexa 594 donkey anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA). Finally, slices incubated for 1 hour at 25˚C in 1:100 neurotrace (Invitrogen, Carlsbad, CA, USA) in PBS, then washed, mounted and visualized with a Zeiss LSM 510 META confocal microscope or a Nikon E600 inverted microscope.

RESULTS

NT has bidirectional effects on NMDA Current

Ten minute bath application of NT (10 nM) produced a 19.2 ± 1.3% increase in NMDA-mediated EPSCs in VTA dopamine neurons ($n = 6$, $p < 0.001$, Figure 1A, C and E,). At higher concentrations (100-500 nM), NT produced a reduction in NMDA current that remained depressed after washout (100 nM: 85.1 ± 2.2%, $n = 5$, $p < 0.001$; 300 nM: 66.6 ± 4.5%, $n = 5$, $p < 0.001$; 500 nM: 77.7 ± 1.2%, $n = 5$, $p < 0.001$, pre- versus post-drug exposure). Example traces of NT-induced potentiation and reduction of NMDA current are shown in Figure 1F. The NT receptor 1 (Nts1) antagonist, SR48692 (100 nM), prevented NT-induced potentiation of NMDA currents ($n = 5$, $p = 0.001$, comparing the ten minutes following NT application in the absence and presence of SR48692, Figure 1G). Interestingly, SR48692 attenuated, but did not eliminate, the NMDA current reduction induced by 100 nM NT (NT 100 nM reduces current to 89.7 ± 0.9% in the presence of the antagonist, $n = 8$, and 85.1 ± 2.2% in the absence of antagonist, $n = 5$, $p = 0.002$). This suggests that 10 nM NT activates Nts1 to enhance signaling at the NMDA receptor, but higher concentrations of the peptide reduce NMDA-mediated current via Nts1 and another unidentified receptor. To confirm these antagonist studies, we studied NT 1 receptor knockout (Nts1KO) mice. In agreement with antagonist studies, no NMDA

potentiation was observed after 10 minute bath application of NT (10 nM) in the absence of Nts1 signaling, however the NT-induced reduction at higher concentrations remained intact (Figure 3A and B). The paired-pulse ratio experiments conducted to investigate whether NT produced a presynaptic change in glutamate release were not significant and given the unreliability of this measure, insufficient to draw conclusions (Figure 2F).

NT reduces AMPA-mediated EPSCs

We next determined the effect of NT on AMPA-mediated current. NT reduces AMPA current in a dose-dependent manner (10 nM: 74.4 ± 3.5%, $n = 9$, $p < 0.001$; 100 nM: 60.9 ± 8.7%, $n = 4$, $P < 0.001$; 500 nM: 59.3 ± 3.3%, $n = 6$, $p < 0.001$, Figure 2A and B). Interestingly, this reduction is not due to Nts1 signaling, as SR48692 does not prevent NT from reducing AMPA-mediated EPSCs (Figure 2C, $n = 5$). Also, the NT-induced depression is observed at higher concentrations in NtsR1KO mice (Figure 3C and D). NT reduced AMPA currents at a holding potential of -70 mV (Figure 2A) as well as at +40 mV in the presence of AP5 to block NMDA receptor signaling (71.2 ± 4.3%, $n = 6$, $p < 0.001$, Figure 2D), indicating that the effect is not dependent on driving force.

The 10 nM concentration of NT exerts opposing effects on NMDA and AMPA currents, which raises the question of the net effect of NT on the combined EPSC. We then measured the EPSC approximately 2.5 ms after the stimulus artifact, a measurement point where glutamatergic EPSCs are carried by both AMPA- and NMDA-mediated components (Figure 4A). NT potentiates the combined EPSC (Figure 4C and E), revealing that the overall effect of NT on the glutamate current is excitatory. We then compared the early time point of the EPSC to the late-phase component mediated solely

by the NMDA receptor (25 ms after the stimulus artifact). The combined EPSC increased significantly less than the isolated NMDA current in the same neurons, suggesting that the AMPA-mediated, early phase component of the EPSC is reduced. In fact, we show that AMPA-mediated currents recorded at +40 mV in the presence of AP5 are reduced by NT (Figure 2D), however this effect is overshadowed by potentiation of NMDA-mediated current (NMDA: 123.4 ± 5.2, Combined EPSC: 116.4 ± 3.5%, n = 8, p = 0.037, Figure 4B-F).

Endogenous NT is released at LH to VTA synapses

Given that NT is released predominately from lateral hypothalamic terminals in the VTA, we next asked whether NT exerts differential effects at LH to VTA synapses versus nonspecific, electrically-stimulated synapses. Thus, optogenetic techniques were used to selectively activate lateral hypothalamic terminals forming synapses onto VTA dopamine neurons. The adeno-associated virus coding for channelrhodopsin-2 under control of the CaMKII promoter successfully transfected neurons in the lateral hypothalamus and the terminals extending into the VTA (Figure 5A and B). A horizontal hemisection of the mouse brain (50 µm thickness) shows the rostral LH site of injection in the green AAV-ChR2 panel with clear projections to the VTA. The red channel depicts neurons positive for tyrosine hydroxylase (TH) immunoreactivity in the ventral tegmental area and the *substantia nigra*. The merged inset illustrates that a dense network of ChR2 axons entangle dopamine neurons (Figure 5B). This anatomical connection carries excitatory signals given that a 5 ms pulse of blue light aimed at the VTA excited lateral

hypothalamic terminals and produced both AMPA- and NMDA-mediated EPSCs in VTA dopamine neurons (Fig 5B).

Given the pronounced NT-induced potentiation of electrically-evoked current, NMDA EPSCs were optically stimulated at isolated LH to VTA synapses by replacing the bipolar stimulating electrode with an optic fiber aimed at the recorded neuron. We initially hypothesized that NT would have a greater effect at LH to VTA synapses due to either an increased density of NT receptors at these synapses, or an abundance of endogenously released NT, however we observed the opposite. 10 nM NT decreased optically-evoked NMDA current in the VTA ($70.1 \pm 6.1\%$, $n = 6$, $p < 0.001$, Figure 5C and D). To verify that this effect was not due to recording from two distinct neuronal populations, the experiment was repeated by recording responses to alternating electrical and optical stimulation in the same cell. One pulse of either electrical current or blue light was applied in the VTA 100-200 μm from the cell body at 0.1 Hz. Within the same neuron, NT (10 nM) concomitantly potentiates electrically evoked NMDA current and reduces current at optically-evoked LH to VTA synapses ($n = 8$, $p < 0.001$, Figure 5E and F).

One possible explanation for this divergent effect is that NT release is evoked at LH to VTA synapses, and the addition of NT 10 nM by bath application reproduces the EPSC inhibition observed at high NT concentrations (e.g. Figure 1B and D). To test this hypothesis, we isolated NT-containing neurons in the LH by expressing a double-floxed AAV coding for channelrhodopsin-2 in NT-cre mice. The Cre-lox system ensures that the ChR2 virus is translated solely in NT-containing neurons. If NT is released at lateral hypothalamic NT to VTA synapses, then the Nts1 antagonist, SR48692 (500 nM), would

reduce NMDA-mediated current. In agreement with our hypothesis, we observe that SR48692 lowers optically-evoked NMDA-mediated EPSCs at LH to VTA synapses (Figure 6), suggesting that the NT peptide is in fact released at these synapses. The antagonist has no effect on electrically-evoked current, suggesting that the overwhelming majority of electrically activated glutamatergic axons do not contain NT. Taken together these results indicate that NT potentiates NMDA-mediated current and is endogenously released at LH to VTA synapses.

DISCUSSION

Here we demonstrate that NT enhances glutamate signaling at VTA dopamine neurons. Conventional electrical stimulation techniques were used to perform a dose response study and identify the receptors responsible for this effect. We have also utilized optogenetic techniques to functionally isolate lateral hypothalamic input to the VTA and produce NT release in a slice electrophysiology preparation.

NT modulates glutamate signaling in the VTA

Whole-cell patch clamp recordings of dopamine neurons in the VTA demonstrate that 10 nM NT potentiates NMDA-mediated current via Nts1. NMDA signaling is necessary and sufficient for certain forms of burst firing in the VTA (Zweifel et al., 2009) and for the induction of certain forms of long-term potentiation (Ungless et al., 2001). Both are mechanisms by which NT, via NMDA-receptor potentiation, could induce the VTA neuron excitation needed to elicit robust ICSS and other forms of goal-directed behavior.

Interestingly, NT modulation of glutamate signaling in the VTA is not solely excitatory. At higher concentrations, 100 – 500 nM, NT reduces the inward current mediated by NMDA and AMPA receptors. Experiments conducted in the absence of Nts1 signaling, via pharmacological blockade and in Nts1KO mice, demonstrate that NT-induced synaptic depression is not Nts1-dependent. Given that the Nts2 receptor and has a lower binding affinity than Nts1 (Mazella et al., 1996; Vincent et al., 1999), it is likely that at higher concentrations, binding at Nts2 exerts inhibitory action similar to an autoreceptor. In fact, NT does not produce excitation at Nts2 when expressed in Chinese hamster ovary (CHO) cell lines (Yamada et al., 1998). This may protect VTA neurons from pathological states of hyperactivity such as depolarization block or excitotoxicity. Brain regions containing Nts1 but not Nts2, such as the prefrontal cortex, have been linked to NT-induced glutamate excitotoxity (Antonelli et al., 2004). Nts2 is present in the VTA but not on dopaminergic neurons (Binder et al., 2001), suggesting that Nts2 may prevent overexcitation at high NT concentrations via presynaptic NT action. The peptide may reduce presynaptic glutamate release or promote GABA release in the region. The latter possibility would not explain our results given that picrotoxin was present in all experiments. Although it cannot be ruled out that higher doses of NT activate targets other than NT receptors, the anatomical and functional evidence supports the hypothesis that actions of higher NT concentrations are mediated by Nts2.

It is important to note that at positive potentials where NMDA receptors are activated, +40 mV, the NT-induced NMDA potentiation outweighs the AMPA reduction. Measurement of EPSCs at a time point comprised of both NMDA and AMPA-mediated currents shows overall enhancement, though not as great as the current carried solely by

NMDA receptors at the late phase of the EPSCs. This indicates that while there is a slight reduction in AMPA current at 10 nM, it is not sufficient to significantly reduce or reverse the NT-induced potentiation at +40 mV.

NT is released at LH to VTA synapses

Electrical stimulation in VTA slice preparations nonselectively activates unidentified glutamatergic afferents. We have utilized optogenetic techniques to selectively stimulate lateral hypothalamic terminals in the VTA. NMDA currents evoked at LH to VTA synapses were inhibited, not potentiated by bath applied NT. According to the dose response curve data, higher concentrations of NT also produce this inhibitory effect.

To determine whether endogenous NT was released at the LH to VTA synapse, we further isolated this projection and solely stimulated NT-containing LH neurons. The NMDA current evoked by optical stimulation of NT-containing terminals in the VTA was significantly reduced by bath application of the Nts1 antagonist, SR48692. The difficulty in demonstrating peptide release in slice electrophysiology experiments may arise from electrically activating heterogeneous afferents that do not release the peptide of interest. We have provided evidence consistent with the idea that peptide release can be evoked by optically isolating NT-containing neurons that project to the VTA. Along these lines, a possibility consistent with our data is that there is an inhibitory NT autoreceptor on the NT-glutamatergic terminals of LH cells that project to the VTA. In fact, Nts2 receptors are found in the VTA but not on dopaminergic neurons (Binder et al., 2001). It is also possible that the inhibitory effect on optogenetic glutamate release is indirect, through the activation of neurons or terminals within the VTA that release an

inhibitory neurotransmitter or neuromodulator that acts selectively on LH NT terminals. However, this is unlikely given that: (1) the fiber optic only produced optical responses when aimed directly at the recorded neuron, indicating a small stimulation area and (2) picrotoxin, a GABA antagonist was present in all experiments.

The use of NT-cre mice with a double-floxed ChR2 virus was essential for isolating NT-containing neurons. In addition to developing the NT-cre mouse line, the Myers laboratory also generated a NT-cre-GFP line confirming that NT/GFP expression was in register with the GENSAT NT map (unpublished findings). The NT-cre mouse line enabled us to functionally demonstrate that lateral hypothalamic NT neurons co-release glutamate in the midbrain.

The relative difference in strength of NT activity at nonspecific versus LH to VTA synapses carries significant implications for our understanding of synaptic transmission. The finding highlights the functional heterogeneity of region-specific synapses and provides a mechanism by which neuromodulators tune activity of terminals arising from distinct sources. Using optogenetic techniques, we were able to dissociate a level of specificity that not only represents a more physiological form of stimulation, but also enables the functional characteristics of distinct synaptic subgroups to be explored. Taken together, these results provide a greater understanding of the mechanisms by which NT modulates excitatory synaptic transmission at midbrain dopamine neurons.

Figure Legends

Figure 1

A and B. Single cell examples of evoked NMDA-mediated current in a VTA neuron recorded via whole-cell, voltage-clamp experiments at a holding potential of +40 mV. NMDA currents were measured 25 ms after the stimulus artifact. NT active peptide fragment, 8-13, was bath applied for ten minutes before being washed out. A = NT 10 nM, B = 100 nM.

C and D. Population response to varied doses of NT. C = 10 nM (red): 119.2 ± 1.3%, $n = 6$, $p < 0.001$; D = 100 nM (green): 85.1 ± 2.2%, $n = 5$, $p < 0.001$; 300 nM (blue): 66.6 ± 4.5%, $n = 5$, $p < 0.001$; 500 nM (purple): 77.7 ± 1.2%, $n = 5$, $p < 0.001$, pre- versus post-drug exposure.

E. Average current change produced by doses of NT. Values were averaged between minutes 32 and 40. One-way ANOVA indicates significant effect of the treatment. Using Tukey's posthoc analysis, all concentrations were significantly different from one another ($p < 0.001$ for all comparisons except 100 nM versus 500 nM in which $p < 0.01$)

F. Example traces of NT-induced potentiation at 10 nM and inhibition by 300 nM NT. Approximately 12 sweeps were averaged per trace. The stimulus artifact was removed from the trace example.

G and H. The NT 1 receptor antagonist, SR48692, was bath applied 10 minutes prior to and 10 minutes after NT superfusion. Antagonist concentrations were higher than agonist in each experiment to ensure receptor occupancy by the antagonist. G = SR48692 100 nM blocked NT-induced potentiation at 10 nM (99.2 ± 1.3%, $n = 5$, p =

0.001 compared to NT 10 nM potentiation in the absence of SR48692). H = SR48692 500 nM attenuated NT-induced potentiation at 100 nM (89.7 ± 0.9% in the presence of the antagonist, n = 8, p = 0.002 compared to 85.1 ± 2.2% reduction in the absence of antagonist).

Figure 2

A and B. Dose responses of evoked AMPA-mediated EPSCs to various NT concentrations. 10 nM (red): 74.4 ± 3.5%, n = 9, p < 0.001; 100 nM (green): 60.9 ± 8.7%, n = 4, p < 0.001; 500 nM (blue): 59.3 ± 3.3%, n = 6, p < 0.001, Holding potential was -70 mV. Average values were determined from minutes 16 – 22. One-way ANOVA indicates the treatment was a significant factor (p = 0.009). Tukey's posthoc analysis revealed that the 10 nM change was significantly different from both 100 nM and 500 nM (p < 0.05), however 100 nM and 500 nM were not statistically different from one another (p > 0.05).

C. SR48692, the Nts1 antagonist, was bath applied prior to and following NT application. AMPA-mediated current reduction of 72.5 ± 5.4%, n = 4 was not statistically distinct from the 74.4 ± 3.5% current reduction observed by 10 nM NT in the absence of the antagonist, p = 0.57).

D. AMPA-mediated currents were recorded at a holding potential of + 40 mV in the presence of NMDA-receptor antagonist, AP5 (71.2 ± 4.3%, n = 4, p < 0.001).

E. Response of a single neuron to electrical stimulation in the absence (black) and presence (red) of NT. The stimulus artifact was removed from the trace example.

F. Paired-pulse ratios of two AMPA EPSC peaks evoked 50 ms apart. Cells perfused with 10 nM NT shown in red, 100 nM shown in blue, no significant difference ($p = 0.631$ and $p = 0.475$, respectively).

Figure 3

A and B. Effect of NT on NMDA-mediated EPSCs in NT 1 receptor knockout mice. A = 10 nM (91.1 ± 2.2%, n = 7, $p < 0.001$ from baseline, $p < 0.001$ compared to effect of NT 10 nM in wildtype); B = 100 nM (85.6 ± 2.4%, n = 4, $p < 0.001$ from baseline, $p = 0.823$ compared to effect of NT 100 nM in wildtype).

C and D. Effect of NT on AMPA-mediated EPSCs in NT 1 receptor knockout mice. A = 10 nM (97.1 ± 6.1%, n = 5, $p = 0.114$ from baseline, $p < 0.001$ compared to effect of NT 10 nM in wildtype; B = 100 nM (80.9 ± 1.3%, n = 4, $p < 0.001$ from baseline, $p = 0.004$ compared to effect of NT 100 nM in wildtype).

Figure 4

A. Illustration demonstrating that the EPSC amplitude was noted approximately 2.5 ms after the stimulus artifact to measure current carried by both the AMPA and NMDA receptors, the early- and late-phase components of the EPSC, respectively. The NMDA component alone was measured 25 ms after the stimulus artifact (black). The AMPA-mediated current revealed by bath application of NMDA antagonist AP5 decays quickly (grey).

B. Bar graph plotting average values of potentiation measured at the two points is shown in A. NMDA: 123.4 ± 5.2%, AMPA + NMDA = 116.4 ± 3.5%, n = 8, $p = 0.037$).

C and D. Raw data, single cell responses to AP5 (50 μM), NMDAR antagonist, and DNQX (10 μM), AMPAR antagonist when measured at different time points.

E and F. NT response in a population of VTA dopamine neurons: E = NMDA only (123.4 ± 5.2%), F = combined AMPA + NMDA peak (116.4 ± 3.5%), n = 8.

Figure 5

A. A graphic representation of the gene sequence of the adeno-associated virus. CaMKII promoter, channelrhodopsin2 (ChR2) gene, followed by the enhanced yellow fluorescent protein (EYFP).

B. Horizontal hemisection of mouse brain (50 μm): rostral is at the top of image, midline is along the right side. Neurotrace (blue) was used to visualize background tissue, AAV-induced ChR2-EYFP signal is shown in green (no antibody), tyrosine hydroxylase (TH) antibody was visualized on the red channel to highlight dopamine neurons. Inset shows VTA dopamine neurons surrounded by ChR2-transfected neurons. Scale bar = 20 μm. Example trace shows NMDA current optically-evoked by aiming a fiber optic directly at the recorded neuron in the VTA.

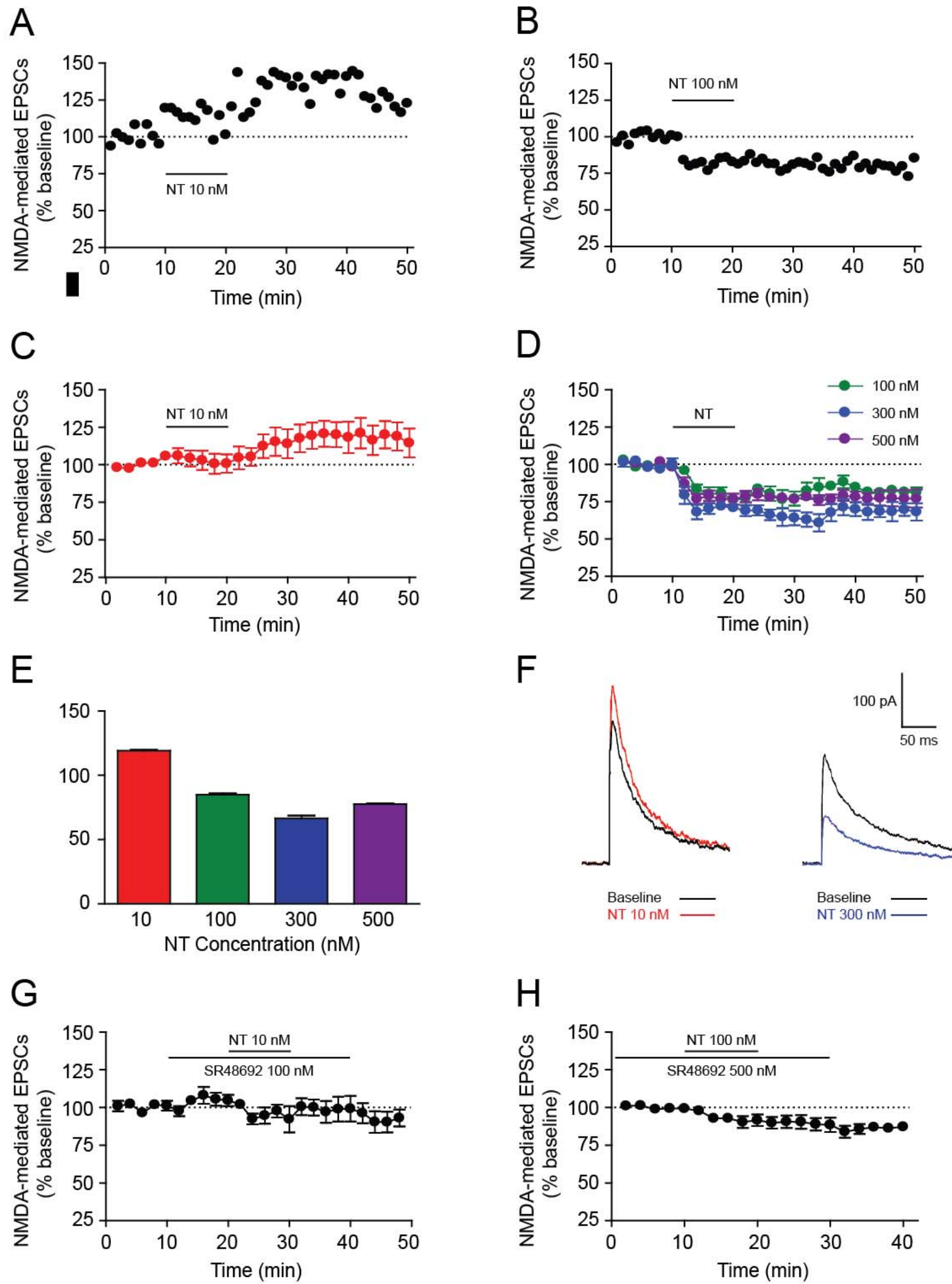
C and D. Comparison of electrically-evoked EPSCs (nonspecific, NS) versus EPSCs evoked by optically stimulating LH terminals (LH) in the VTA. Recordings were performed in separate experiments, however electrical and optical stimulation were both delivered at 0.1 Hz. NS stimulation: 119.2 ± 1.3%, n = 6; LH stimulation: 70.1 ± 6.1%, n = 6, $p < 0.001$).

E and F. The experiment in C and D was repeated by recording alternating electrical and optical responses in the same cell. The neuron received 0.1 Hz stimulation, however every other pulse was delivered by a bipolar stimulating electrode or a fiber optic coupled to a laser, such that each method of stimulation was delivered at 0.05 Hz. NS stimulation: 117.1 ± 5.6%; LH stimulation: 81.8 ± 4.8%, $n = 8$, $p < 0.001$).

Figure 6

A and B. NT-containing neurons in the LH were selectively transfected with ChR2 via the Cre-lox system in mice. A double-floxed ChR2 virus encoding a lox site was injected into the LH of NT-cre mice. Optical stimulation of LH neurotensin-containing neurons in the VTA produced NMDA currents that were reduced by SR48692, Nts1 antagonist (67.2 ± 4.5%, $n = 6$, $p < 0.001$). SR48692 did not produce the same reduction at electrically-evoked synapses (98.4 ± 1.4%, $n = 9$).

Figure 1



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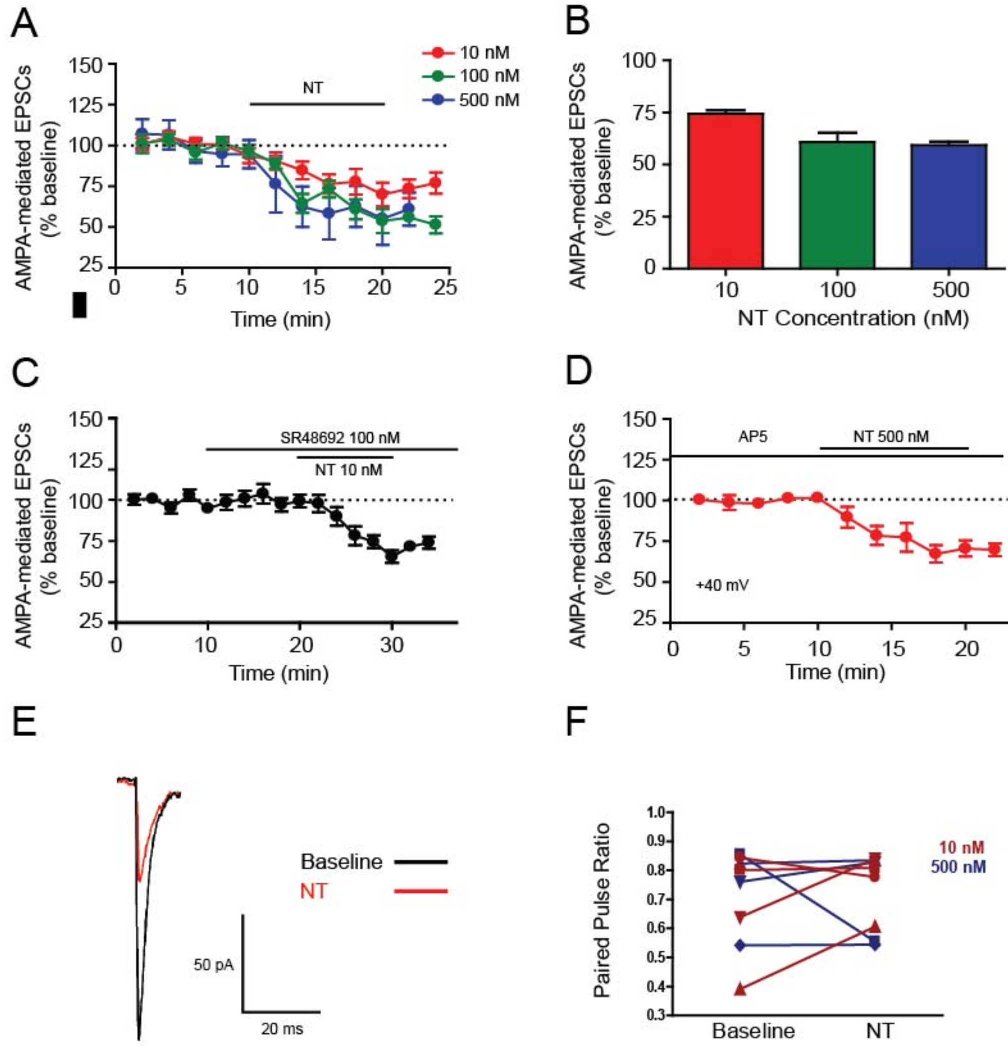

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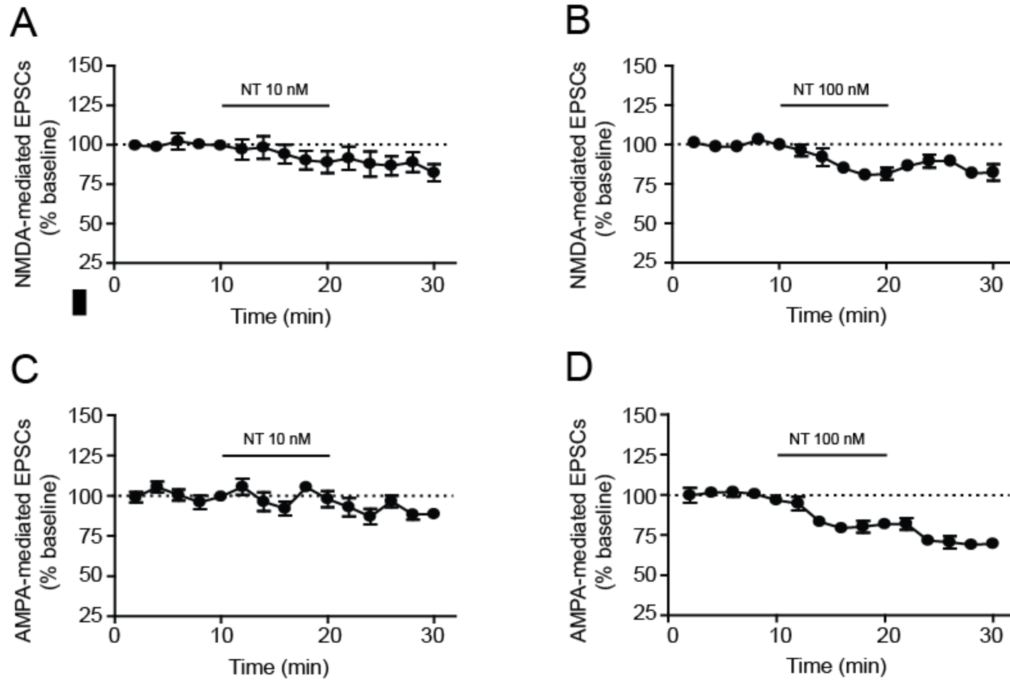
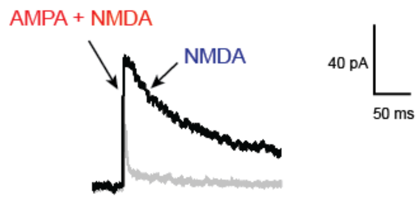


Figure 4

A



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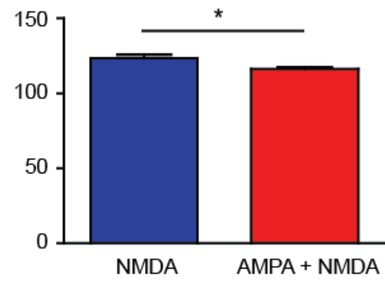

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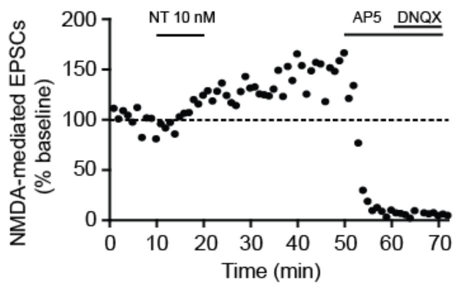

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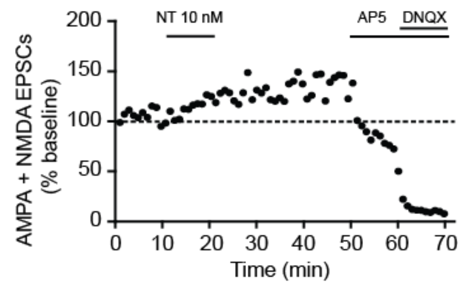

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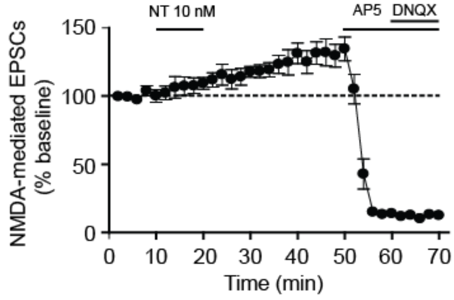

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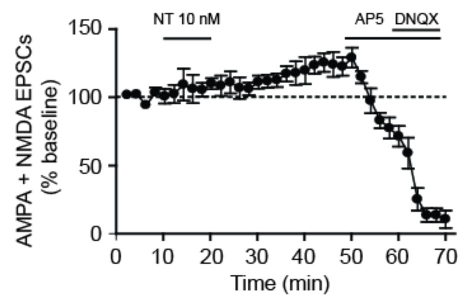


Figure 5

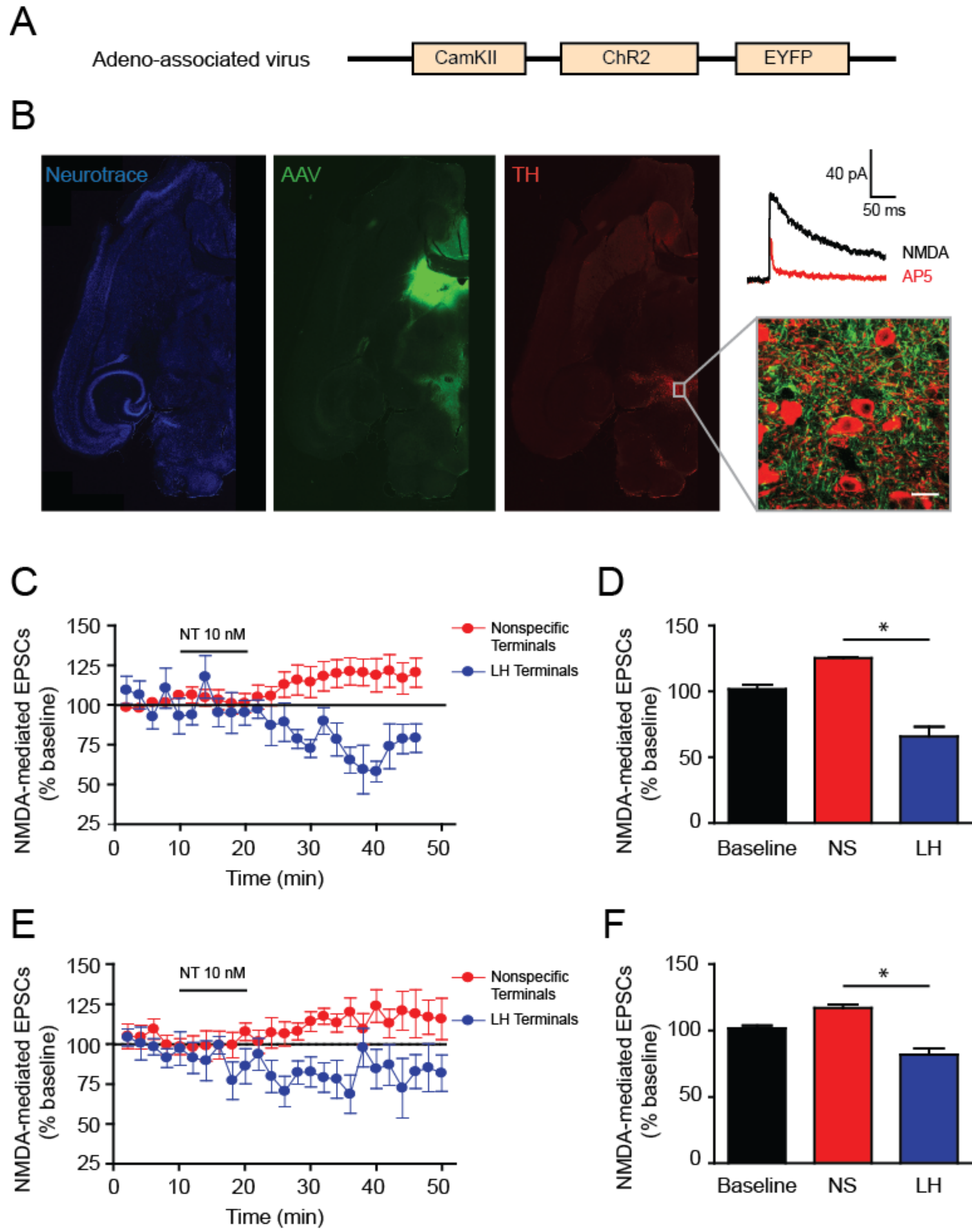
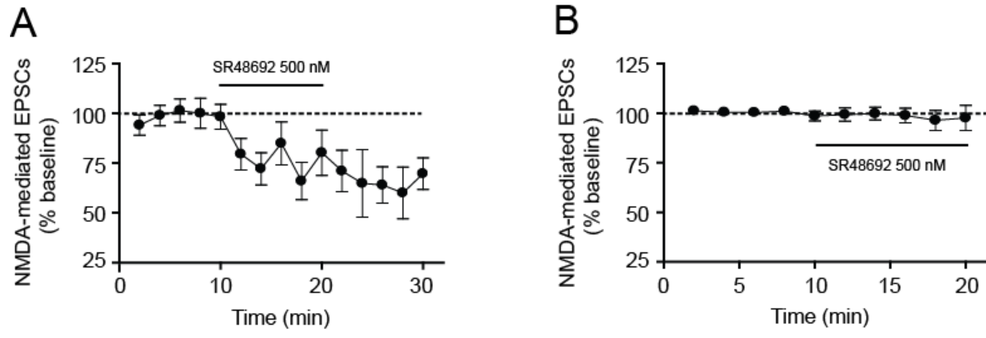


Figure 6



Chapter 3

NT enhances the behavioral effect of optical intracranial self-stimulation of the LH to VTA pathway

INTRODUCTION

The LH, a brain region most commonly associated with metabolism and homeostatic regulation, is as also a critical structure in reward-related behavior (Hess and Akert, 1955; Harris et al., 2005; de Lecea et al., 2006). The LH provides one of the largest sources of glutamatergic and peptidergic input to the VTA, placing the LH in prime position to drive the mesolimbic dopamine system (Geisler and Zahm, 2006; Geisler et al., 2007). VTA dopamine release promotes increased locomotor behavior, is required for certain behavioral models of drug abuse, and is critical for encoding cue-reward associations (Wise, 2004; Flagel et al., 2011). Although competing theories debate the role of dopamine in mediating reward, reinforcement, incentive-salience and hedonic value, there is general agreement that VTA dopamine neuron activation promotes motivated, goal-directed behavior (Berridge and Robinson, 1998; Wise, 2004; Fields et al., 2007). Much emphasis has been placed on elucidating the role of dopamine release in reward-related actions, however the synaptic mechanisms by which specific afferents drive this midbrain region remain poorly understood.

Dating back to the mid-1900s, intracranial self-stimulation studies identified the LH as a reward center in the rodent brain (Hoebel and Teitelbaum, 1962; Margules and Olds, 1962). In this classic instrumental learning paradigm, the high rate of active lever-

pressing to deliver direct electrical stimulation to the LH demonstrates the rewarding properties of the brain region. One major limitation of this work is that electrical stimulation of the lateral hypothalamus simultaneously activates fibers of passage coursing in the MFB. Descending inputs from forebrain structures such as the nucleus accumbens, prefrontal cortex, ventral pallidum and septum are included in this diverse projection to the VTA (Wise and Bozarth, 1984). It is fitting that this “reward highway” promotes such robust seeking activity, however the distinction between these fibers of passage and lateral hypothalamic neurons are blurred by the technical inability to selectively activate LH neurons (You et al., 2001). Therefore, the current study focuses on isolating the effect of LH cell bodies on downstream VTA targets.

Neurons containing the tridecapeptide NT comprise 30% of all LH neurons projecting to the VTA (Fadel and Deutch, 2002). Additionally, up to 66% of NT terminals are within 5 μm of dopamine cell bodies (Beaudet and Woulfe, 1992). Close to half of all NT-containing fibers in the VTA originate in the lateral hypothalamic area-preoptic area continuum (Geisler and Zahm, 2006).

NT is an anatomical and functional powerhouse within the VTA. Intra-VTA infusion of NT promotes locomotor activity, supports conditioned place preference, is actively self-administered, and induces cross-sensitization to cocaine (Kalivas and Duffy, 1990; Glimcher et al., 1984; Elliott and Nemeroff, 1986; Rompre et al., 2006; Glimcher et al., 1987). These psychostimulant-like behavioral effects beg the question of whether lateral hypothalamic NT drives reward-related actions by intra-VTA release. Independently, the VTA and LH are required for certain motivated behaviors, however defining the role of LH terminals in the VTA has been hampered by the inability to functionally isolate the

pathway *in vivo* (Arvanitogiannis et al. 1999). Here we utilize optogenetic techniques to selectively activate lateral hypothalamic afferents within the VTA to demonstrate the behavioral function of this LH-VTA projection.

METHODS

Animals and Surgery

Adult male C57Bl/6 mice were obtained from Jackson Laboratories and maintained in accordance with the IACUC of the Ernest Gallo Clinic and Research Center guidelines. Animals 8-12 weeks old were anesthetized with ketamine/xylazine prior to the surgical procedure. An adeno-associated virus (AAV) coding for the light-sensitive cation channel, channelrhodopsin-2 (ChR2) and enhanced yellow fluorescent protein (EYFP) under control of the CaMKII promoter were used (courtesy of Karl Deisseroth). The AAV (0.3 μl over 3 minutes) was injected unilaterally in the rostral lateral hypothalamus (AP: -0.4, ML: +/-1.0, DV: -4.9) with an ipsilateral 4 mm guide cannula implanted above the ventral tegmental area (AP: -3.2, ML: +/-0.5, DV: -4.0). Both the fiber optics and the microinjectors were designed to project 1 mm beyond the cannula. Behavioral experiments were conducted at least three weeks after surgery.

Optical Intra-Cranial Self-Stimulation

A fiber optic cable (Thor Labs) attached via FC/PC connector to a 430-473 nm laser (blue light, Laserglow Inc.) was inserted into and secured to the guide cannula aimed at the VTA prior to each optical ICSS session. Mice were placed into Med Associates Inc. operant boxes in which they had a choice of two nosepokes. The inactive nosepoke

produced no result, while each active nosepoke activated delivery of a 20 Hz stimulus train of 5 ms pulses of blue light for 3 seconds, presumably depolarizing excitatory lateral hypothalamic terminals in the VTA. Each active nosepoke was also accompanied by a light and tone. Nosepoke activity was recorded with MedPC software and visually monitored via an infrared camera aimed at the operant boxes. Animals were limited to 4.5 g of food per day beginning the day before the first training session. Up to five daily training sessions were allowed for animals to reach the 40-600 nosepokes per session criterion. After reaching criterion, mice were given up to two additional baseline sessions prior to either drug or vehicle administration. Pharmacological agents (0. 3 µl over 3 minutes) were delivered directly into the VTA ten minutes prior to the session start. AP5 (0.5 µg) was obtained from Tocris Inc., SR48692 (500 nM) was ordered through the National Institutes of Mental Health Chemical Synthesis and Drug Supply Program, and the vehicle for both drugs consisted of 0.1% DMSO in saline. The optical intensity was measured prior to and after each session to ensure consistent optical output of approximately 20 mW. The time of each nosepoke was recorded and used for subsequent analysis of operant behavior.

Immunohistochemistry

For immunohistochemical staining, animals were perfused with phosphate-buffered saline (PBS) for 5 min, followed by 4% paraformaldehyde. Brains were dissected, refrigerated at 4˚C in fixative overnight and then transferred to 30% sucrose solution until saturated. 50 µm sections were prepared on a Leica cryostat (CM3050). Slices were washed with PBS and refrigerated until pre-blocking the tissue with PBT (0.3% triton in

PBS) and 10% normal donkey serum at 25°C for 30 min on a shaker. Slices incubated at 4°C for 16 hours with 1:100 rabbit anti-tyrosine hydroxylase (Chemicon, Temecula, CA, USA). Slices were then washed and blocked with 2% normal donkey serum prior to incubating in Alexa 594 donkey anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA). Finally, slices incubated for 1 hour at 25˚C in 1:100 neurotrace (Invitrogen, Carlsbad, CA, USA) in PBS, then washed, mounted and visualized with a Nikon E600 inverted microscope and a Zeiss LSM 510 META confocal microscope.

RESULTS

Activation of lateral hypothalamic terminals in the ventral tegmental area promotes robust nosepoking behavior

We have isolated LH terminals in the VTA of animals performing an operant learning task. Figure 7A shows ChR2-EYFP-transfected terminals in the mouse VTA, indicated by TH immunoreactivity. Mice produced robust nosepoking behavior when given the opportunity to instrumentally activate optical stimulation of LH neuron derived VTA axonal terminals (Figure 7B). On average, animals nosepoked for optical intra-cranial self-stimulation (ICSS) 354 ± 54 times within one hour, with only 9 ± 2 nosepokes at the inactive nosepoke during baseline sessions (n = 8). In all tested groups, there was no significant difference in nosepoke totals between baseline and vehicle infusion (data not shown). To verify that nosepoking was due to optical stimulation in the VTA and not solely in response to the cues associated with the nosepoke, two representative animals were given an extinction session during which the laser was turned off ten minutes after the session start (Figure 9). The rate of nosepoking slowed after stimulation ceased,

demonstrating that optical stimulation was required for enhanced nosepoking, and cue presentation alone was not sufficient to maintain the nosepoking rate in the absence of optical stimulation.

Blockade of endogenous NT in the VTA attenuates self-stimulation of LH to VTA synapses

Given that NT, one of the most abundant peptides in the LH to VTA projection, is actively self-administered into the VTA in rats, we sought to determine whether NT is implicated in this anatomical isolated mouse paradigm (Glimcher et al., 1987). Although NT binds to four known receptors, Nts1-4, Nts1 is the high-affinity receptor thought to underlie the majority of NT-induced behaviors. Mice that received an intra-VTA infusion of SR48692 500 nM, the Nts1 antagonist, prior to the optical ICSS session nosepoked significantly less than vehicle-treated animals at the active nosepoke, with no significant difference at the inactive nosepoke (2-way ANOVA, SR48692 ($n = 5$): 116 ± 37 active, 9 ± 3 inactive; vehicle ($n = 3$): 393 ± 155 active, 12 ± 2 inactive; Treatment: $F(1,12) = 5.12$, $p = 0.043$; Nosepoke Type: $F(1,12) = 15.57$, $p = 0.002$; Interaction: $F(1,12) = 4.90$, $p = 0.047$, Bonferroni post-test: treatment effect at the active lever $p < 0.05$, treatment effect at the inactive lever $p > 0.05$, Figure 7B). In both vehicle and antagonist-treated groups there was a significant difference between the active versus inactive nosepokes (2-way ANOVA, $p = 0.002$, Figure 7B). Nts1 antagonist-treated animals also showed a lower percentage of nosepokes than vehicle-treated animals (SR4892: $33.0 \pm 9.3\%$ baseline, $n = 5$; vehicle: $99.0 \pm 19.6\%$ baseline, $n = 3$; $p = 0.007$). While previous studies have investigated the effect of NT infusion in the VTA (Kalivas and Taylor, 1985), this is the

first demonstration that Nts1 signaling is critical for the operant behavior produced by activation of the LH to VTA pathway.

To determine whether the pattern of nosepoking was altered in the presence of the NT 1 receptor antagonist, SR48692, we plotted the cumulative nosepokes of sessions in the presence of the vehicle versus SR48692 (example animal shown in Figure 8A). Interestingly, animals showed distinct periods of continuous nosepoking punctuated by long pauses. A histogram of the inter-nosepoke intervals, or time between each nosepoke, suggested a bimodal distribution of times between nosepokes (Figure 8B). We therefore defined a bout, or cluster of higher-rate nosepoke activity, as a series of three or more nosepokes with an inter-nosepoke interval less than 30 seconds. Interestingly, the histogram of the representative animal shows that less time was spent in periods of low inter-nosepoke intervals in the Nts1 antagonist condition (red) versus the vehicle condition (blue). This suggests that animals are spending less time in periods of higher frequency nosepoking, or bouts. The raster plots in Figure 8C illustrate a notable difference in bout activity in the absence of NT signaling. The number of bouts, bout duration, and number of nosepokes per bout were marginally, but not significantly, lower during SR48692 trials ($p = 0.643$, $p = 0.132$, $p = 0.120$, respectively, Figure 8D). The preservation of intra-bout nosepoke frequency serves as a motor control, demonstrating that animals were physically able to nosepoke at the same rate whether infused with SR48692 or vehicle ($p = 0.840$, Figure 8D). Taken together these data suggest that the NT 1 receptor antagonist reduced the number of nosepokes per bout, the length of each bout, and the overall number of bouts, to yield a significantly lower number of nosepokes per session.

NMDA receptor signaling in the VTA promotes self-stimulation of LH to VTA synapses

We have demonstrated that NT potentiates N-methyl-D-aspartate (NMDA) receptor-mediated excitatory postsynaptic currents in VTA dopamine neurons. NMDA receptors are required for certain forms of burst firing in dopamine neurons and are critical for the development of synaptic plasticity in the VTA (Bonci and Malenka, 1999; Ungless et al., 2001; Zweifel et al., 2008). To identify whether glutamate signaling at the NMDA receptor is necessary for LH to VTA optical self-stimulation, the NMDA receptor antagonist, AP5, was infused into the VTA prior to the ICSS session. Compared to vehicle, AP5 significantly reduced nosepoking at the active nosepoke (2-way ANOVA, AP5: 48 ± 19 active, 7 ± 4 inactive, $n = 4$; vehicle: 290 ± 104 active, 14 ± 6 inactive, $n = 4$; Treatment: $F(1,12) = 5.64$, $p = 0.035$; Nosepoke Type: $F(1,12) = 9.04$, $p = 0.011$; Interaction: $F(1,12) = 4.97$, $p = 0.046$, Bonferroni post-test: treatment effect at the active lever $p < 0.05$, treatment effect at the inactive lever $p > 0.05$, Figure 7C) as well as percentage of nosepoking ($11.1 \pm 3.7\%$, $n = 4$, $p = 0.011$ compared to vehicle), and did not impair the ability of the rodent to explore the operant chamber or physically perform a nosepoke (Figure 7C). The placements of cannulae aimed at the VTA for the AP5 and control groups of animals are shown in Figure 10. Taken together, these results indicate that glutamate release from the LH to the VTA promotes nosepoking behavior, in large part, via both Nts1 and NMDA receptor activation.

DISCUSSION

Our results demonstrate that LH terminals in the VTA promote motivated behavior via activation of both the NT 1 receptor and the NMDA receptor. To the best of our knowledge, this is the first study to functionally isolate excitatory terminals in the ventral tegmental area that originate from cell bodies in the lateral hypothalamus. Both brain regions play important roles in generating reward-related behavior, and utilization of optogenetic techniques allowed us to demonstrate that lateral hypothalamic innervation of the VTA promotes nosepoking behavior via peptidergic and glutamatergic signaling.

Rewarding peptides in the lateral hypothalamus

Our data indicate that NT receptor activation within the VTA promotes robust appetitive behavior. Numerous studies implicate the peptide in motivated behaviors and animal models of drug addiction. Other peptides such as hypocretin, MCH and CART in the lateral hypothalamus have also been connected to reinforcement and reward (DiLeone et al., 2003). This raises the question of whether other peptides convey salient physiologically-relevant information and if so, what role multiple peptide systems play in goal-directed behavior. It is important to note that the LH receives both central and peripheral signals. Given the neuronal heterogeneity in the LH and the diversity of inputs and targets of those populations, it would be advantageous for numerous peptide systems to modulate specific drives for the generation of the appropriate behavioral output. For instance, NT and hypocretin are anorectic and orexigenic, respectively, when injected intracerebroventricularly (Haynes et al., 1999; Luttinger et al., 1982), however both appear to promote reinforced behaviors when endogenously released in the VTA. This

dichotomy may enable animals in varied metabolic states to produce the behavioral response needed to satiate the associated physiological need.

NMDA receptor activation in the VTA and reinforcement

NMDA receptors promote burst firing in dopamine neurons and are essential for the development of specific types of long term potentiation in the VTA. Here we demonstrate that activation of VTA NMDA receptors also promote intracranial optogenetic operant activation of LH to VTA synapses. The blockade of NMDA receptors may have reduced burst firing in VTA neurons, reducing the release of dopamine in target structures such as the nucleus accumbens. Such a reduction could yield a decrease in motivation to seek the stimulus, a devalued experience with the optical stimulation, or perhaps a break in the association between the nosepoke action and the receipt of intracranial stimulation. It is not likely that the learned connection between the nosepoke and the stimulation was obliterated given that animals continued to poke the active port significantly more than the inactive nosepoke even in the presence of AP5. Further studies are required to elucidate whether the AP5-induced reduction was due to decreased motivation, diminished hedonic value, or increased incentive salience.

Peptidergic modulation of motivated behavior

Operant learning paradigms model certain aspects of goal-directed behavior in humans. Our studies suggest that NT is a key lateral hypothalamic peptide that directly mediates reward-related behavior. Here we demonstrate that SR48692, a small molecule non-peptide antagonist of the NT 1 receptor, reduces self-stimulation without inducing

locomotor deficits. This finding highlights the possible role of this small molecule in reducing excessive amounts of reinforced behavior, while maintaining lower levels of stimulation-seeking. A potential application of this compound is a therapeutic pharmacological agent to reduce forms of pathological reward-seeking in humans such as drug addiction.

Figure Legends

Figure 7

A. Images showing cannula placement aimed at the VTA. Tyrosine hydroxylase (red) indicates dopamine neurons, ChR2 transfected neurons are shown in green (AAV). In the merged image, the asterisk indicates the cannula location, and arrow denotes path of cannula towards the VTA. Scale bar = 500 μm.

B. Rates at which mice nosepoke for optical stimulation in the VTA during a one-hour session. Vehicle is 0.1% DMSO in saline. Day 1 = baseline, Day 2 = vehicle, Day 3 = SR48692, the Nts1 antagonist (n = 5). A separate group of control animals were tested with vehicle on Day 3 to ensure effect was not due to repeated intra-VTA drug delivery (n = 3). Top: raw nosepoke data. SR48692: 116 ± 37 active, 9 ± 3 inactive, n = 5; vehicle: 393 ± 155 active, 12 ± 2 inactive, n = 3; p = 0.043. Bottom: normalized nosepoke data. SR4892: 33.0 ± 9.3% baseline, n = 5; vehicle: 99.0 ± 19.6% baseline, n = 3; p = 0.007.

C. Rates at which mice nosepoke for optical stimulation in the VTA. Animals received either AP5, the NMDA receptor antagonist, or vehicle the day after baseline. Top: raw nosepoke data. AP5: 48 ± 19 active, 7 ± 4 inactive, n = 4; vehicle: 290 ± 104 active, 14 ± 6 inactive, n = 4; p = 0.046. Bottom: normalized nosepoke data. AP5: 11.1 ± 3.7% baseline, n = 4, vehicle: 130.8 ± 38.4% baseline, n = 4; p = 0.011.

Figure 8

A. Cumulative nosepokes plotted for a representative animal after infusion of vehicle and SR48692, the Nts1 receptor antagonist. Each square represents one nosepoke.

B. A histogram depicting the amount of time the representative animal spent executing various inter-nosepoke intervals. Vehicle is shown in blue, Nts1 antagonist is shown in red. The bimodal distribution of time-between-nosepokes indicates that 30 sec is an appropriate definition of what constitutes a bout, or high-frequency period of nosepoking. Bout = three or more nosepokes with and inter-poke-interval less than 30 seconds (dotted line).

C. Raster plots of bout activity from the representative animal. Each red tick represents one nosepoke, every line is one bout within a given session.

D. Analysis of bout activity in all animals treated with SR48692 (n = 5). The average number of bouts in the session, the number of nosepokes within a bout, length of bout and frequency of poking within a bout were plotted. Reduction trend, though not significant, in all areas accept the intra-bout frequency, a motor control.

Figure 9

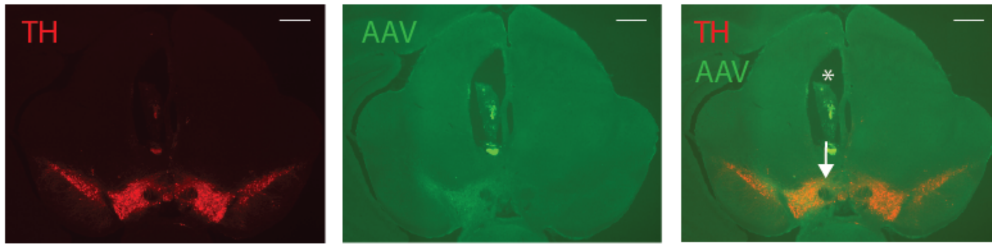
A - D. Nosepoke rates of two example animals during baseline (A and C). Reduced nosepoke activity after optical stimulation was turned off at minute 10, dashed line (B and D).

Figure 10

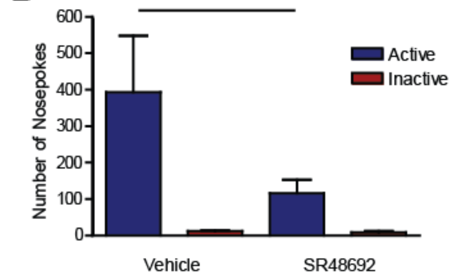
The placements of cannulae aimed at the VTA in the AP5 and control groups are illustrated by ovals on coronal sections of the mouse brain. Microinjectors and fiber optics extended 1 mm beyond the cannulae. The distance from bregma is noted for each section.

Figure 7

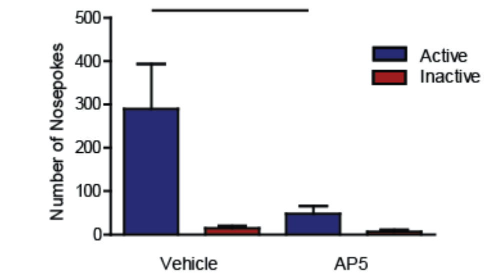
A

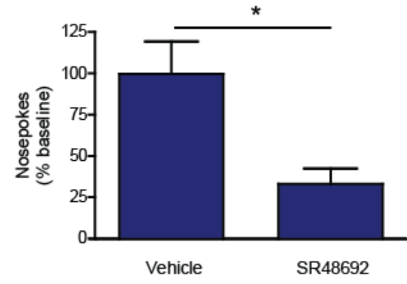


B



C





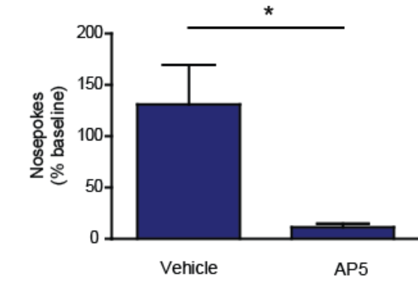


Figure 8

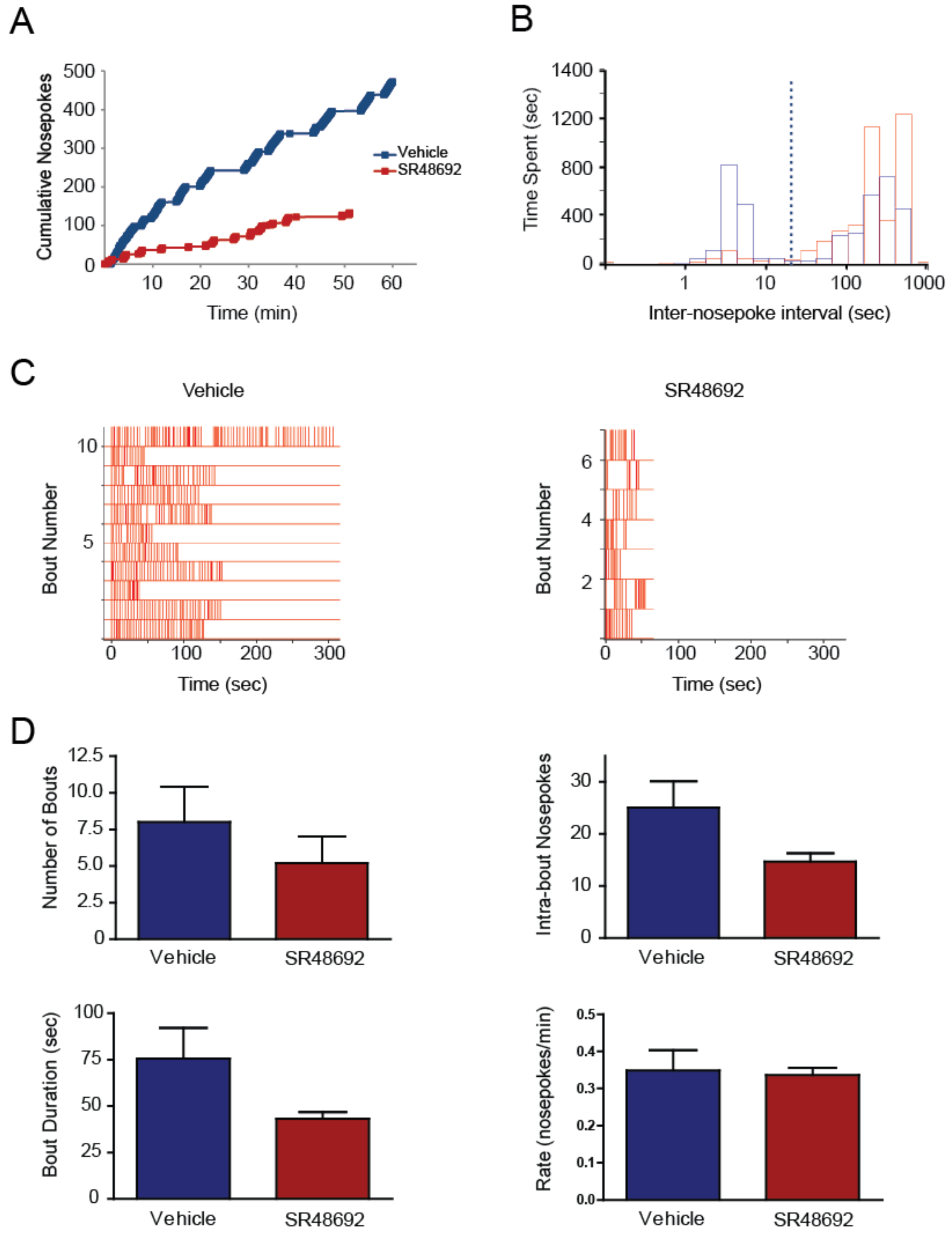


Figure 9

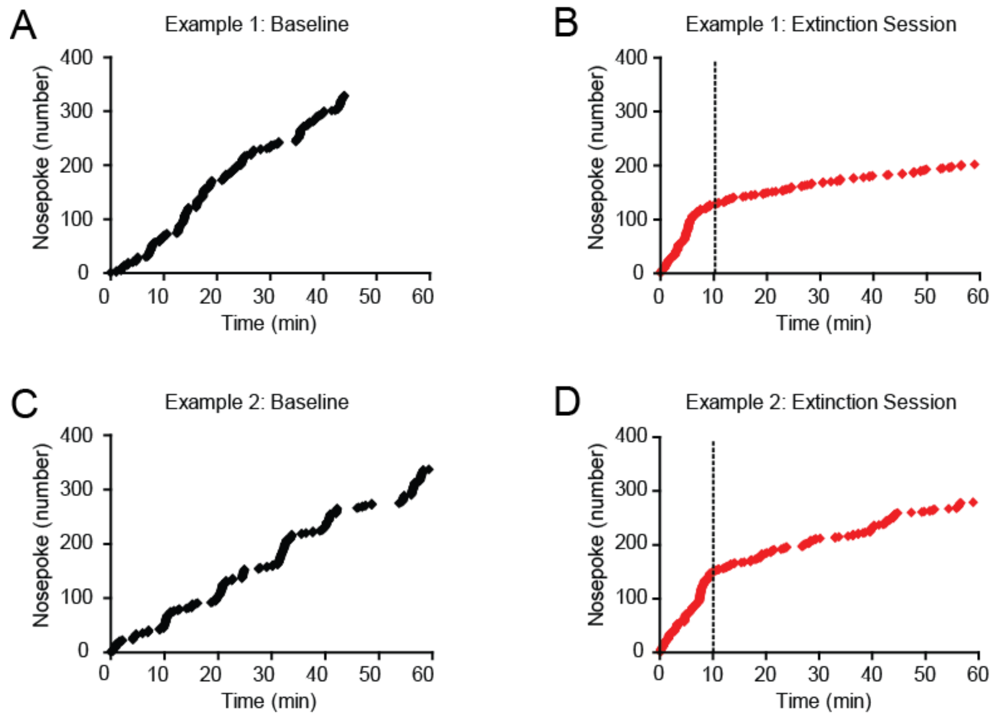
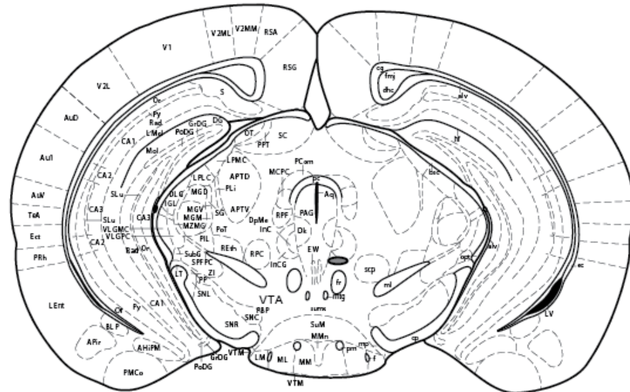
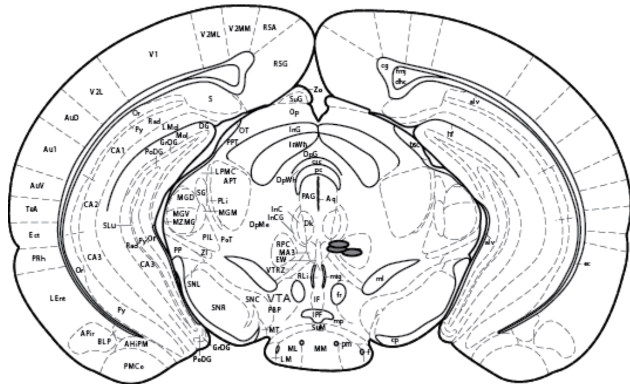


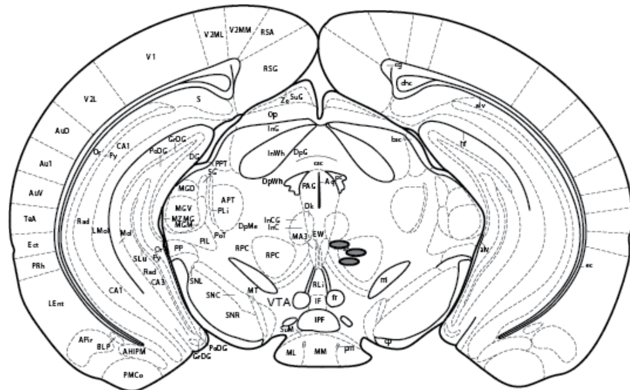
Figure 10



Bregma -2.92 mm



Bregma -3.08 mm



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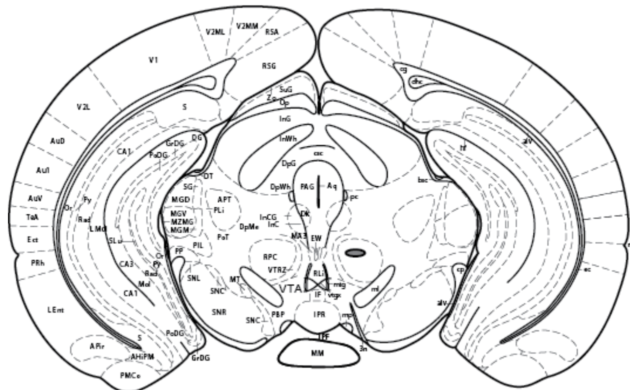

Bregma -3.28 mm

Chapter 4

Discussion

We have shown that NT potentiates NMDA-mediated currents. This excitatory action carries a number of implications given the role of NMDA receptors in the VTA. For one, NMDA receptors are necessary and sufficient for promoting burst firing in dopamine neurons (Phillips et al., 2003). The ability to produce a long-lasting depolarization in neurons, combined with other factors such as calcium influx and potassium channel conductance give rise to the bursting phenomenon (Overton and Clark, 1997). Burst firing is thought to promote dopamine release in VTA projection targets, a salient physiologically-relevant signal (Willuhn et al., 2010). In addition to burst firing, NMDA receptors are necessary for the induction of specific forms of LTP in the VTA (Bonci and Malenka, 1999; Ungless et al., 2001). Therefore, NT may promote the induction of LTP at VTA synapses and/or render VTA neurons more sensitive to glutamatergic inputs such as those derived from activity in lateral hypothalamic neurons.

Although NT enhances NMDA-mediated EPSCs, a reduction in AMPA current is also observed in VTA neurons and this effect is independent of the Nts1 receptor. Given that Nts2 receptors are also present in the VTA (Geisler and Zahm, 2001), we propose that the inhibitory action is via Nts2 activation. In fact, NT is an antagonist at Nts2 in cultured neurons (Yamada et al., 1998). NT has a lower affinity for Nts2 versus Nts1 (Mazella et al., 1996), potentially explaining why greater suppression of both NMDA and AMPA currents are observed at higher NT concentrations. This inhibition of EPSCs may

be mediated pre- or postsynaptically. However, a reduction in presynaptic glutamate release would decrease both AMPA- and NMDA-mediated components of the EPSCs. Given that the paired-pulse ratio experiment did not indicate a reduction in presynaptic glutamate release, and given that 10 nM NT has opposite effects on NMDA and AMPA current, we propose that at least one of these two effects is postsynaptic. If the effect is mediated by a postsynaptic Nts2 receptor, it is possible that the Nts2 receptor acts similarly to an autoreceptor. It would serve a protective role and prevent VTA neurons from excessive NT concentrations. If so, NT would function as a true modulator that enhances signals in a physiological range and prevents loss of salient information that may occur during overexcitation.

However, further experiments are needed to identify which receptor produces NT-induced current decreases. For instance, miniature EPSCs could be recorded to identify the potential source of altered glutamate signaling. Unfortunately, a specific Nts2 receptor antagonist has not yet been developed, however this can be circumvented by using the Nts2 knockout mouse or by creating a Nts2 receptor conditional knockout mouse to avoid compensatory changes that may develop in knockout mice.

The antagonist studies reported here strongly suggest that NT is released in the VTA by optical stimulation of LH derived terminals. Peptide release is quite challenging to demonstrate in slice preparations. There are a number of potential reasons why NT release could be demonstrated here. For one, we have isolated NT-containing neurons using the NT-cre mouse with a double-floxed ChR2 virus. Sole activation of NT-containing neurons may have produced a measurable local concentration of peptide that may have been otherwise undetectable with concurrent activation of non-NT terminals.

Another possible explanation is that optogenetic techniques activate different intracellular machinery than standard electrical methods. ChR2 is a cation channel that permits calcium influx and may increase calcium levels significantly more than electrical stimulation (Nagel et al., 2005). It has been demonstrated that increased intracellular calcium levels promote dense core vesicle release (Elhamdani et al., 2000).

It is possible that NT release from glutamatergic fibers derived from the LH did not exert direct action on dopamine neurons, but evoked NT release from intra-VTA sources. It has been reported that NT colocalizes with dopamine neurons in the VTA of rats (Bayer et al., 1991), but not in mice (Roubert et al., 2004) or humans (Berger et al., 1992). If NT release was evoked from non-LH terminals, then electrical stimulation should have also induced NT release, which was not observed in these experiments.

The heterogeneity of activity at distinct synapses is well known in the synaptic plasticity field (Zalutsky and Nicoll, 1991; Tye et al., 2008), however direct demonstration of synapse-specific peptide modulation is not as well described. Given that NT produces opposing effects at distinct synapses onto the same cell, it is likely that NT modulates afferent-specific information, albeit directly or indirectly. This suggests that NT could differentially modulate inputs such as descending frontal cortex fibers the VTA, while maintaining basal levels of transmission at LH to VTA synapses. Levy and colleagues demonstrate that high-frequency stimulation of the prefrontal cortex reduces cocaine-seeking, but not sucrose consumption (2007). In theory, this could provide a mechanism by which animals are able to assign priority to specific drives and potentially develop habitual reward-seeking for substances like drugs of abuse while maintaining normal consummatory behavior for foods.

NT antagonists reduce optical ICSS of LH to VTA synapses. The numerous dopamine hypotheses call into question which aspect of the goal-directed behavior NT is modulating. The decrease in nosepoking for optical ICSS may have resulted in reduced incentive salience of the stimulation or altered hedonic value. The fact that animals nosepoke at the same rate within a bout suggests that the stimulus-outcome association is intact and that the stimulation is still reinforcing. It may also indicate that once a nosepoke has been performed, the motivation to repeat the action is unchanged. This suggests that the motivation to perform the first nosepoke in a given bout may vary between control and NT antagonist groups.

Additional behavioral tests are needed to gain further insight. Conditioned place-preference studies would identify whether the decrease in ICSS due to Nts1 receptor blockade was associated with an enhanced hedonic value. If NT decreases the hedonic value of the stimulation, the animal should spend less time on the half of the chamber paired with stimulation in the presence of the antagonist. Also, testing animals in the operant chamber on a progressive ratio schedule will indicate whether animals are willing to exert less effort for the stimulation after Nts1 receptor blockade. I predict that NT blockade in the VTA would reduce motivation to nosepoke and therefore lower the number of nosepokes animals will perform to receive stimulation. However, dopamine-independent reinforcement is possible and experimental models should not exclude the possible actions of peptides on non-dopaminergic neurons (Tzchentke, 2000; Margolis et al., 2003; Fields et al., 2007)

An avenue of future research is to repeat the optical ICSS experiment with NT-Cre animals to selectively activate NT-containing neurons. Also, placing ChR2 under control

of various peptide promoters would enable direct comparison of the effects of other lateral hypothalamic peptides in this paradigm. Not only would that identify the relative contribution of LH peptides to mediating this goal-oriented behavior, but it opens doors for other behavioral studies that can identify how activation or inhibition of parallel systems mediate reward-seeking. It should be noted that as a part of this work, a lentivirus placing ChR2 under control of the NT promoter was created. The virus was not selective for NT neurons, in fact, it solely transfected non-NT neurons. However, it would be advantageous to alter the NT promoter fragment to obtain cell specificity for future studies. This would enable NT-containing neuron activation in wildtype animals versus various strains of cre mice.

In addition to cell-specific activation of the lateral hypothalamus, the ICSS paradigm could be expanded to evaluate underlying aspects of the behavior. A wide range of frequency of optical stimulation frequencies and light intensity could be measured, enabling the experimenter to plot the nosepoke rate against the stimulation frequency. The effect of various peptide antagonists can then be used to determine in which direction the curves are shifted. This will indicate whether the peptides are more reinforcing or not. Given the robust nosepoking behavior, one could ask whether this stimulation seeking would be reduced by punishment. If animals continue to nosepoke for optical ICSS in the face of negative consequences, this may be a model of compulsive behavior.

Given the relative novelty of optogenetic approaches in the field (Nagel et al., 2005), a number of fundamental questions have yet to be answered. For instance, the physiological range of optical stimulation duration, frequency and intensity *in vivo* and *in vitro* have not been fully identified. Anecdotally, animals are sensitive to changes in

optical stimulation intensity. Nosepoking for intracranial self-stimulation was significantly reduced when the output of the optic fibers failed during an experimental session. With an increasing number of optogenetic studies being published, standardization across the field would facilitate comparison across experimental paradigms. Also, the use of the CaMKII promoter to drive ChR2 is a highly beneficial tool, however the greatest level of specificity would be obtained via use of promoter-specific approaches such as the cre-lox system or by using more discretely-expressed promoters. I believe that the use of optogenetic techniques will significantly advance the neuroscience field and the understanding of the circuits that drive behavior.

In terms of the larger implication of this work, it is fitting that neuromodulators such as neurotensin mediate synaptic transmission in a synapse-specific manner. I propose that parallel or overlapping networks of modulators would be necessary for creating the appropriate physiological landscape in a heterogeneous brain region like the VTA. For instance, if an animal is hungry, being chased by a predator, and craving a reward like a drug of abuse it must be able to prioritize its needs and identify which actions require immediate attention and action. This is a complex decision-making task which requires synchronous processing in a number of neural networks, however lateral hypothalamic stimulation has been linked to motivated behaviors relating to all of these drives (de Lecea et al., 2006). Hunger may activate orexigenic peptides such as hypocretin and CART to promote food-seeking, while CRF, a stress-related hormone floods the VTA in response to fear. NT release could signal the motivation to seek out the drug of abuse. Under conditions that are not pathological, CRF would prompt escape behavior, followed by food consumption by hypocretin, with NT playing little role in those actions.

However, in pathological states such as drug addiction, NT release could potentially override the other drives to disregard negative consequences and promote drug-seeking.

Numerous peptides have been identified for playing critical roles in reward-related behaviors, leaving the question unanswered of how parallel VTA-stimulating peptides convey salient, behaviorally appropriate information. I propose that specific internal drives and external signals act on the hetereogeneous LH neurons and contribute to the synapse-specific modulation in the VTA to produce motivated actions via distinct targets.

Summary

This dissertation supports the hypothesis that neurotensin functions as a reward-related peptide. Neurotensin potentiates NMDA-mediated excitatory synapses onto VTA dopamine neurons via the Nts1 receptor. Optogenetic techniques can be used to isolate specific synapses and promote peptide release in a slice preparation. Endogenous release of the peptide derived from lateral hypothalamus neurons enhances glutamate signaling in the VTA and promotes optical intracranial self-stimulation of the LH to VTA pathway. This evidence suggests that lateral hypothalamic peptide antagonism may be a therapeutic target for overactive reward-seeking such as drug addiction. This body of work highlights the critical actions of lateral hypothalamic peptides in mediating motivated behaviors and encourages rigorous dissection of distinct neuronal populations within the neural circuits underlying reinforced behavior.

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