Université de Montréal

Neurotensinergic modulation of glutamatergic neurotransmission in VTA neurons.

par
Poulomee Bose

Département de Psychaitrie
Faculté de Médecine

Thèse présentée à la Faculté de Médecine

en vue de l'obtention du grade de Philosophie Doctor (Ph.D)
en Sciences Biomedicale.

Juillet 2015

Université de Montréal

Faculté des études supérieures et postdoctorales

Cette thèse intitulée:

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Présentée par:

Poulomee Bose

a été évaluée par un jury composé des personnes suivantes :

Jean Seguin, président-rapporteur

Richard A Warren, directeur de recherche

Valerie Mongrain, membre du jury

Andrew Chapman, examinateur externe

Roger Godbout, représentante du doyen de la FESP.

Résumé

L'aire tegmentaire ventrale (VTA) contient une forte densité de terminaisons neurotensinergiques ainsi que des récepteurs à la surface des neurones dopaminergiques et non-dopaminergiques. Le VTA a été impliqué dans des maladies comme la schizophrénie, les psychoses et l'abus de substance. Les drogues d'abus sont connues pour induire le phénomène de sensibilisation - un processus de facilitation par lequel l'exposition à un stimulus produit une réponse augmentée lors de l'exposition subséquente au même stimulus. La sensibilisation se développe dans le VTA et implique mécanismes dopaminergiques et glutamatergiques. Il a été montré que les antagonistes neurotensinergiques bloquaient le développement de la sensibilisation et certains mécanismes de récompense et ces effets pourraient être médiés indirectement par une modulation de la neurotransmission glutamatergique. Cependant, on connaît peu les mécanismes de modulation de la transmission glutamatergique par la neurotensine (NT) dans le VTA.

Le but de la présente thèse était d'étudier la modulation neurotensinergique de la neurotransmission glutamatergique dans les neurones dopaminergiques et non-dopaminergiques du VTA. Pour ce faire, nous avons utilisé la technique du patch clamp dans la cellule entière dans des tranches horizontales du VTA pour étudier les effets de différents agonistes et antagonistes neurotensinergiques. Les neurones ont été identifié comme I_h^+ (présumés dopaminergiques) ou I_h^- (présumés non-dopaminergiques) selon qu'ils exprimaient ou non un courant cationique activé par l'hyperpolarisation (I_h). Des techniques d'immunocytochimie ont été utilisées pour marquer les neurones et vérifier leur localisation dans le VTA.

Dans une première étude nous avons trouvé que la neurotensine indigène (NT1-13) ou son fragment C-terminal, NT8-13, induisait une augmentation comparable des courants postsynaptiques excitateurs glutamatergiques (CPSEs) dans les neurones I_h^+ ou I_h^- du VTA. L'augmentation induite dans les neurones I_h^+ par la NT8-13 a été bloquée par le SR48692, un antagoniste des récepteurs NTS1, et par le SR142948A, un antagoniste des récepteurs NTS1 et NTS2, suggérant que l'augmentation était médiée par l'activation des récepteurs NTS1. Dans les neurones I_h^- l'augmentation n'a été bloquée que par le SR142948A indiquant une implication des récepteurs NTS2.

Dans une deuxième étude, nous avons testé les effets de la D-Tyr[11]NT (un analogue neurotensinergique ayant différentes affinités de liaison pour les sous-types de récepteurs neurotensinergiques) sur les CPSEs glutamatergiques dans les neurones I_h^+ et I_h^- en parallèle avec une série d'expériences comportementales utilisant un paradigme de préférence de place conditionnée (PPC) menée dans le laboratoire de Pierre-Paul Rompré. Nous avons constaté que la D-Tyr[11]NT induisaient une inhibition dépendante de la dose dans les neurones I_h^+ médiée par l'activation de récepteurs NTS2. En revanche, la D-Tyr[11]NT a produit une augmentation des CPSEs glutamatergiques médiée par des récepteurs NTS1 dans les neurones I_h^-. Les résultats des expériences comportementales ont montré que des microinjections bilatérales de D-Tyr[11]NT dans le VTA induisait une PPC bloquée uniquement par la co-injection de SR142948A et SR48692, indiquant un rôle pour les deux types de récepteurs, NTS1 et NTS2. Cette étude nous a permis de conclure que i) la D-Tyr[11]NT agit dans le VTA via des récepteurs NTS1 et NTS2 pour induire un effet de récompense et ii) que cet effet est dû, au moins en partie, à une augmentation de la neurotransmission glutamatergique dans les neurones non-dopaminergiques (I_h^-).

Dans une troisième étude nous nous sommes intéressés aux effets de la D-Tyr[11]NT sur les réponses isolées médiées par les récepteurs N-méthyl-D-aspartate (NMDA) et acide α-amino-3- hydroxy-5-méthyl-4-isoxazolepropionique (AMPA) dans les neurones du VTA. Nous avons constaté que dans les neurones Ih^+ l'amplitude des CPSEs NMDA et AMPA étaient atténuées de la même manière par la D-Tyr[11] NT. Cette modulation des réponses était médiée par les récepteurs NTS1 et NTS2. Au contraire, dans les neurones I_h^-, l'amplitude des réponses NMDA et AMPA étaient augmentées en présence de D-Tyr[11]NT et ces effets dépendaient de l'activation des récepteurs NTS1 localisés sur les terminaisons glutamatergiques. Ces résultats fournissent une preuve supplémentaire que le NT exerce une modulation bidirectionnelle sur la neurotransmission glutamatergique dans les neurones du VTA et met en évidence un nouveau type de modulation peptidergique des neurones non-dopaminergiques qui pourrait être impliqué dans la sensibilisation.

En conclusion, la modulation neurotensinergique de la neurotransmission glutamatergique dans les neurones dopaminergiques et non-dopaminergiques du VTA se fait en sens opposé soit, respectivement, par une inhibition ou par une excitation. De plus, ces effets sont médiés par différents types de récepteurs neurotensinergiques. En outre, nos études mettent en évidence une modulation peptidergique de la neurotransmission glutamatergique dans le VTA qui pourrait jouer un rôle important dans les mécanismes de lutte contre la toxicomanie.

Mots-clés : dopamine, aire tegmentaire ventrale, glutamate, neurotensine, courant postsynaptique excitateur, patch-clamp.

Abstract

The ventral tegmental area (VTA) contains a high density of neurotensin (NT) terminals and receptors that are expressed on dopaminergic (DA) and non-DA neurons. This area of the brain is strongly implicated in disorders like schizophrenia, psychosis and drug abuse. Drugs of abuse induce behavioural sensitization- a facilitatory process whereby exposure to a stimulus results in an enhanced response to a subsequent exposure of the same stimulus. Sensitization develops in the VTA and involves glutamatergic neuroadaptations in VTA DA neurons. NT antagonists prevent the development of sensitization and reward mechanisms and this could be mediated through a modulation of glutamatergic neurotransmission in the VTA. However, how NT modulates glutamatergic neurotransmission in VTA neurons remains unclear.

The present thesis was aimed at investigating the NTergic modulation of glutamatergic neurotransmission in VTA DA and non-DA neurons. Whole cell patch clamp electrophysiology in acute VTA horizontal slices was used to study the effects of different NTergic agonists on VTA neurons. Neurons were classified as either I_h^+ (putative dopaminergic neurons) or I_h^- (putative non-dopaminergic neurons) based on the presence or absence of a hyperpolarisation activated cationic current (I_h) respectively. Immunohistochemical techniques were routinely used to label neurons and confirm their location in the medial VTA.

In the first study we report that native neurotensin (NT1-13) or its C-terminal fragment, NT8-13 induced comparable increases in the amplitude of glutamatergic excitatory post-synaptic currents (EPSCs) in VTA neurons. The NT8-13 induced augmentation in I_h^+ neurons was

blocked by SR48692 (NTS1 antagonist) and SR142948A (NTS1/NTS2 antagonist), suggesting that the augmentation effect was mediated by NTS1 receptors. In I_h^- neurons, however, only SR142948A blocked the increase in the EPSC amplitude, indicating the involvement of NTS2.

In the second study we tested the effects of D-Tyr[11]NT (an active NT analog with differential binding affinities for NT receptor subtypes) on glutamatergic EPSCs in I_h^+ and I_h^- neurons and conducted (by Romrpe's Lab) a parallel series of behavioral experiments using a conditioned place preference (CPP) paradigm. We found that D-Tyr[11]NT induced a dose dependent inhibition of EPSCs in I_h^+ neurons that was mediated by the activation of NTS2 receptor. In contrast, D-Tyr[11]NT dose dependently enhanced glutamatergic EPSCs through an NTS1 receptor involvement in I_h^- neurons. Results from behavioural experiments show that bilateral VTA microinjections of D-Tyr[^{11}]NT induced a CPP that was blocked only by co-injection of SR142948A and SR48692, indicating a role for NTS1. This study allowed us to conclude that i) NT acts on VTA NTS1 receptors to induce a rewarding effect and ii) that this effect is due, at least in part, to an enhancement of glutamatergic inputs to non-dopamine (I_h^-) neurons.

The third study entailed investigating the effects of D-Tyr[11]NT on isolated n-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor mediated EPSCs in VTA neurons. We found that in I_h^+ neurons both NMDA and AMPA EPSC amplitudes were attenuated by D-Tyr[11]NT. This attenuation appeared to be mediated by both NTS1 and NTS2 receptors. In Contrast, in I_h^- neurons both NMDA and AMPA EPSC amplitudes were enhanced by an NTS1 dependent mechanism. Additionally, the enhancement effect resulted from a presynaptic potentiation of glutamatergic inputs. These results provide additional evidence that NT exerts a bidirectional modulation on glutamatergic

neurotransmission in VTA neurons and highlights a novel peptidergic modulation of non-DA neurons that might be implicated in sensitization mechanisms. Altogether, our studies allowed us to conclude that the NTergic modulation of glutamatergic neurotransmission in VTA DA and non-DA neurons is oppositely regulated by NTS2 and NTS1 receptors respectively. Additionally it highlights a peptidergic modulation of glutamatergic inputs to VTA non-DA neurons that might be crucial for addiction mechanisms.

Keywords: dopamine, VTA, glutamate, NT, EPSCs, patch-clamp.

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ACSF: Artificial cerebrospinal fluid

AHP: Afterhyperpolarisation

AM251: *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Amyg: Amygdala

ANOVA: Analysis of Variance

AP: Action Potential

APDs: Antipsychotic drugs

APV: (2R)-amino-5-phosphonovaleric acid

ATP: Adenosine triphosphate

BLA: Basolateral amygdala

B_{max}: Maximum specific binding

BMI: Bicuculline methiodide

BNST: Bed Nucleus of Stria Terminalis

CCK: Cholescystokinin

cGMP : Cyclic guanosine monophosphate

CHO: Chinese Hamster Ovary cell lines

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CNS: Central nervous system

CPP: Conditioned Place Preference

D_2R: Dopamine receptor subtype 2

DA: Dopamine

DAG: Diacylglycerol

DAT: Dopamine transporter

DMSO: Dimethyl Sulfoxide

DOPAC: 3,4-dihydroxyphenylacetic acid

DR: Dorsal Raphe

EC: Entorhinal cortex

EGPF: Enhanced green fluorescent protein

EGTA: Ethylene glycol tetraacetic acid

EPSC: Excitatory Post Synaptic Current

GABA: ɣ aminobutyric acid

GAD67: Glutamic acid decarboxylase

GIRK: G-protein coupled inwardly rectifying potassium channels

GLT1: Glutamate Transporter subtype1

GTP: Guanosine triphosphate

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HVA: Homovanillic acid

i.c.v : intracerboventricular

I_h : Hyperpolarisation activated cationic current

IP3: Inositol triphosphate

KA: Kainic acid

K_d: Dissociation constant

KOH: Potassium hydroxide

LDTg : Laterodorsal tegmentum

LH: Lateral hypothalamus

LS: Lateral Septum

LTD: Long term depression

MAPK: Mitogen activated protein kinases

mGluR: metabotropic Glutamate receptor

mPFC: medial prefrontal cortex

nAcb: Nucleus accumbens

NMDA: n-methyl-D-aspartate

NN: neuromedin N

NR1: NMDA receptor subtype1

NT: Neurotensin

NTS: NT receptor subtype

NTS1: Neurotensin receptor subtype1

NTS2: Neurotensin receptor subtype2

NTS3: Neurotensin receptor subtype3

PAG: Periaqueductal gray

PBP: Parabrachial pigmented area

PFC: Prefrontal cortex

PFR: Para fasciculus retroflexus area

PKC: Protein Kinase C

PLC: Phospholipase C

PN: Paranigral nucleus

PPR: Paired pulse ratio

PPTg: Pedunculopontine tegmentum

QX314: *N*-Ethyllidocaine

RMTg: Rostromedial tegmental nucleus

SK: Small conductance calcium activated potassium channels

SN: Substantia nigra

SNC: Substantia nigra pars compacta

SorLA: Sortilin

STN: Sub thalamic nucleus

TH: Tyrosine hydroxylase

VGluT: Vesicular glutamate transporter

vHipp: Ventral hippocampus

VP: Ventral pallidum

VTA: Ventral tegmental area

VTT: Ventral tegmental tail

Dedicated to

My parents for making my dream – theirs.

Shubho- for believing in me.

Pour

Mes parents pour faire mon rêve leur.

Shubho- de croire en moi.

Acknowledgements

It is almost impossible to complete a PhD program alone. Every aspiring PhD student is steered by a team of people who constantly motivate and push the student to do his/her best. I express my heartfelt gratitude to my supervisor, Dr. Richard A Warren for having me under his tutelage for my doctoral studies. He has always been there, leading me to think scientifically, patiently listening to my questions, supporting me during the most difficult times, and always motivating me to achieve more. Without Dr. Richard, this surely would not have been possible.

I sincerely thank all the jury members for having taken out time from their busy schedule to read and revise the manuscript. I express my sincere gratitude to Dr. Pierre Paul Rompre, who has constantly picked me up when I stumbled upon a difficulty and lent me priceless advice on being focused. A special note of thanks to Dr. Valerie Mongrain and Dr. Daniel Levesque for being a part of the comité de parrainage for my PhD. Their comments and inputs have indeed added perspective and direction throughout my studies at the Université de Montreal.

I would like to thank all my friends- colleagues and members of other labs, with who I have had the pleasure of working. A special mention to Claude Bouchard for his ever willing help and Alexandra Gallo, for always saying “You will “. I also take the opportunity to thank my brother, Jaideep Mallick for his relentless inspiration and support.

Finally, a huge thank you to my parents, Pulak Kumar Bose and Swapna Bose for making my dreams theirs. Their faith in me has always instilled the confidence to strive for excellence and without them I would not be where I am. Last but not the least, I thank my husband,

Shubhashish Dhole, for his unconditional support and sacrifice. He has always believed in me and instilled the courage to persist, work hard and achieve.

Introduction

This thesis focuses on the modulation of glutamatergic neurotransmission by neurotensin (NT) in the ventral tegmental area (VTA). NT is a thirteen amino acid endogenous neuropeptide that modulates neurotransmission in several brain regions like the VTA, the prefrontal cortex (PFC) and the nucleus accumbens (nAcb)(Binder et al. 2001a). The VTA is a midbrain dopamine (DA) rich region and plays a role in sensitization, reward, motivation cognition and processes information about emotion(Nestler 2013). The neurotensinergic projections from surrounding brain areas (eg. PFC, lateral hypothalamus(LH),) densely innervate the VTA and 80-90%percent of the VTA neurons express NT receptors, the activation of which is known to modulate DA cell activity and consequently DA dependent behaviors(Rompre et al. 1998). The VTA DA neurons also receive glutamatergic inputs containing NT terminals and receptors from limbic brain regions such as the PFC(Vezina and Queen 2000a).

Drugs of abuse elicit motor stimulant effects that enhance with repeated drug administration, and this sensitized behavioral response can endure for months after the last repeated drug administration(Robinson and Berridge 1993). The sensitized behavioural response is a bipartite phenomenon, consisting of initiation and expression phases. The VTA serves as the key anatomical substrate for the initiation of sensitization. Furthermore, the glutamatergic inputs to the VTA are essential for the development of drug induced synaptic plasticity on DA neurons (Bellone and Luscher 2006;Ungless et al. 2001). It was found that blockade of ionotropic glutamate receptors disrupted the development of psychostimulant induced behavioural sensitization. Interestingly, it was also found that blockade of NT receptors

disrupted the development of sensitization. Since the VTA is heavily innervated by NT receptors and terminals and often these terminals are found on glutamatergic efferents from limbic regions such as the PFC, it is possible that NT modulates these inputs to the DA neurons of the VTA and thereby play a role in the development of sensitization. However, how glutamatergic neurotransmission is modulated by NT in the VTA at the cellular level remains elusive. This study was aimed at characterising the effects of NT on glutamatergic neurotransmission in VTA DA and non-DA neurons using electrophysiological and immunohistochemical techniques.

In my attempt to create a background for my readers, in the subsequent sections, I will draw an overview of the VTA and its projections that are relevant to this study ; its neuronal composition Following this, I will focus on the properties of NT as a neuropeptide neurotransmitter, the distribution of NT neurons and terminals in the midbrain, the mechanism of action of NT ,effects of NT administration in the VTA, effects of NT on glutamatergic neurotransmission and the relevance of a role for NT in reward mechanisms and schizophrenia.

Chapter 1: VTA anatomy, neuronal composition and projections.

The VTA serves as a key anatomical substrate of neuroadaptive changes that result in reward and addiction mechanisms and is also crucial for the development of sensitization. Drugs of abuse induce synaptic plasticity on VTA DA neurons by modulating glutamatergic inputs to the VTA. The present study involves characterizing the neurotensinergic modulation of glutamatergic inputs to VTA neurons. This section therefore focusses on the anatomical organisation of the VTA, its neuronal composition, projections and the functions.

1.1 VTA Anatomy:

In 1984, Lindvall and Bjorklund (Lindvall et al., 1984) grouped the DA containing nuclei of the midbrain and named them A1-A17. The VTA or the A10 group of cells was further divided into four subzones that were called the paranigral nucleus (PN), the parabrachial pigmented area (PBP), the parafasciculus retroflexus area (PFR), and the ventral tegmental tail (VTT). The cell density in these sub-regions is estimated by TH (tyrosine hydroxylase- rate limiting enzyme for DA synthesis) immunocytochemistry (Kohler et al. 1983). The PFR and VTT, border the VTA rostrally and caudally respectively and contain a low density of dopaminergic cell bodies that are small in size. Laterally the VTA is marked by the PN. Both PN and PBP are rich in dopaminergic cells, and comprise mainly of medium to large sized TH-positive cell bodies (Binder et al. 2001a).

1.2 Neuronal composition of the VTA

The VTA is majorly comprised of dopaminergic, glutamatergic and GABAergic neurons. Among other peptidergic neurons that are found in the VTA, the neurotensinergic neurons are particularly relevant in the present study. While there is a controversy over the exact number of each neuronal sub-population in the VTA, there seems to be a general agreement in most studies that in the VTA DA neurons constitute about 60-65%, the ɣ-aminobutyirc acid (GABA) neurons account for 30-35% and the glutamatergic neurons constitute about 5 % of the total cell population (Margolis et al. 2006a;Nair-Roberts et al. 2008;Yamaguchi et al. 2011). About 1-2% of the VTA neurons are neurotensinergic (Binder et al. 2001a). The following section describes the characteristics of each of these subpopulations of VTA neurons.

1.2.1 DA neurons:

Using TH immunohistochemistry Margolis and group, morphologically classified VTA TH-positive DA neurons as fusiform, round, multipolar or elliptical. The fusiform and elliptical dopaminergic neurons, are approximately equal in number and each population constitute about 32% of the VTA DA neurons. Both fusiform and elliptical DA neurons have an elliptical cell body with the exception that fusiform neurons have two dendrites at opposite ends of the major axis whereas elliptical dopaminergic neurons lack a readily identifiable number of dendrites. DA cells with round or multipolar soma constitute 20% and 17% of the VTA dopaminergic neurons. The size of TH positive dopaminergic neurons varied between 200-600μm^2 in horizontal tissue sections of the rat VTA (Margolis et al. 2006a). The dendrites

of VTA DA neurons branch out in forks or tufts and these have 2-3 fine processes which sometimes wrap around another terminal. The VTA dendrites are also possessed with irregular spinule like projections which can possibly serve as dendritic DA release sites. *In vivo* recordings of VTA DA neurons display two modes of spike firing: tonic single spike activity and burst spike firing (Goto et al. 2007;Grace and Bunney 1983a;Grace and Bunney 1983b;Grace and Onn 1989;Koyama and Appel 2006). However, when recorded *in vitro*, in slices, VTA DA neurons display only the tonic pacemaker activity of 0-5Hz (as shown in figure 1B) while the bursts are absent due to the transection of afferent synaptic inputs required to promote a DA neuron to the burst firing mode (Mao et al. 2011;Ungless and Grace 2012).

The resting membrane potential in DA neurons recorded *in vitro* in VTA slices from the rat vary between -44mV and -47mV and the action potential (AP) threshold lies between -24 to-28mV (Margolis et al. 2006a). Recent studies nominate the AP width as a criteria to identify DA neurons. *In vitro* recordings of VTA DA neurons display a broader AP width (than non-DA neurons) of >2 ms as shown in Figure 1C (Mao et al. 2011;Ungless and Grace 2012).

VTA DA neurons are characterized by the presence of a hyperpolarisation activated cationic current (I_h) (Ferrario et al. 2005;Kempadoo et al. 2013a;Margolis et al. 2006a). The I_h current results from an activation of non-specific cationic conductance at hyperpolarised potentials, when the voltage of the neuron is stepped from -40mV to -120 mV and the inward current reflects as a long sag at the most hyperpolarised potential. Figure 1D shows an example of an I_h current in a DA neuron recorded *in-vitro* from a rat VTA slice preparation.

Based on the presence or absence of I_h and TH immunohistochemistry, VTA dopaminergic neurons are classified as $I_h^+TH^+$, $I_h^+TH^-$ and $I_h^-TH^-$ cells. Figure 1 A shows a double labelled

recorded neuron with TH immunohistochemistry (left panel shows: recorded neuron; middle panel: TH positive neuron; right panel: merged). The $I_h^+TH^+$ cell group defines the dopaminergic cell type whereas the $I_h{}^+TH^-$ represent either the glutamate or GABA neurons (Lammel et al. 2014;Li et al. 2013;Margolis et al. 2006a;Margolis et al. 2012a;Ungless and Grace 2012). However there is no ambiguity about I_h^- cells representing the non-DA cell type, the exact neurotransmitter identity of which is still elusive (Lammel et al. 2014;Margolis et al. 2006a).

In addition to the firing pattern of DA neurons, Johnson and North (Johnson and North 1992a) included hyperpolarisation of DA neurons by dopamine D_2 receptor (dopamine receptor subtype 2 which mediates inhibitory actions by inhibiting adenylyl cyclase activity) agonist, quinpirole as a property typical to DA neurons. However recent studies suggest that only 55% of TH^+ DA neurons are hyperpolarised by quinpirole (Margolis et al. 2006a). Additionally, about 20% of TH^+ DA neurons in the VTA are insensitive to quinpirole application. DA neurons are also characterised by the presence of DA transporter (DAT), D_2 autoreceptors and G protein regulated inward rectifier potassium channel subtype 2 (GIRK2) however none of the characteristics unequivocally characterize DA neurons (Bellone and Luscher 2006;Saal et al. 2003;Wanat et al. 2008a).

The use of different parameters for characterizing a DA neuron in different studies, has led to the understanding that the VTA DA neurons are a heterogeneous group of neurons and there exist controversies in the identification and classification of this neuronal population (Ungless and Grace 2012).This issue is addressed in detail in the discussion section of the thesis.

Figure 1 shows the properties of *in vitro* recorded DA neurons in the VTA.

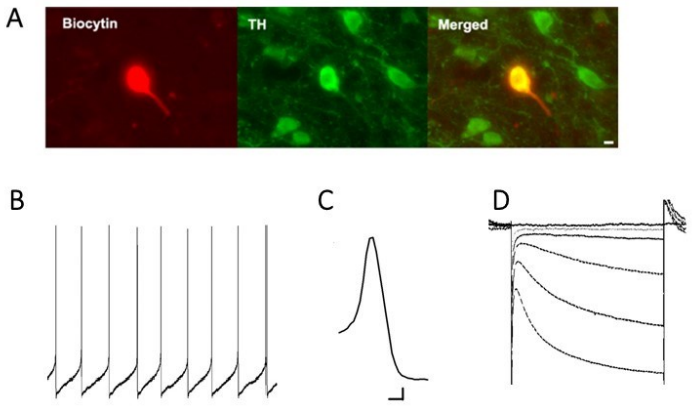


Figure 1: Properties of *in vitro* recorded VTA DA neurons. A, Immunohistochemistry of a VTA neuron filled with biocytin via the recording electrode. Left, Biocytin staining; middle, TH staining; right, co-labelling of biocytin and TH. B, Representative whole-cell recording of regular spontaneous action potentials (tonic pacemaker activity). C, Representative trace of an action potential from a DA neuron. DA neurons were associated with action potential duration of >2 ms. D, A representative trace of I_h induced by hyperpolarizing steps from −40 to −55 mV through −115 mV in 10 mV increments. Scale bar: A, 10 μm. Calibration: B, 350 ms, 6.25 mV; C, 1.0 ms, 10 mV; D, 80 ms, 80 pA. Adapted from Mao D et al., 2011.

1.2.2 GABA neurons:

GABAergic neurons constitute 30-35% of the total neuronal population in the VTA, and their main function in the VTA was assigned as providing inhibitory input to dopaminergic cells by Johnson and group(Johnson and North 1992a). GABA neurons have been studied using glutamic acid decarboxylase (GAD67) immunohistochemistry and like DA neurons, GABA neurons exist in fusiform (44%), round (11%), multipolar (38%) and elliptical shapes (7%). GABA neurons possess fewer dendritic processes and are usually larger than DA neurons with a diameter greater than 30µm. DA neurons were reported to have an approximate diameter of 24-26µm(Grace and Onn 1989;Margolis et al. 2006a).

GABA neurons are grouped into two sub populations based on their firing rates; one neuronal population is characterized by a relatively high frequency firing (≈ 8 Hz), while the second cluster consists of slow-firing cells (≈ 0.7 Hz) (Korotkova et al. 2002;Korotkova et al. 2004). However, a later study reported a spontaneous firing rate of around 5 Hz for GAD67 identified GABA neurons in the VTA (Margolis et al. 2006a). It is important to note that in the former study, the GABA neurons were identified by the lack of TH, smaller AP width and a smaller amplitude of I_h. Recent studies suggest that a small or no I_h and the lack of TH signal also qualify as characteristics for glutamatergic neurons (Hnasko et al. 2012). Therefore it is possible that the two population of GABA neurons also contain a subset of glutamatergic neurons.

The resting membrane potential of GABA neurons recorded *in vitro* is similar to that of DA neurons and range between -44 and -46mV. The AP threshold for GABA neurons is more hyperpolarised than DA neurons, and vary between -30 and -32 mV. However when compared

between the two groups of GABA neurons (as identified by difference in firing rates) the spike threshold is more negative for the fast firing cells (Korotkova et al. 2004). The AP duration for *in vitro* recorded GABAergic neurons of the VTA is smaller than DA neurons and the narrow AP width of <3 ms is often used to identify a GABAergic neuron (Margolis et al. 2012a;Ungless and Grace 2012). Both subpopulations of GABA neurons have little or no I_h current. The amplitude of I_h GABA neurons is smaller than that of DA neurons and range between 5-100pA(Margolis et al. 2012a).

Although initially GABA neurons were differentiated from DA neurons by their sensitivity to opioid peptides and insensitivity to quinpirole (Johnson and North 1992a), recent evidences suggest that GAD67 positive GABA neurons do not unequivocally adhere to these characteristics(Margolis et al. 2012a). In this latter study the authors report that a significant proportion of GABA neurons (7 out of 13) were not hyperpolarised by opioid peptides. Most of these neurons confirmed by GAD67 immunohistochemistry, were found to be I_h^+ and only a small proportion to be I_h^-(4 out of 31 cells). Furthermore, about 30% of GABA neurons are inhibited by quinpirole. However, Korotkova et al.,(Korotkova et al. 2004) report that GABA neurons (as identified by a difference in their firing rates) have little or no I_h, therefore a subset of neurons n the two populations is truly GABAergic as Margolis et al.,(Margolis et al. 2012a) reports that a small proportion of GABA neurons were also I_h^-. Therefore it appears that at least some GABA neurons and some glutamatergic neurons are I_h^- and it is reliable to designate a non-dopaminergic identity to these neurons. In summary, although some I_h^- neurons are GABA, whether GABA neurons are exclusively I_h^- is still debatable as there are reports of contradiction as explained above. However, it is important to note that within the scope of our studies the neurotranmistter content of I_h^+ and I_h^- neuron can only be speculated.

Nonetheless categorizing neurons as I_h^+ or I_h^- does enable us in characterizing effects induced by NT (discussed in Section 4.4).

1.2.3 Glutamatergic neurons:

The identification of glutamatergic neurons in the VTA is comparatively recent and glutamateric neurons are located primarily to the medial aspects of the VTA (Kawano et al. 2006;Lammel et al. 2014). The glutamatergic neurons represent 2-4% of the total VTA neuronal population and share resemblance in terms of electrophysiological and morphological properties to DA neurons of the same area. The glutamatergic neurons of medial VTA are said to have little or no I_h current and have reduced D_2 receptor sensitivity (Root et al. 2014). Since in the present study the location of cell recordings was restricted to the medial VTA, it is possible that a subset of our neurons are glutamatergic. Glutamatergic neurons are characterized by the presence of vesicular glutamate transporters (VGluT- that pack glutamate into synaptic vesicles). All glutamatergic neurons in the VTA contain VGluT2. VGluT2 neurons sometimes colocalise TH and these neurons may co-release dopamine and glutamate from their projection terminals (Hnasko et al. 2012) and are mostly found in the medial VTA (Sanchez-Catalan et al. 2014).

VgluT2 neurons form local synapses on VTA DA and non-DA neurons (Dobi et al. 2010). Furthermore, VGluT2 neurons project to the mPFC (Yamaguchi et al. 2011), which indicates that this population of cells possibly modulate dopaminergic activity not only through direct synaptic contacts but also affect dopaminergic activity indirectly by modulating PFC neurons

that project back to the VTA and provide feedback to dopaminergic neurons (Dobi et al. 2010).

1.2.4 Neurotensinergic neurons:

NT positive cell bodies are located in the dorsolateral aspect of the VTA and there are few NT only neurons in the VTA. Most VTA neurotensinergic neurons co-localise TH and cholecystokinin (CCK) and are significant in number when compared to the substantia nigra pars compacta(SNc) (where NT positive cell bodies do not co-localise TH) (Jennes et al. 1982a;Uhl et al. 1979). The VTA is densely innervated by NT terminals from surrounding brain regions and NT receptors are abundantly found on the cell bodies, dendrites of VTA neurons (Binder et al. 2001a). However NT terminals rarely form direct synaptic contacts on VTA neurons and less than 10 % of these synaptic contacts are with DA neurons. Nevertheless, about 60% of the NTergic terminals are in close vicinity of DA cells (within5µm) thus enabling NT to act on DA cells by volume or paracrine transmission (Woulfe and Beaudet 1989;Woulfe and Beaudet 1992).

1.3 VTA projections and functions:

1.3.1 VTA Projections

The dopaminergic projections from the VTA innervate the PFC, ventral pallidum (VP), nAcb, amygdala, thalamus and the hippocampus and activation of dopaminergic neurons in the VTA lead to DA release in these terminal fields (Oades and Halliday 1987;Sotty et al. 2000a). The dopaminergic projections are implicated in reward, reinforcement, memory

formation, processing information about emotion and drug seeking behaviour (Britt and Bonci 2013;Russo and Nestler 2013). The dopaminergic projections of the VTA are shown in green in Figure 2.

The dopaminergic neurons of the VTA send out projections to different brain regions and often these regions send back projections, thus connecting reciprocally and forming loops (Watabe-Uchida et al. 2012). The concerted activity of such projections provide a positive feedback mechanism that make the VTA a control centre for certain behaviours. For example, psychostimulant induced DA release from VTA DA neurons cause an increase of NT expression in the nAcb shell on neurons that selectively project back to the VTA (Geisler and Zahm 2006). Since NT increase the excitability of DA neurons (Binder et al. 2001a)(discussed in chapter 2), the enhanced release of NT in the VTA, causes DA release in the nAcb which results in enhanced NT release in the VTA. This leads to a positive feedback mechanism, which may contribute to facilitate or lock in neuroadaptive changes associated with psychostimulant drug addiction (Geisler and Wise 2008).

The glutamatergic projections to the VTA come from the PFC, thalamus, amygdala, lateral habenula, hypothalamus, pedunculopontine and laterodorsal tegmentum (PPTG/LDT) and the dorsal raphe (DR). The glutamatergic projections to the VTA from these anatomical substrates are shown in blue in Figure 2. The glutamatergic afferents modulate DA cell firing and activation of glutamatergic neurons in the efferent areas increase the rate of DA cell firing and eventually takes the DA neurons to a burst firing mode that leads to DA release and DA dependent behaviour (Geisler and Wise 2008). For example, PFC glutamatergic neurons

project to DA neurons, that send back efferents to the PFC. Also the PFC projections possess NT receptors at its terminals which may modulate glutamatergic neurotransmission in the VTA and this is particularly crucial to the development of sensitization induced by the psychostimulant –amphetamine (Cador et al. 1999a;Kim and Vezina 1998).

There is also evidence for peptidergic innervation of the VTA. For example, the lateral hypothalamus in addition to sending glutamatergic inputs to the VTA also send orexinergic, and neurotensinergic projections to the VTA (Aston-Jones et al. 2010). The lateral hypothalamus send an abundant source of peptidergic input to the VTA and is particularly relevant for its implication in reward (Tyhon et al. 2008). Recent studies show that lateral hypothalamic NTergic projections to VTA promote reward by modulating glutamatergic neurotransmission in VTA DA neurons (Kempadoo et al. 2013a) (discussed in detail in Chapter 2).

Local interneurons provide the major inhibitory inputs to DA neurons (Tan et al. 2012). The other inhibitory afferents to the VTA arise from the nAcb and the VP (Britt and Bonci 2013;Xia et al. 2011). However, recent studies suggest that the tail of the VTA or the rostromedial tegmental nucleus (RMTg) sends GABAergic projections to the VTA DA neurons and this inhibitory input serves as the " master brake" for VTA dopaminergic pathways (Barrot et al. 2012).The inhibitory inputs to the VTA are shown in red in Figure 2.

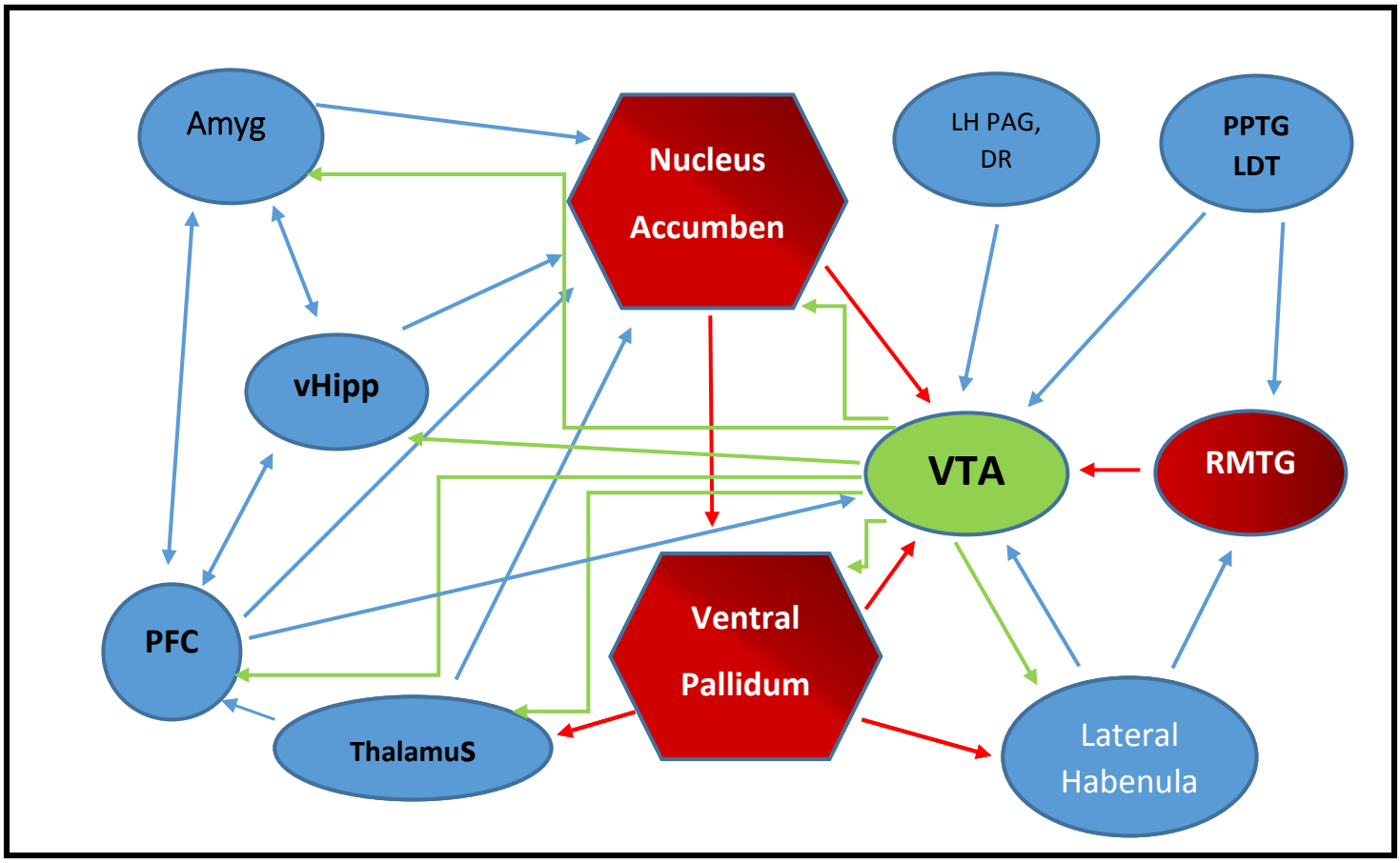


Figure 2: Schematic of the principal brain regions that innervate the VTA. Green: Dopaminergic projections; Blue: excitatory projections; Red: inhibitory projections (Modified from *Britt and Bonci 2013*). *Abbreviations*: Amyg, amygdala; vHipp, ventral hippocampus; LH, lateral hypothalamus; PAG, periaqueductal gray; DR, dorsal raphe; PPTG/LDT, pedunculopontine and
Laterodorsal tegmentum; RMTG, rostromedial tegmental nucleus.

1.3.2 Functions of the VTA:

Being one of the major substrates of the limbic system, the VTA is implicated in reward, working memory formation, cognition, motivation and drug addiction. The functional role of VTA depends on its afferent inputs and efferent outputs. Since majority of the VTA neuronal population is accounted for by DA neurons, most of its functions also involve these neurons.

The dopaminergic projection from the VTA to the nucleus accumbens is vital for the development of drug addiction ,sensitization and reward mechanisms (Britt and Bonci 2013) (Grueter et al., 2012; Britt et al., 2013). In an elegant study by Deisseroth and colleagues (insert citation) it was shown that optogenentic stimulation of VTA DA neurons induced intracranial self-stimulation in rats, promoted conditioned place preference to psychostimulants whereas stimulation of GABAergic interneurons in the VTA disrupted reward and promoted conditioned place aversion(Tan et al. 2012;van et al. 2012;Witten et al. 2011). Enhanced DA cell firing in the VTA, leads to release of DA in the terminal fields and this release augments with repeated activation of DA neurons induced by the action of psychostimulants.

The dopaminergic projection to the PFC is critical for working memory formation and learning (D'Ardenne et al. 2012). Normal cognitive functions, motivated behaviour, emotions as well as pathological manifestations for e.g. schizophrenia and ADHD (attention deficit hyperactivity disorder) have also been implicated in abnormal functioning of these projections (Lammel et al. 2008;Volkow et al. 2011). Prefrontal cortical dopamine tone is characterized by an inverted U shaped dose response curve, suggesting that too much or too little DA perturbs working memory formation and other PFC functions (Arnsten 2009;Arnsten and Li

2005). This suggests that functionally distinct sets of DA neurons projecting to different cortical layers are activated at either ends of the inverted U curve, thereby mediating the dual roles. It is possible for example, that DA neurons that have a role in working memory project selectively to cortical layers associated with primary sensory cortices, thereby keeping the continuum of representation of a stimulus even in its absence(Chandler et al. 2014).

On the other hand, afferents from the LHb to the VTA synapse on DA neurons that project to the medial PFC and induce aversion (Lammel et al. 2012).Thus it is possible that these DA neurons selectively project to PFC regions associated with limbic structures and not sensory structures.

Lateral hypothalamic projections to the VTA and NAcc have been associated with pain modulation. Stimulation of LH efferents (orexinergic) to the VTA can induce antinociception, thus suggesting the involvement of VTA DA neurons. Recent studies highlight a preferential role for D2 receptors in the NAcc over VTA D2 receptors in antinociception. However, a role for D1 receptors in the VTA has been attributed to antinociception suggesting the involvement of a concerted D1-D2 mechanism in antinociception (Moradi et al. 2015a;Moradi et al. 2015b).

LH VTA projections are also implicated in reward. Activation of NT neurons in the LH that project to the VTA were found to augment locomotor activity , induce prolonged dopamine efflux in the ventral striatum and transient increase in VTA NT levels. Intra VTA injections of NT antagonist, attenuated DA efflux in the NAcc, suggesting that lateral hypothalamic afferent induced transient NT release in the VTA links LH signalling to prolonged DA release in the NAccc, thereby affecting reward and mesolimbic functions (Patterson et al. 2015).

Recent studies report that selective activation of VTA DA neurons rescue depression like symptoms in mice subjected to chronic mild stress (Chaudhury et al. 2013). Owing to the heterogeneity of DA neurons in the VTA, VTA functions emerges as a direct readout of functioning of distinct neuronal populations within the VTA.

Chapter 2: Neurotensin synthesis, receptors and effects.

An action of NT in modulating glutamatergic neurotransmission in the ventral midbrain is required for the development of amphetamine sensitization. Since NTergic innervation is dense in the VTA and VTA neurons possess NT receptors, NT may modulate DA neural activity either directly or by modulating inputs to VTA neurons. This section aims to discuss NT as a neuropeptide neurotransmitter, the distribution of NT in the midbrain, the mechanism of action of NT, effects of NT administration in the VTA, effects of NT on glutamatergic neurotransmission and the relevance of a role for NT in reward mechanisms and schizophrenia.

2.1.1 Discovery and synthesis:

NT is a tridecapeptide that was originally isolated and sequenced from the bovine hypothalamus in 1973(Carraway and Leeman 1973). The NT gene encodes a 170-amino acid precursor protein that contain both the tridecapeptide NT and a closely related hexapeptide, neuromedin N (NN). In the brain, NT and NN are produced by the action of the prohormone convertase PC2(Kitabgi 2010).

In neurons, NT is stored in dense core vesicles and is released in a traditional calcium dependent manner. NT transmission is arrested by the cleavage of NT by endopeptidases.

2.1.2 NT as a neuropeptide neurotransmitter:

NT is an endogenous neuropeptide that serves as a neuromodulator and neurotransmitter in the central nervous system(CNS) (Vincent et al. 1999). In the CNS, NT is known for its role in reward mechanisms, pain modulation and regulation of body temperature (Kleczkowska and Lipkowski 2013) . The neuromodulating role of NT in dopaminergic and glutamatergic systems is implicated in diseases like Parkinson's disease and schizophrenia (Binder et al. 2001a;Tanganelli et al. 2012). NT is believed to act both as a psychostimulant and a neuroleptic in the CNS as NT administration produced similar dopamine dependent behaviours in animals that receive exposure to psychostimulants (Dobner et al. 2003;Fadel et al. 2006). On the other hand NT also increases glutamate levels in the thalamocortical system (projections from the thalamic nucleus to the prefrontal cortex) that is hypothesized to ameliorate negative symptoms such as cognitive deficits associated with schizophrenia (Borroto-Escuela et al. 2013).

2.1.3 NT receptors:

NT has three well characterised receptors, NT receptor subtype (NTS) 1 to 3. NTS1 and 2 belong to the G protein coupled receptor family, with 7 transmembrane domain. NTS 3 belong to the single transmembrane domain receptor type 1 and is mainly located intracellularly (Mazella et al. 1998;St-Gelais et al. 2006a)

The possibility of the existence of a fourth NT receptor , SorLA/LR11, has been proposed, which like NTS 3 receptors belong to the single transmembrane domain receptor type 1(St-Gelais et al. 2006a).

NTS1 receptors:

NTS1 receptor was first cloned in rat and is the high affinity receptor for NT (K_d = 0.1-0.3nM). NTS1 receptor is expressed in dendrites, cell bodies and terminals in the VTA. NTS1 is the predominant receptor subtype in DA cells of the VTA and exist at presynaptic as well as postsynaptic sites to VTA neurons (Binder et al. 2001a). Functionally, NTS1 is coupled to phospholipase C (PLC), inositol triphosphate (IP3), mitogen activated protein kinases (MAPKs) and the production of diacylglycerol (DAG). These signalling cascades are linked to an elevated level of intracellular calcium and suggest induction of excitatory effects to depolarise the neuron (St-Gelais et al. 2006a;Trudeau 2000). NTS1 receptor activation is also linked with an enhanced formation of cyclic guanosine monophosphate (cGMP) and production of arachidonic acid (Binder et al. 2001a;Binder et al. 2001c). The properties of NTS1 receptors in terms of its size, location, receptor type, agonists and antagonists have been summarized in Table 1.

Once NT binds to NTS1, the receptor ligand complex is internalized into neurons that express NTS1 and the NTS1 receptors reach the lyzosomes for degradation (Beaudet et al. 1994;Vandenbulcke et al. 2000). Recent studies suggest that prolonged exposure to NT agonists might result in a second gene activation process, resulting in recycling of some NTS1

receptors to the cell surface and this process is mediated by NTS2 receptors(Perron et al. 2006).

NTS2 receptors:

NTS2 receptor is a low affinity NT receptors (K_d =3-10nM)(Binder et al. 2001a). Table 1 summarizes the properties of NTS2 receptors in terms of its location, size, receptor classification, agonists and antagonists. While the NTS2 receptor share sequence homology with NTS1 receptor, these receptors functionally coupled to different downstream signalling cascades. NTS2 receptor does not stimulate cytosolic calcium mobilization or IP3 accumulation but is linked to mitogen activated protein kinases (MAPK) and are suggested to elicit inhibitory effects when cloned human NTS2 receptors are expressed on Chinese Hamster Ovary cell lines(CHO) cell lines (Sarret et al. 2002). However, when the human NTS2 receptors were transfected in COS cells, IP3 production was reported to be constitutive. This constitutive activity was enhanced by almost 50% by an NTS1 antagonist (SR48692), not affected by NT concentrations of up to 10μM and decreased below constitutive levels by levocabastine (a histaminergic antagonist which is known to bind to NTS2 receptors, thus suggesting a weak partial inverse agonist activity. Additionally, NT, concentration dependently reversed the effect of SR48692 back to constitutive levels, suggesting that it acts like a neutral antagonist. Therefore, whether NT acts as an agonist, inverse agonist or neutral antagonist for NTS2 receptors is undetermined (Richard et al. 2001).

In contrast to NTS1, NTS2 receptors once sequestered into the cell as NT-NTS2 complex, preferentially reaches the recycling complex and efficiently recycles back to the cell surface (Botto et al. 1998).

NTS3 receptors:

NTS3 receptors are also called gp95/ sortilin owing to its 100% homology with the previously cloned gp95 protein that is involved in receptor sorting (Mazella et al. 1998;Vincent et al. 1999). NTS3 receptors are located in glia, neurons and adipocytes and only 5-10% of these receptors are found on the cell surface (Mazella et al. 1998). Table 1 summarizes the characteristics of NTS3 receptors in terms of its size, receptor classification, location, agonists and antagonists. These receptors recognize NT only after it is translocate to the plasma membrane and acts like a scavenger protein to sequester extracellular NT. This receptor is predominantly associated with the Golgi apparatus and the endoplasmic reticulum (Mazella and Vincent 2006b) but is known to heteromize with NTS1 after translocation to the cell surface and modulate NTS1 activity in terms of activating MAPK (Sarret et al. 2003b).

Table 1 summarizes the characteristics of the different NT receptor subtypes, their locations, agonist and antagonists.

NT receptor subtype	***Size***	***Receptor classification***	***Location***	***Agonist***	***Antagonists***
NTS1	50-60 kDa	G protein coupled- 7 transmembrane spanning regions	Neurons Glia (astrocytes)	• NT (Kd=0.1-0.3nM) • NN • Xenopsin	• SR48692 • SR142948A
NTS2	45 kDa	G protein coupled- 7 transmembrane spanning regions	Neurons Glia (astrocytes)	• NT(Kd=3-10nM) • NN • Xenopsin • SR48692 (expressed in oocytes) • SR142948A (expressed in oocytes or CHO cells) • Levocabastine (expressed in oocytes or CHO cells)	• NT(expressed in oocytes or CHO cells) • SR142948A • Levocabastine
NTS3	100 kDa	Type I amino acid receptor single transmembrane spanning region	Neurons Glia (astrocytes) Intracellular vesicles containing GluT4 glucose transporter NT triggers insertion of the receptor into the membrane.	• NT (Kd=0.10.3nM) • Receptor associated protein(40kDa endoplasmic reticulum associated protein) • Cleaved sortilin propeptide	Uncleaved sortilin propeptide functional antagonist blocks agonist binding until cleaved from receptor.

2.1.4 NT analogs:

Neurotensin analogs are derived from cleavage of the native NT peptide and since 1975 when Carraway and Leeman(Carraway and Leeman 1973) confirmed the importance of the carboxy terminal domain in conferring the biological activity and binding of NT, the first few analogs that were synthesized were variants of the C-terminal domain. Interestingly, NT analogs respond differently in the same anatomical substrates or the same analog behaves differently in various regions of the brain (Sotty et al. 2000a). Since, the binding affinity of these analogs to NT receptors have different orders of potency as does it depend on the dose of the analog used, the physiological effects that translate from these binding events also vary. In the present study, three NT analogs have been used to evaluate their effects on glutamatergic transmission in DA and non-DA neurons of the VTA. NT1-13(the native peptide), NT8-13 (the C terminal hexapeptide) and D-Tyr [11] NT1-13 (the native peptide substituted at the 11 th residue by a D-tyrosine which renders the peptide more resistant to cleavage by endopeptidases). The following section aims to describe the properties of each of these analogs, and the similarity and differences in the effects mediated by them.

NT 1-13 and NT8-13: NT1-13 is the 13 amino acid, native neurotensin peptide which is endogenous in the brain, mostly found in the dopamine rich regions (for example the VTA) and most effects mediated by this peptide are through its C terminal region. When applied exogenously, NT1-13 fails to cross the blood brain barrier and is not resistant to peptide degradation. AT the rat NTS 1 receptor, NT1-13 has a K_d of 1.97nM while NT 8-13 has a K_d of 1.60E-01. NT1-13 is less potent than NT8-13 but more than D-Tyr [11] NT1-13 at NTS 1

receptors. However, at the NTS 2 receptors, NT1-13 has a similar potency as that of NT8-13(Kitabgi et al. 1980a;Labbe-Jullie et al. 1994).

NT1-13 when locally applied increases dopamine cell firing in the VTA (Seutin et al. 1989;Shi and Bunney 1991b), stimulates dopamine metabolism and consequentially dopamine release in the terminal fields of DAergic projections (Cador et al. 1995;Kalivas and Taylor 1985a) and that these effects are similarly mimicked by NT8-13.

NT8-13 or the hexapeptide C terminal fragment produces most of the known effects of NT and therefore most peptide agonists for NT receptors are analogs of this hexapeptide. There are discrepancies on the similarity in effectiveness of NT1-13 and NT 8-13. For example, a study by Rompre et al., (Rompre and Boye 1993) suggests, both these peptides are equally effective in operant responding for brain stimulation reward paradigms. This observation is further supported by the similarity of both these peptides in terms of binding affinities and receptor activation(Kitabgi et al. 1980a). Interestingly, there are reports of differential actions of these two peptides too. For example, ventromesencephalic tegmental microinjections of NT1-13 but not NT81-13 induce conditioned place preference (an experimental paradigm that reflects sensitization)(Glimcher et al. 1984a). Another, evidence comes from the evaluation of dopamine efflux using electrochemical methods in different brain regions upon different NT analog administration. In the rostral nucleus accumbens, the effect of NT and D-Tyr [11] NT 1-13 (another NT analog, described in detail in the following paragraphs) were found to be similar, whereas NT8-13 was less potent (Sotty et al. 2000a). In glial cells, NT 8-13 was reported to cause an increase in both internal and external Ca^{2+} levels that implicated both external and internal, and that the initiation of this release is mediated from calcium sources

dependent on inositol triphosphate (IP3)(Trudeau 2000). Another study in the VTA of guinea pigs, revealed two types of responses, a fast and short duration and a long and slow duration inward current that were induced on application of NT. While both the responses could be induced, by NT 1-13 only the fast/ short inward current could be induced by NT8-13. Additionally, NT was found to be more potent in reducing the DA induced inhibition in the VTA (Nalivaiko et al. 1998a). Therefore, although a general consideration might be made that NT8-13 is more or equally potent compared to NT in exerting its effects, there are studies that report otherwise, which suggests, that there are multiple receptor types that are activated by each of these peptides and that this activation is concentration sensitive. In the present studies, NT1-13 and NT8-13 have been used to test their effectiveness in modulating glutamatergic responses in VTA neurons and NT antagonists have been used in identifying the receptors involved in mediating these responses.

D-Tyr [11]NT1-13 is a neurotensin analog that has the 13 amino acids of the peptide intact but the 11^{th} position is substituted by a D-tyrosine residue that makes it more resistant to cleavage by endopeptidases(Checler et al. 1983a). In fact, after an intracerebroventricular injection of NT, 98% of the NT was cleared and degraded in brain tissues during a 30 min period after the injection. Under the same conditions,33% of D-Tyr [11]NT1-13 was retained, thereby suggesting a half-life 1.5 times greater than that of NT(Checler et al. 1983a). D-Tyr [11]NT1-13 is known to stimulate DA release *in vitro* and *in vivo* , because of the close interplay between interacting DA and NTergic systems majorly in the limbic system(Steinberg et al. 1995). This peptide is known to sensitize to the locomotor effects of amphetamine sensitization, when injected in the VTA and has similarity in effectiveness when compared to NT but has a greater potency, thus possibly explaining its property of being resistance to

peptidase degradation(Rompre 1997a). A similar sensitization effect to cocaine, possibly mediated by NMDA receptors in the VTA has also been reported(Rompre and Bauco 2006a). In a more recent study, a role for D-Tyr [11]NT1-13 in induction of both context dependent and independent , amphetamine sensitization has been elucidated(Rouibi and Rompre 2014).In comparison to NT and NT8-13, D-Tyr [11]NT1-13 has a more stable metabolic profile and that in the presence of thiorphan, a peptidase inhibitor D-Tyr [11]NT1-13 induces larger locomotor effects caused by elevated levels of extracellular dopamine concentrations, than NT alone(Steinberg et al. 1995) . However, it is important to note that thiorphan by itself increases extracellular dopamine concentration, and therefore the evaluation of effects metabolically unstable NT analogs, in terms of increasing dopamine concentration is not possible (Labbe-Jullie et al. 1994). However, the failure of D-Tyr [11] NT1-13 in stimulating dopamine release in the PFC when injected in the VTA cannot be explained by the metabolically stable profile whereas both NT8-13 and NT in similar range of concentrations have been effective in causing dopamine release. Interestingly, in the same study, differential effects of D-Tyr [11] NT1-13 are reported in the rostral and caudal aspects of the nucleus accumbens. For example, in the caudal aspects, NT8-13 and NT were more potent than D-Tyr [11] NT1-13, whereas, in the rostral aspects NT8-13 was less potent than D-Tyr [11] NT1-13 and NT. Therefore the effects of these three peptides in limbic regions specially seem to be varied and dependent on (i) the concentration in which it is applied, (ii) the binding affinity of NT analogues at multiple NT receptors and their consequent activation (Sotty et al. 2000a).

2.2 NT in the Midbrain:

In the VTA NT colocalizes with TH and these mixed NT/DA neurons project to the PFC, Entorhinal cortex(EC), nAcb, BLA and lateral septum (LS) (Fallon 1988). The incoming NT afferents to the VTA do not colocalize TH suggesting their non-dopaminergic origin. In rats, these fibers were reported to originate from the bed nucleus of the stria terminalis (BNST), lateral hypothalamus (LH), LS and the preoptic area (Zahm et al. 2001). Neurotensinergic efferents from the VTA project to the EC, amygdala, nAcb, piriform cortices, LS and pre-optic area. Figure 3 shows the neurotensinergic projections and NT rich regions of the rat brain. Evidence for local direct synaptic connections between NT axon terminals from adjacent brain regions and TH positive cells and dendrites within the VTA, indicate a possible presynaptic mode of action of NT owing to the vast majority of NT positive terminals as evidenced by electron microscopic autoradiography (Woulfe and Beaudet 1992).

Eighty to ninety percent of midbrain NT receptors are located on DA neurons of the VTA and these are predominantly NTS1 receptors. The remaining NT receptors are found on projection neurons (glutamatergic or GABAergic), non-Dopaminergic axon terminals and glial cells (Fassio et al. 2000;Nicot et al. 1994;Szigethy and Beaudet 1989). There are also reports of NTS2 mRNA in the midbrain, however their exact cellular location has not been verified (Lepee-Lorgeoux et al. 1999;Walker et al. 1998).

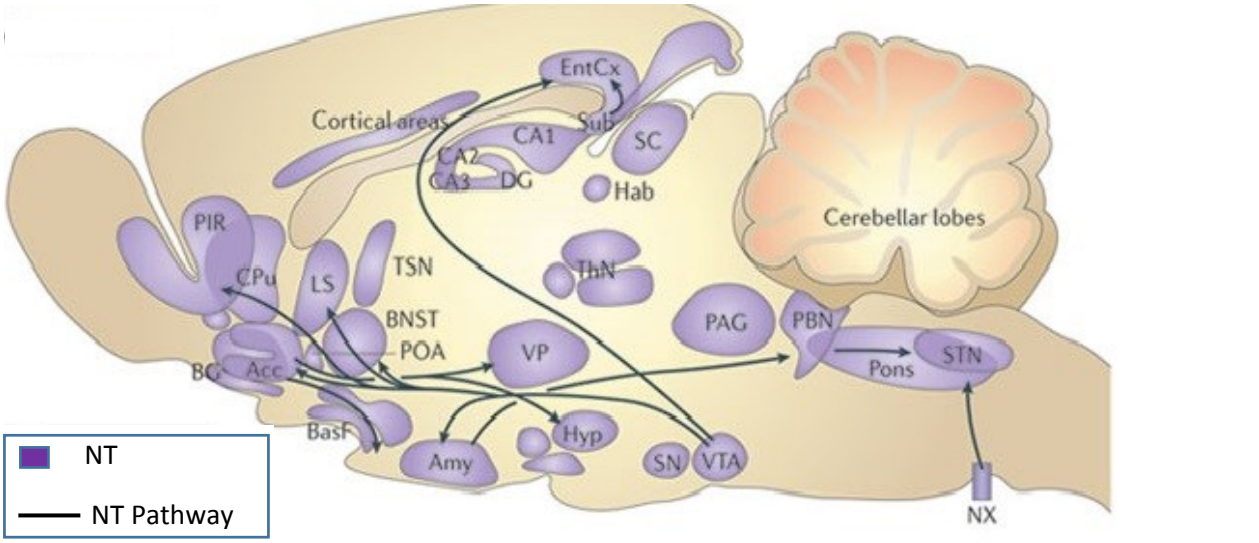


Figure 3: NT distribution and NTergic projections of the rat brain. Acc, accumbens nucleus; Amy, amygdala; BasF, basal forebrain; BG, basal ganglia; BNST, bed nucleus of the stria terminalis; CA1, hippocampal field CA1; CPu, caudate putamen; DG, dentate gyrus; EntCx, entorhinal cortex; Hab, medial habenula; Hyp, hypothalamic nucleus; LS, lateral septum; MPA, medial proptic area; NX, dorsal motor nucleus of the vagus; PAG, periaqueductal grey; PBN, parabrachial nucleus; PIR, piriform area; POA, preoptic area; Sub, subiculum; SN, substantia nigra; STN, solitary tract nucleus; ThN, thalamic nucleus; VP, ventral pallidum; VTA, ventral tegmental area. Adapted from (Griebel and Holsboer 2012).

2.3 Modulation of DA neurotransmission by NT:

The localization of NT receptors on DA neurons in the VTA raises the possibility of a functional interaction between these two neurotransmitter systems (Binder et al. 2001a). Once NT binds to NT receptors on DA cells, an NT –NTS complex is formed and NT receptors are activated. The section below describes these events and their consequent effect on DA cells.

1. Effects of NT-NTS complex formation

Once NT binds to NT receptors, the NT-NTS complex is internalized and depending on the receptor type that was activated, the receptor is either recycled to the cell surface or degraded in the lysosome (Mazella et al. 1998;Mazella and Vincent 2006b). The internalized NT ligand, eventually moves to surround the nucleus and has been reported to increase TH gene expression in DA neurons (Burgevin et al. 1992). Step1 of Figure 4 shows the formation of the NT-NTS complex.

The NT-NTS complex decreases the agonist binding affinity of D_2 dopamine receptors for DA and DA agonists as shown in Step2 of Figure 4. This leads to a decrease in DA autoinhibition (binding of DA to D_2 receptors increase K^+ conductance and hyperpolarise the neuron) and shifts the activity of post synaptic cells to D1(dopamine receptor subtype 1 , that depolarises the neuron and increase firing) mediated transmission. The mechanism of this decrease is a concerted effect of direct receptor interactions between NTS1 and D2; and activation of second messenger pathways. At the receptor level, NT via allosteric receptor/receptor interactions and second messenger dependent pathways decreases the dissociation constant (K_d) of D_2 receptors without affecting the total density of receptors (B_{max}), which indicates

that the dynamics of the receptors are altered, leaving the density of functional receptors unaltered (Fuxe et al. 1992;Tanganelli et al. 1993). Although, NTS1, has been the only well characterised receptor known to modulate D2 function, the potency of NT analogs in decreasing D2 receptor agonist binding affinity is incongruent with their binding affinities for NTS1. NT is more potent than NT8-13 in decreasing agonist binding affinity at D_2 receptors, however the binding affinity at NTS1 is higher for NT8-13 than NT. This indicates the possible involvement of another NTR, in addition to NTS1 in mediating this effect (Kitabgi et al. 1980a;Li et al. 1993a;Li et al. 1993b).

2. Effects of NT receptor activation

Activation of NTS1 by intracerebroventricular (i.c.v) injections of NT depolarised and increased the firing rate of midbrain DA neurons as evidenced from extracellular singe unit field recordings. This depolarisation and increase in firing rate culminate in an increase in the number of active DA neurons (Kobayashi et al. 1977;Pinnock 1985;Shi and Bunney 1991a;Shi and Bunney 1991c).

NT-NTS1 binding decreases the binding affinity of DA agonists for D2 receptors (Figure 4 step1), thus removing the inhibitory effect of D2 receptors that consequentially led to DA cell firing. Using patch clamp recordings of rat midbrain DA neurons that were identified with TH immunohistochemistry it was reported that NT and D_2 receptors oppositely regulate the same K^+ conductance. While NT decrease the K^+ conductance resulting in depolarisation and enhance the firing rate of the neuron, D2 receptors enhance it to hyperpolarize the neuron (Farkas et al. 1997). However, another study argues against the involvement of the same K^+

channel conductance, as in extracellular recordings of firing of DA neurons, NT attenuated the inhibitory effect of the D_2R agonist, at concentrations that were insufficient to promote augmentation in firing rates (Werkman et al. 2000a). NT induced attenuation of D2 inhibition is not a result of antagonizing general excitation as glutamate (neurotransmitter that increases DA cell firing) failed to mimic this effect (Shi and Bunney 1990).

The cell depolarisation induced in step 2 leads to a two component inward current. While the first component is a fast excitation, the second component is a slow excitation. The fast component comprised an increase in the non-selective cationic conductance mediated by the activation of $G_{\alpha q}$ and $G_{\alpha 11}$ (G protein subtypes) and IP3 and involved NTS2 as shown in Step4 of Figure 4. The slow component is comprised of a decrease in an inwardly rectifying K^+ channel conductance (I_h) and involves PKC activation and activation of NTS1 (Cathala and Paupardin-Tritsch 1997;Chien et al. 1996;Farkas et al. 1996;Nalivaiko et al. 1998a). This reflects as a decrease in I_h current. Step 3 of figure 4 shows the decrease of I_h. Additionally, only NT8-13 was able to induce the fast response, however NT8-13 has a higher affinity for NTS1 than NTS2 (Kitabgi et al. 1980a). The NT initiated inward current was blocked by the NTS1 antagonist SR48692, was equally permeable to Na^+ and K^+ ions and blocked externally by Ca^{2+} and Mg^{2+} ions. (Farkas et al. 1996;Nalivaiko et al. 1998a)

As described earlier, it is important to note that most of the work on the mechanism of NT action on DA cells have focussed on NTS1, however in the VTA NTS2 exist on the cell body, dendrites of DA neurons and terminals from afferent areas, and therefore NTS2 receptor activation will also have consequences on DA cell activity (Jennes et al. 1982a;Woulfe et al. 1992;Woulfe and Beaudet 1992). However little is known about the effects of NTS2 receptor

activation in neurons. In other expression systems activation of NTS2 have resulted in inhibitory effects (Sarret et al. 2002).For details see section on NTS2.

Figure 4 is a schematic that shows the effects of NT on DA cells as described in the section above.

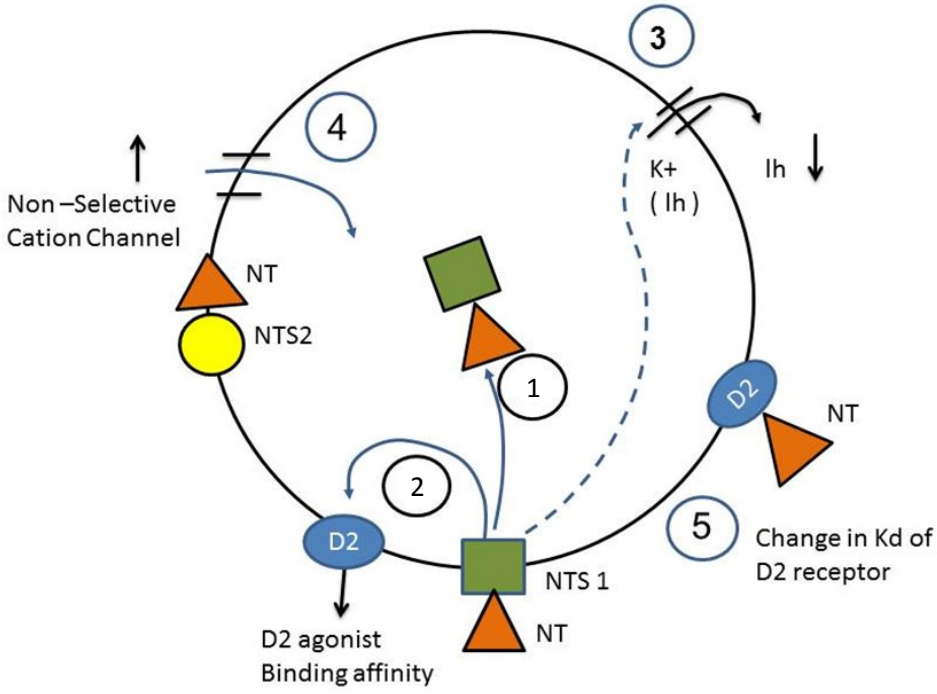


Figure 4: Mechanism of action of NT on DA cells. **2**: once NT binds to the NT_1receptor, the NT-NT_1 complex is rapidly internalized. **1**: the NT-NT_1complex decreases the agonist binding affinity of the DA D_2receptor. **3**: binding of NT to NT_1 decreases Ih. **4**: NT binding to the NTR increases the conductance of a nonselective cation channel, transduced by activation of Gαq and/or Gα-11 G-protein subtypes and IP_3. **5**: NT interacts with the extracellular portion of

the D_2 receptor via hydrophobic mode matches. This interaction leads to a change in the K_d of D_2 receptor antagonist binding. Adapted from(Binder et al. 2001a).

2.4 Effects of NT administration in the VTA

As described in the section above, the effects of NT are concentration dependent. Bath application of NT to VTA slices in guinea pigs at a concentration of 0.5μM or lower induced reduction of I_h in DA neurons in culture, resulting in a slow depolarisation (Chien et al. 1996;Nalivaiko et al. 1998a). Bath application of NT induced increase in DA cell firing in VTA cultured neurons at a potentially non-physiologic dose (1μM) when the conductance of the non-selective cation channel is increased (Farkas et al. 1997). At even higher concentrations, DA cell firing as recorded *in vitro* in extracellular field recordings is arrested due to the overexcitation resulting from depolarisation inactivation (Pozza et al. 1988;Seutin et al. 1989).

The excitatory effects of NT on DA neurons of the VTA, lead to increased DA cell firing and eventually translate to increased DA release in the terminal areas of projection of the DA neurons. For example NT administration in the VTA leads to increased levels of DA efflux in the nAcb and the PFC. At the cellular level, the DA release is measured as a function of increase in homovanillic acid (HVA-a catecholamine metabolite associated with dopamine levels) and 3,4-dihydroxyphenylacetic acid (DOPAC- a metabolite obtained from the degradation of dopamine). A schematic representative of this effect is shown in Figure 5 A and B. Behaviourally this relates to potentiation of DA dependent behaviours such as increased

locomotor activity, circling that finally culminate in sensitization and rewarding effects (Sotty et al. 2000a).

Figure 5 shows the summary of the electrophysiological effects of NT injection in the VTA.

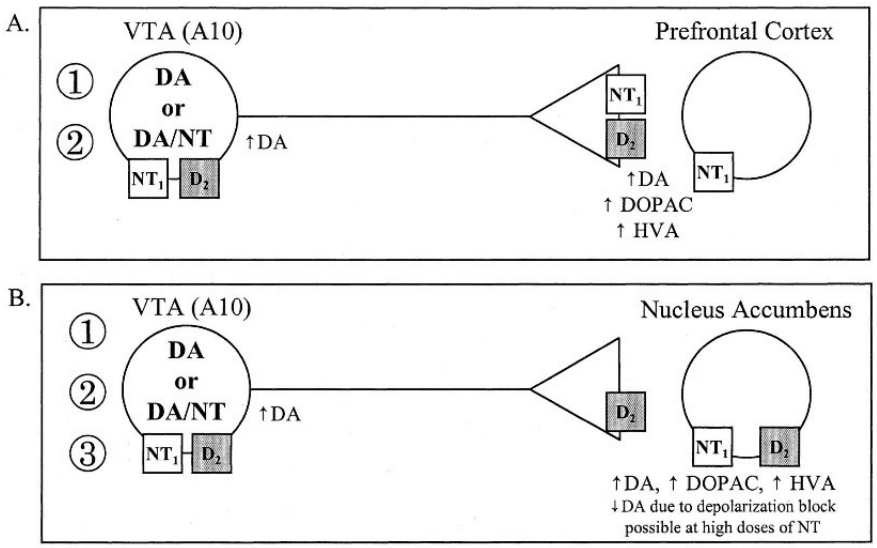


Figure 5: Summary of the electrophysiological and neurochemical effects of NT administered in the VTA. Effects are shown at the sight of injection (VTA) and in the prefrontal cortex (A) and nucleus accumbens (B). Low dose (0.5µM or less) of NT depolarize DA neurons without increasing cell firing (1). Moderate dose (1µM) of NT increases the number and rate of spontaneously firing DA neurons (2) which leads to increased DA turnover in the terminal regions. At high doses (>1µM) NT decreases the

firing rate of DA neurons in the VTA that project to the nucleus accumbens (3). Adapted from (Binder et al. 2001a).

2.5 Effect of NT on glutamatergic neurotransmission

Glutamatergic neurotransmission in the VTA play an important role in modulating VTA neuronal activity and is vital for the functioning of the VTA. Release of glutamate from glutamatergic terminals, depolarise VTA DA neurons and increase their excitability (Nestler 2013). Activation of glutamatergic inputs to DA neurons (for e.g. from the PFC, LdTg) induce burst firing and consequentially increases DA release in terminal areas of projection (for e.g. in the nAcb) (Seutin et al. 1989). Glutamatergic inputs to the VTA are also crucial to the development of sensitization and blockade of glutamatergic receptors in the VTA impair this process (Rompre and Bauco 2006b). Furthermore, synaptic plasticity at excitatory synapses on DA neurons is a key neural adaptation that contributes to addiction (Geisler and Wise 2008). Therefore the modulation of such inputs are pivotal to the understanding of neuroadaptations involved in sensitization, reward and addiction related processes.

The VTA is an NT rich region in that it receives a dense innervation of NT fibers from cortical and subcortical structures and possesses NT receptors on VTA neurons. Amphetamine induced sensitization requires a role for NT and blockade of NMDA receptors in the VTA disrupted this process. This suggests a possible role for NT in modulating glutamatergic neurotransmission in the VTA (Vezina and Queen 2000b).

Early studies that focussed on the effect of NT on glutamatergic neurotransmission at the cellular level, were performed in the striatum or the cortex (Ferraro et al. 1995;Ferraro et al.

2000). Although, the anatomical substrate in our study is different, it would give us a general idea about the possible modes of interaction between these two interacting systems.

A functional interaction between NTS1 and D_2 receptors at the cortico-striatal glutamatergic terminal comes from an in-vivo microdialysis study in awake rats. In the presence of a high concentration of K^+ in the Ringer's solution, extracellular glutamate levels increased, which was blocked by the D_2 receptor agonist quinpirole. This inhibitory effect was counteracted by the addition of NT8-13 or NT thus indicating NTS1 receptor modulation at the cortico-striatal glutamate terminal(Ferraro et al. 1995). Another study in rat cortical slices, reported that NT enhanced glutamate release in the cortex and that this effect was further enhanced in the presence of NMDA, leading to excitotoxicity induced cell death. The pattern of enhancement followed a bell shaped curve and that the maximal effect was observed at a concentration of 10nM and thereafter at even higher concentrations (100nM and 1000nM) the enhancement effect progressively declined. Since this effect was blocked by the NTS1 antagonist -SR48692 , an involvement of NTS1 receptors was suggested (Ferraro et al. 2000). However, in contrast to the observation in these studies, another electrophysiological study in the nAcb of rats, suggests that NT at higher concentrations (0.5-1μM) reduced glutamate release from terminals and that this action required the activation of D2 and group I metabotropic glutamate receptors. Since, SR142948A (the non-selective antagonist for NT receptors) blocked the inhibitory effect, the authors suggest a role for NTS1. However, it is important to note that SR142948A is an antagonist for both NTS1 and 2 receptor subtypes. Additionally, the depression also recruited CB1 receptors (Yin et al. 2008).A similar *in vitro* electrophysiological study in VTA slices from TH EGFP (green fluorescent protein) transgenic mice, reported a reduction of glutamatergic EPSCs in VTA DA neurons produced

by NT8-13 that involved NTS1 receptors. Moreover, AM251, a CB1 antagonist, blocked this synaptic depression and the NTergic effect was independent of intracellular calcium dynamics but dependent on PLC and G protein activation. The endocannabinoid responsible for this effect was identified as 2AG, the same molecule known to gate long term potentiation in the VTA (Kortleven et al. 2011;Kortleven et al. 2012a). In a more recent study in rats (Kempadoo et al. 2013a) optogenetic techniques was used to selectively activate hypothalamic NTergic afferents to the VTA. This study suggested that NT 8-13 potentiates composite glutamatergic EPSCs and isolated NMDA at low concentrations (10nM) and inhibited pharmacologically isolated AMPA EPSCs at equimolar concentrations of NT in DA neurons that were identified using TH immunohistochemistry and the presence of I_h. However, at higher concentrations (100nM and 500nM), NT 8-13 inhibited both AMPA and NMDA EPSCs. While the potentiation of the NMDA currents was mediated by NTS1 receptor activation, the reduction of NMDA EPSCs at higher concentrations was attenuated but not eliminated by SR48692, suggesting the involvement of another receptor (possibly NTS2) in addition to NTS1. The reduction in AMPA mediated EPSC was not NTS1 dependent as SR48692 failed to block or attenuate the depression (Kempadoo et al. 2013a).

Not much is known about the effect of NTergic modulation of glutamatergic inputs to non-DA neurons of the VTA. A behavioural study reported that blockade of NMDA receptor1 (NR1) on non-DA neurons in the VTA, by using NR1DATCre transgenic mice (NR1DATCre - Cre-loxp mice lacking functional NMDARs in DA neurons expressing Cre recombinase under the control of the endogenous dopamine transporter gene) impair the development of sensitization to cocaine (Luo et al. 2010a) . Since non-DA neurons of the VTA receive efferent

glutamatergic inputs and express NT receptors, the possibility of NTergic modulation of these neurons and its inputs are likely to play a role in sensitization mechanisms.

2.6 Relevance of a role for NT in behavioural disorders

The close anatomical and functional interaction of NT with the mesolimbic system prompts NT to be studied in relation to the adverse effects implicated by this system, including reward, addiction and schizophrenia. In fact, NT produces behavioral effects (for example enhanced locomotor activity) similar to that produced by psychostimulants when directly injected in the VTA (Kalivas et al. 1981;Kalivas 1993;Kalivas and Taylor 1985a). It induced conditioned preference for a place that was previously paired with the use of NT, indicating a reinforcing effect (Glimcher et al. 1984a) and the rats subsequently self-administer NT. In rats, NT also sensitizes to the locomotor effect of amphetamine (Cador et al. 1999a) and NT antagonists disrupt the development of NT induced sensitization to amphetamine (Panayi et al. 2002;Rompre and Perron 2000a). The behavioural manifestation of sensitization effects relates to the rewarding properties of drugs of abuse or psychostimulants which finally translate to long lasting effects reflecting a compulsive pattern of drug intake.

One of the corner stones of the DA hypothesis of schizophrenia is that schizophrenia is a disorder of overactivity of DA systems and all clinically effective APDs (antipsychotic drugs) that are used to treat schizophrenia have high affinity for DA D2 receptors (Dobner et al. 2003). Interestingly, NT administration in the nACb have antipsychotic like effects or D2 antagonistic effects. For example, NT injected into the nAcb reduced hyperlocomotion induced by DA agonists and SR48692 blocked this effect (Boules et al. 2014;Dobner et al.

2003;Feifel et al. 1999;Jolicoeur et al. 1984;Robledo et al. 1993). Furthermore, amphetamine induced locomotor activity is also attenuated by NT administration in the nAcb (Ervin et al. 1981;Feifel et al. 1997a;Feifel et al. 1997b). In addition to reducing the DAergic tone, the antipsychotic like effect of NT has also been evaluated in its potential to increase inhibitory (GABA) transmission in the striato-pallidal pathway (part of the indirect pathway of the basal ganglia that controls movement; projects from the striatum to the VP and then the sub thalamic nucleus (STN)) (Chen et al. 2006;Ferraro et al. 2011;Matsuyama et al. 2003). This restores the glutamate tone of the thalamo-cortical pathway (the other part of the indirect pathway of the basal ganglia; projections from the sub thalamic nucleus to the cortex) that is reduced in schizophrenic patients (Antonelli et al. 2007;Ferraro et al. 2001). Since NT produces a bell shaped and concentration dependent enhancement in cortical glutamate levels (Ferraro et al. 2000), it is suggested that the antipsychotic-like effects of NT may be mediated in part by its modulatory effect on glutamate (Boules et al. 2013). Recent studies reveal that NT peptide analogs hold a great promise in treating disorders like psychosis, schizophrenia, drug abuse and autism (Boules et al. 2014).

Chapter 3. Questions and Hypothesis

Psychostimulants or drugs of abuse induce motor stimulant effects that endure with repeated drug administration and lead to long lasting effects that culminate in behavioural sensitization and subsequently addiction. The VTA is involved in the initiation of drug induced sensitized behavioural response and glutamatergic neuroadaptations of VTA DA neurons contribute to this phenomenon. Disruption of the development of sensitization by blockade of NMDA receptors in the VTA, support a role for glutamatergic neurotransmission. Additionally, blockade of NT receptors in the VTA also leads to the disruption of the development of sensitization to drugs. These led us to think that the role of NT in behavioural sensitization mechanisms possibly resides in its ability to modulate glutamatergic neurotransmission in VTA neurons. However, how NT modulates glutamatergic neurotransmission of VTA neurons remain largely unknown.

Hypothesis

Since the VTA is heavily innervated with NT terminals and NT receptors colocalize in most DA neurons, most actions of NT in the VTA involve the activity of DA neurons. This led us to hypothesize that 1) activation of ventral midbrain NT receptors (sub-type 1 (NTS1) and/or 2 (NTS2)) by endogenous NT increases DA neuronal activity; 2) that results in activation DA receptors; 3) which in turn increases local glutamate which activates NMDA and/or non-NMDA receptors; 4) activation of these receptors leads to activation of intracellular signaling pathway(s) that lead to long lasting functional changes that sub-serve sensitization. The model focuses on the possibility that NT acts directly or indirectly on

ventral midbrain non-DA neurons and/or nerve terminals from glutamatergic efferents. A schematic of the hypothesis is outlined in Figure 6.

Specific hypotheses:

Specific hypothesis 1:

The effect of NT on DA neurons reflects as a biphasic inward current comprising of a fast excitation followed by a slower longer excitation involving the increase in non-selective cationic conductance and the reduction of K^+ conductance respectively (Farkas et al. 1996). However, only the slower component is generated by the C terminal fragment NT8-13 whereas the fast excitation is elicited by both NT1-13 and NT8-13 suggesting that different NT analogs generate varied responses. D-Tyr[11]NT1-13 , another NT analog with a d-tyrosine substitution in the 11^{th} position is more resistant to cleavage by endopeptidases. D-Tyr[11]NT also has a higher affinity for NTS2 than NTS1, thus enabling characterisation of effects that are attributed to these two different receptor subtypes.

Since NT analogs generate varied responses, we first sought to characterize the effects of different NT analogs (NT1-13, NT8-13 and D-Tyr[11]NT1-13) on glutamatergic EPSCs from VTA DA and non-DA neurons and identify the receptors mediating the effects. To test this hypothesis, in the first study we used three different NT analogs; NT1-13, NT8-13 and D-Tyr[11]NT1-13 and tested their effects on glutamatergic EPSCs from DA and non-DA neurons of the VTA. To identify the receptor associated in mediating the effects produced by these analogs, we used SR142948A (the non-selective NTs antagonist) and SR48692 (NTS1 antagonist) to test their effectiveness in blocking the effects.

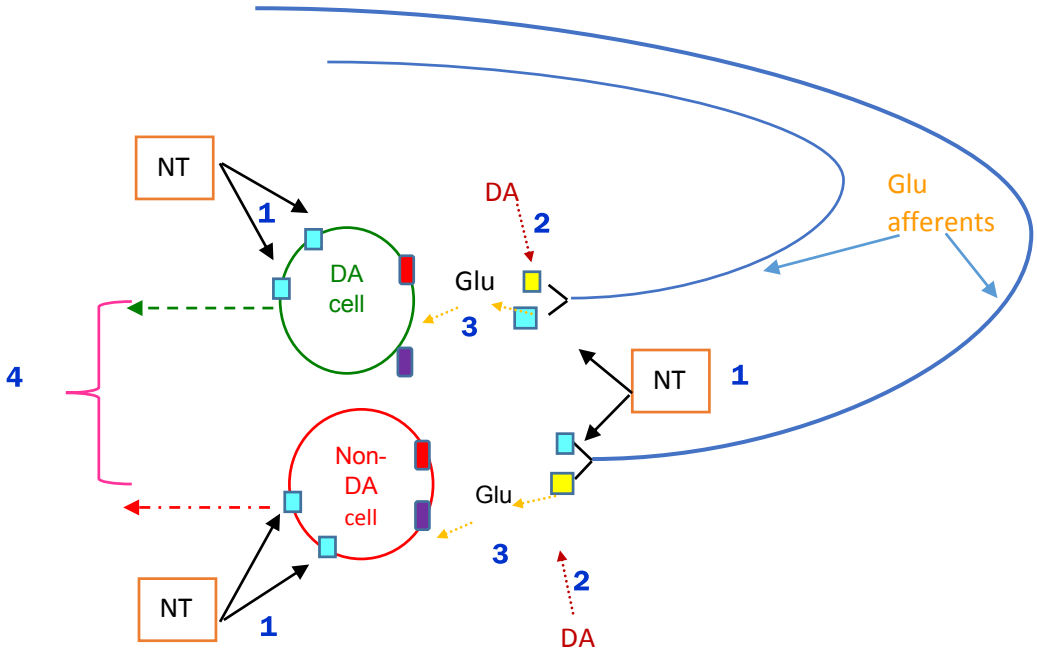
Specific Hypothesis 2:

Since NT receptors within the VTA are located not only on neuron somata and on dendrites, but also on axon terminals, activation of these receptors could possibly modulate neurotransmitter release as well as induce postsynaptic effects. Our model proposes two pathways through which NT can modulate glutamatergic neurotransmission: 1- Directly by modulating glutamate release via the activation of NT receptors located on glutamatergic terminals **(**see Figure 7A**)** and 2- indirectly through the activation of DA neurons, which will release DA that can act on D1or D2 receptors on glutamatergic terminals **(**see Figure 7B**).** The second study is aimed at testing these hypotheses and identify the locus of glutamatergic neurotransmission modulation. Bath application of NT agonists will activate both mechanisms, and therefore, we proposed, in a first series of experiments, to study the modulatory actions of D-Tyr[11]NT1-13 on evoked NMDA and non- NMDA EPSCs from DA and non-DA neurons and estimate the contribution of NMDA vs non- NMDA modulatory effects. NT antagonists would be tested in a separate set of experiments to identify the NT receptor subtype mediating the modulatory effect. Paired pulse protocols would be administered in all experiments to isolate presynaptic effects if any.

The studies entailed above would enable a better understanding of the neurotensinergic modulation of glutamatergic neurotransmission of VTA neurons and highlight a role for non-DA neurons in sensitization and addiction mechanisms.

The studies entailed above would enable a better understanding of the neurotensinergic modulation of glutamatergic neurotransmission of VTA neurons and highlight a role for non-DA neurons in sensitization and addiction mechanisms.

Figure 6- schematic of the proposed model.



1. Activation of NT receptors on cell bodies or terminals.

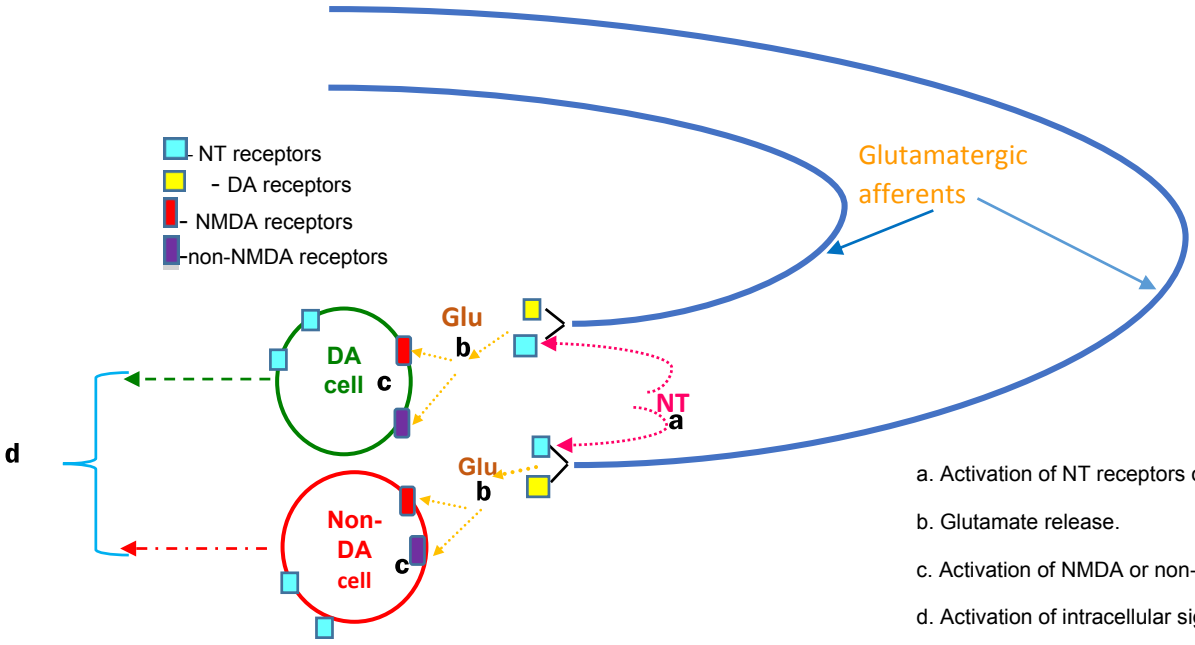
2. Activation of DA receptors on terminals or cell bodies, causing DA release.

3. Glutamate release

4. Activation of intracellular signalling pathways

- NT receptors
- DA receptors
- NMDA receptors
- non-NMDA receptors

Figure 7A: Direct pathway



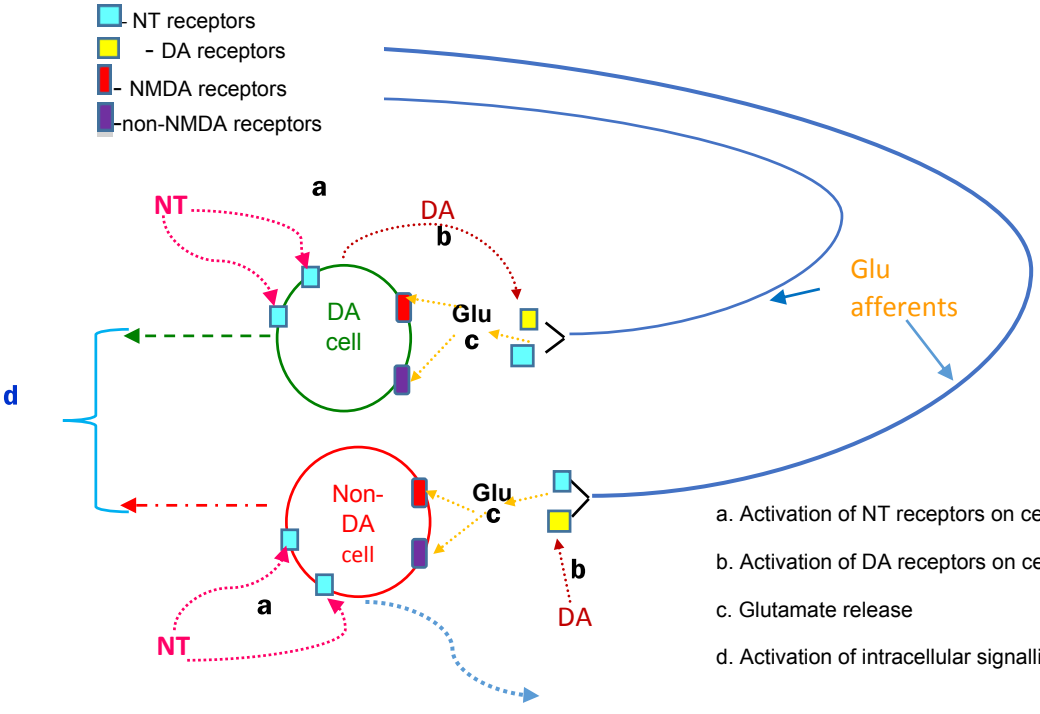
a. Activation of NT receptors on glutamatergic terminals.

b. Glutamate release.

c. Activation of NMDA or non-NMDA receptors.

d. Activation of intracellular signalling pathways.

Figure 7B: Indirect pathway



a. Activation of NT receptors on cell bodies

b. Activation of DA receptors on cell bodies, causing DA release.

c. Glutamate release

d. Activation of intracellular signalling pathways

???

Results

Contribution of co-authors

The results of the studies undertaken during my PhD has resulted in 3 articles. These 3 articles are presented below. I am the first author of all the 3 articles. The second article has been co-first authored by Khalil Rouibi* (post-doctoral fellow at Dr. Pierre Paul Rompre's Lab). This implies that I realized most of the experiments presented in the 3 articles that I have written.

This work was majorly supervised by Dr. Richard A Warren. A special mention to Dr. Pierre Paul Rompre for helping me write the manuscripts of the 1st and 2nd articles.

Article 1

Neurotensin enhances glutamatergic EPSCs in VTA neurons by acting on different neurotensin receptors.

Poulomee Bose, Pierre-Paul Rompré, Richard A Warren

Author contribution:

Conceived and designed the experiments: Richard A Warren and Poulomee Bose

Performed the experiments: Poulomee Bose

Analyzed the data: Poulomee Bose and Pierre-Paul Rompre

Wrote the paper: Poulomee Bose, Pierre Paul-Rompre and Richard A Warren.

Article 2

Spotlight on the role of ventral midbrain neurotensin receptors in reward behavior.

Rouibi K., Bose, P., Rompré, P.-P., Warren, R.A.

Author contribution:

Conceived and designed the experiments: Pierre Paul Rompre, Rouibi Khalil, Richard A Warren and Poulomee Bose

Performed the experiments: Poulomee Bose and Rouibi Khalil.

Analyzed the data: Poulomee Bose, Pierre-Paul Rompre and Rouibi Khalil.

Wrote the paper: Poulomee Bose, Pierre Paul-Rompre.

Article 3

D-Tyr[11]NT differentially modulates glutamatergic neurotransmission in VTA neurons.

Poulomee Bose and Richard A. Warren.

Author contribution:

Conceived and designed the experiments: Richard A Warren and Poulomee Bose

Performed the experiments: Poulomee Bose

Analyzed the data: Poulomee Bose.

Wrote the paper: Poulomee Bose, Richard A Warren.

Article 1

Neurotensin enhances glutamatergic EPSCs in VTA neurons by acting on different neurotensin receptors.

Poulomee Bose[1], Pierre-Paul Rompré[2], Richard A Warren[1]

[1]Department of Psychiatry, University of Montreal, Quebec, Canada. [2]Department of Neuroscience, University of Montreal, Quebec, Canada

Address for correspondence:

Richard A. Warren
Department of Psychiatry
Faculty of Medecine
University of Montreal
C.P. 6128, succursale Centre-ville
Montreal, QC, Canada
H3C 3J7

Status of the manuscript: Paper published on August 2015 in the Journal of Peptides.
http://www.sciencedirect.com/science/article/pii/S0196978115002284

Abstract

Neurotensin (NT) is an endogenous neuropeptide that modulates dopamine and glutamate neurotransmission in several limbic regions innervated by neurons located in the ventral tegmental area (VTA). While several studies showed that NT also exerts a direct modulation on VTA dopamine neurons less is known about its role in the modulation of glutamatergic neurotransmission in this region. The present study was aimed at characterising the effects of NT on glutamate-mediated responses in different populations of VTA neurons. Using whole cell patch clamp recording technique in horizontal rat brain slices, we measured the amplitude of glutamatergic excitatory post-synaptic currents (EPSCs) evoked by electrical stimulation of VTA afferents before and after application of different concentrations of NT1-13 or its C-terminal fragment, NT8-13. Neurons were classified as either I_h^+ or I_h^- based on the presence or absence of a hyperpolarisation activated cationic current (I_h). We found that NT1-13 and NT8-13 produced comparable concentration dependent increase in the amplitude of EPSCs in both I_h^+ and I_h^- neurons. In I_h^+ neurons, the enhancement effect of NT8-13 was blocked by both antagonists, while in I_h^- neurons it was blocked by the NTS1/NTS2 antagonist, SR142948A, but not the preferred NTS1 antagonist, SR48692. In as much as I_h^- neurons are non-dopaminergic neurons and I_h^+ neurons represent both dopamine and non-dopamine neurons, we can conclude that NT enhances glutamatergic mediated responses in dopamine, and in a subset of non-dopamine, neurons by acting respectively on NTS1 and an NT receptor other than NTS1.

Keywords: Neurotensin, Ventral Tegmental Area, Glutamatergic EPSCs, SR142948A, SR48692.

Introduction

Neurotensin (NT) is an endogenous tridecapeptide that modulates neurotransmission in several limbic nuclei known to play a role in substance use disorder(Binder et al. 2001b). These limbic nuclei are densely innervated by terminals from the ventral tegmental area (VTA), a midbrain region that also contains a dense network of NT fibers(Beaudet and Woulfe 1992). The majority of VTA neurons that constitute the mesocorticolimbic pathway contain dopamine (DA) and nearly 80-90% of them express NT receptors(Szigethy et al. 1990) . Activation of VTA NT receptors stimulate DA cell firing, an effect that is mediated, at least in part, by an increase in non-selective cationic conductance and by inactivation of the somatodendritic auto-receptors(St-Gelais et al. 2006b).Consistent with these findings are neurochemical and behavioral results showing that VTA microinjections of the native NT peptide, NT1-13, or its active C-terminal fragment, NT8-13, stimulate ventral striatal DA release, increase forward locomotion and enhance brain stimulation reward(Kalivas and Duffy 1990;Sotty et al. 2000b) . In addition to playing a role in DA modulation and DA-dependent behaviors, NT also contributes to the induction of neural plastic changes that sub-serve sensitization to the behavioral effects of psychostimulant drugs. For instance, repeated central injections of NT, and its active analog, D-Tyr [11] NT1-13, lead to an enhancement of cocaine- and amphetamine-induced locomotor activity (Rompre 1997b;Rompre and Bauco 2006b) . Moreover, the development of amphetamine sensitization is prevented by

blockade of VTA NT receptors(Panayi et al. 2005a) . The role of NT in sensitization is consistent with the fact that NT, by stimulating VTA DA neurons, increases VTA DA release a phenomenon that is critical for the development of amphetamine sensitization(Vezina et al. 2007) . Other studies have also revealed that VTA glutamate plays a key role in the development of amphetamine sensitization. When sensitization is initiated by either repeated systemic or VTA injections of amphetamine, it is prevented by blockade of VTA N-Methyl-D-Aspartate (NMDA) receptors(Cador et al. 1999b;Vezina and Queen 2000a) . This raises the hypothesis that NT may initiate sensitization by modulating glutamatergic inputs to VTA DA neurons. In support of this hypothesis are recent findings by Kempadoo et al., (Kempadoo et al. 2013b) showing that activation of the efferent hypothalamic NT pathway to the VTA induces a NMDA-dependent rewarding effect, and that NT8-13 enhances NMDA excitatory postsynaptic currents (EPSCs) in putative VTA DA neurons. Using a higher concentration of NT8-13, Kortleven et al., (Kortleven et al. 2012b) reported a suppression of glutamatergic EPSCs in VTA DA neurons. It is possible that these opposite effects are related to the action of the NT8-13 fragment on different NT receptors. Activation of NT receptors with NT1-13 induces two types of excitatory response in DA neurons, a fast and short lasting response mediated by NTS2 receptors that is followed by a slow and longer lasting response mediated by NTS1 receptors (Nalivaiko et al. 1998b). Interestingly, NT8-13 only elicits the fast response suggesting that it preferentially activates NTS2 receptors(Nalivaiko et al. 1998b;Werkman et al. 2000b); this is inconsistent, however, with the high affinity and the high efficacy of NT8-13 for the NTS1 receptor(Tanaka et al. 1990a).

To further characterize the modulating action of NT on VTA glutamatergic neurotransmission, we used whole cell patch clamp recordings to measure the effects of

different concentrations of NT1-13 and NT8-13 on glutamatergic EPSCs measured in putative VTA DA and non-DA neurons. Putative VTA DA and non-DA neurons were distinguished by the presence, or the absence, of a hyperpolarization-activated potassium current (I_h) respectively(Margolis et al. 2006b). The interest in studying VTA non-DA neurons resides in the evidence that activation of NMDA receptors expressed by these neurons is required for the development of VTA neural plasticity that sub-serves drug reward and drug sensitization, two phenomena that are modulated by NT.

Methods

Animals and slice preparation

All experimental procedures were approved by the Institutional Animal Committee in accordance with guidelines of the Canadian Council on Animal Care. All efforts were made to minimize the suffering and number of animals used.

Fourteen to 21-day-old (P14-P21) Long Evans pups of either sex obtained from Charles River (St-Constant, QC) were used. Pups were anaesthetized by inhalation of methoxyfluran vapor in a closed chamber, decapitated and their brain quickly removed and transferred to chilled, oxygenated artificial cerebrospinal fluid (ACSF) in which NaCl had been replaced by equivalent osmolarity of sucrose and containing (in mM) sucrose 252 (NaCl 126 in standard ACSF); KCl, 3; NaH_2PO_4, 1.25; $MgSO_4$ 7 H_2O, 1.3; $CaCl_2$, 2.5; $NaHCO_3$, 26; and glucose, 10, and saturated with a gas mixture of 95% O_2 and 5% CO_2. Two hundred and fifty micrometer thick horizontal slices preserving the VTA afferents (Margolis et al. 2006b) were cut using a vibrating microtome(DSK Microslicer). Slices were transferred to a submerged

recording chamber maintained between 32 to 34°C and superfused with standard ACSF at a rate of 2 ml/min; slices were incubated for at least one hour before recording began.

Drugs and peptides

The following pharmacological agents were applied through the superfusing ACSF: (-) bicuculline methiodide, 6 cyano-7-nitroquinoxaline-2,3-dione (CNQX) (in some experiments) , (+)-2-amino-5-phosphonopentanoic acid (APV) (in some experiments)obtained from Sigma Aldrich(Oakville, Ontario, Canada) ; neurotensin- (1-13), neurotensin-(8-13) were obtained from Bachem (Sunnyvale, CA,USA); SR-48692 [2-(1-[7-chloro-4-quinolinyl]-5-[2, 6-dimethoxyphenyl]-1H-pyrazol-3-)carbonylamino-adamantane-2-carboxylacid]and SR142948A2-[[[5-(2,6-Dimethoxyphenyl)-1-[4-[[[3-(dimethylamino)propyl]methylamino]carbonyl]-2-(1-methylethyl)phenyl]-1*H*-pyrazol-3-yl]carbonyl]amino]-tricyclo[3.3.1.13,7]decane-2-carboxylic acid were obtained from Tocris Biosciences. All drugs were made up as 10 mM stock solutions in distilled water and diluted with ACSF solution to final concentration just before addition to the perfusion medium with the exception of SR48692 and CNQX which was dissolved in DMSO (final concentration 0.1%) and distilled water.

Electrophysiological recordings

Whole-cell configuration was achieved using the 'blind' patch-clamp technique(Blanton et al. 1989a). Pipettes were pulled from thin wall borosilicate capillary glass on a P-87 micropipette puller (Sutter Instrument, Novato, CA, USA. Recording pipettes had a resistance of 3-5MΩ when filled with a solution containing (in mM) potassium gluconate, 140; $MgCl_2$, 2; $CaCl_2$, 0.1; EGTA, 1.1; HEPES, 10; K_2-adenosine trisphosphate (ATP), 2;

guanosine trisphosphate (GTP), 0.5 and biocytin (5%). The pH was adjusted to 7.3 with KOH solution, and final osmolarity was 280 ±5 mosmol/kg. All recorded neurons were labelled to confirm their location in the medial VTA.

Whole-cell recordings were made with an Axoclamp-2B amplifier (Molecular Devices, Sunnyvale, CA, USA) in continuous single-electrode voltage-clamp mode. The output of the amplifier was fed into a LPF 200A DC amplifier/filter (Warner Instruments Corp., Hamden, CT, USA) and digitized at 5 to 10 kHz with a real-time acquisition system (CED 1401 Power). Data acquisition was achieved using the Signal 4.0 software (Cambridge Electronic Design, Cambridge, England). Recording pipette's capacitance was optimally adjusted before whole-cell configuration was achieved. The resting membrane potential was measured just after rupturing the cell membrane and the offset potential, measured upon withdrawal of the electrode from the cell, was accounted for assuming that it drifted in a linear fashion with time from the start of the recording session. We did not correct for liquid junction potential, which for a pipette containing 140 mM potassium gluconate amounts for an additional potential shift of around -10mV(Spigelman et al. 1992).

Synaptic activation and drug application

The presence of I_h was first determined by voltage clamping cells at -60mV and stepping to −40, −50, −70, −80, −90, −100, −110 and −120 mV. Input resistance was monitored with hyperpolarizing pulses in current clamp mode. A monopolar tungsten stimulating microelectrode was placed rostral to the recording site in the medial VTA, on the slice superficial layer, 0.5-1.0 mm from the recording electrode. Excitatory postsynaptic currents were evoked by 0.1ms, 3 to 6V cathodal pulses delivered at 15 sec intervals. In order to

isolate glutamate receptor-mediated EPSCs, all experiments were performed in the presence of (-) bicuculline methiodide (BMI, 10μM) in bath solution to block $GABA_A$ receptor-mediated synaptic currents. BMI was applied 30 min before obtaining whole-cell configuration to ensure a complete diffusion in the slice tissue.

In all experiments the EPSCs were recorded from an online voltage-clamped potential of -70mV. The effects of NT1-13 and NT8-13 on glutamatergic EPSCs were assessed at a holding membrane potential of -70mV.Three concentrations of each peptide (0.01μM, 0.1 μM and 0.5 μM) were tested and only one concentration was tested per cell. Upon agonist application, the change in amplitude of the glutamatergic EPSC was measured. Five minutes of baseline EPSC activity was recorded before superfusion with the peptide. The EPSC amplitudes were recorded during 7 min after the onset of the peptide application and averaged over the last 5 min. A washout period of 15 min was allowed before the amplitude of the recovered EPSC was measured. In some experiments, the control EPSC amplitude was measured for 4 min before a NT antagonist was added to the superfusion medium. However, SR142948A or SR48692 produced no effect on the control EPSC amplitude (n=9; data not shown). Therefore in further experiments where NTS receptor antagonists were used, SR142948 or SR48692 was added to the superfusing medium for 7 min and control response was measured in the presence of the antagonist. Additionally, only one antagonist was tested per cell and each type of antagonist was tested on different cells and their results were pooled together for each neuronal population.

Data analysis

Data analysis was performed using Signal software (Cambridge Electronic Design,

Cambridge, England). Statistical analysis was performed using Sigmaplot 12 software (Systat, San Jose, CA, USA). The magnitude of EPSC recorded after application of the peptide was expressed as percent of baseline and group means were calculated for drug condition. A one-way ANOVA was performed and Bonferroni post-hoc test used to determine significant differences between concentration or drug and peptide condition ($P < 0.05$) when justified.

RESULTS

Whole-cell voltage-clamp recording was obtained from 91 physiologically identified VTA neurons from rat pups aged between P14 and P21 (Margolis et al. 2006b;Margolis et al. 2010a). Neurons were categorized as I_h^+ (n=44) or I_h^-(n=39) based on the presence or absence of the hyperpolarization activated cationic current (I_h). The amplitude of I_h in I_h^+ positive neurons ranged between 81 pA and 407 pA with an average of 151.64pA (data not shown). The action potential and membrane characteristics of all I_h^+ and I_h^- neurons were measured and found to be similar to those reported in previous studies.

NT1-13 and NT8-13 enhance glutamatergic EPSCs.

Application of NT1-13 or NT8-13 produced a dose dependent increase in the amplitude of the glutamatergic EPSCs in both I_h^+ and I_h^- cells. Representative examples of the enhancement effect of NT1-13 (0.5μM) and NT 8-13 (0.5μM) on the glutamatergic EPSC in I_h^+ cell and I_h^- cells are shown in Figure 1 (Panel A and B). The amplitude of the EPSC increased significantly following application of each peptide and returned to baseline level following a washout.

The mean change in EPSC produced by NT8-13 in I_h^+ and I_h^- neurons is shown in panel C of Figure 1. The C-terminal fragment increased the EPSC amplitude by 20 % (0.01μM) to 66% (0.5 μM) while in Ih^- cells the mean percent increase ranges from 17% (0.01μM) to 75% (0.5 μM). As seen with NT1-13, the increase in EPSC was larger in I_h^- than in I_h^+ neurons at the highest dose but the difference did not reach statistical significance. A two-way ANOVA yielded a significant effect of dose ($F_{2, 27} = 29.6$, $p < 0.001$) but no effect of

cell type ($F_{1, 2} = 3.6$, $p > 0.05$) and no cell type by dose interaction ($F_{2, 27} = 0.17$ $p > 0.05$). Post-hoc test confirmed that the EPSC amplitude increased orderly with the dose (medium > low; high > medium).

To determine whether the effects of NT8-13 differ from that of NT1-13, we ran a three-way ANOVA comparing peptide, dose and cell type. Results yielded a significant dose effect as expected ($F_{2,51} = 154.9$, $p < 0.001$) and a significant cell type by dose interaction ($F_{2,51} = 6.35$, $p < 0.01$), but no effect of peptide ($F_{1,51} = 0.76$, $p > 0.05$), no peptide by dose interaction ($F_{1,51} = 0.15$, $p > 0.05$) and no peptide by dose by cell type interaction ($F_{1,51} = 0.37$, $p > 0.05$). This analysis confirmed that NT1-13 and NT8-13 produced a similar enhancement of the EPSC in both cell types. But it also reveals that when we combined the results obtained with each peptide, we observed a significant difference between I_h^+ and I_h^- neurons as a function of the dose. The post-hoc test reveals that this difference is due to a significantly larger increase in EPSC at the highest dose in I_h^- neurons (Figure 1 panel D).

Effects of neurotensinergic antagonists

To determine which subtype of NT receptors mediate the increase in glutamatergic EPSCs in each cell type, we tested the effectiveness of the NTS1 and NTS2 antagonist, SR142948a, and the preferred NTS1 antagonist, SR48692, at blocking the enhancing effect of NT8-13 . Since, Nt1-13 and NT8-13 produced similar effects we tested the antagonists with the more active C terminal fragment, NT8-13. Representative examples for I_h^- neurons are shown in Figure 2 (Panel A and B). The non-selective antagonist, SR1412948a, completely prevented the enhancement effect of NT8-13 (Panel A) while the preferred NTS1 antagonist had no effect. Mean percent change in EPSCs following application of NT8-13 in the presence or

not of each antagonist is shown in panel C of Figure 2. In I_h^+ neurons, application of each antagonist produced a near complete suppression of the enhancing effect of NT8-13. The ANOVA yielded a significant effect of treatment ($F_{2, 16} = 24.1$, $p < 0.001$) and post-hoc test showed that the increase following application of NT8-13 was significantly different than that measured in the presence of SR142948a and SR48692; there was no difference between SR142948a and SR48692 in I_h^+ neurons. In I_h^- neurons, application of SR142948a, but not SR48692, prevented the increase in EPSCs by NT8-13. The ANOVA yielded a significant effect of treatment ($F_{2, 16} = 25.2$, $p < 0.001$) and post-hoc test showed that the increase following application of NT8-13 was significantly different than that measured in the presence of SR142948a but not in the presence of SR48692. There was also a significant difference between SR142948a and SR48692. When applied alone, the antagonists had no effect on the amplitude of the glutamatergic EPSCs neither in I_h^+ nor in I_h^- neurons ($F_{3, 13} = 0.05$, $p > 0.05$; data not shown).

Discussion

The important findings of the present study are that NT1-13 and NT8-13 produced a dose-dependent increase in the amplitude of the EPSCs in rat VTA neurons and that this enhancement is mediated by different NTS1 and NTS2 receptor subtypes in I_h^+ and I_h^- neurons respectively. Initial studies on the interaction of NT with its receptors (now known as NTS1 and NTS2) have shown that the C-terminal segment, NT8-13, is required for binding and for activation of the receptors (Binder et al. 2001b;Schotte et al. 1986). Consistently, VTA microinjections of equimolar concentrations of NT1-13 and NT8-13 produce similar enhancement of locomotor activity, dopamine metabolism and brain stimulation reward (Kalivas and Taylor 1985b;Rompre and Gratton 1993a). One

behavioral study, however, reported that repeated VTA microinjections of NT1-13, but not NT8-13, induce a conditioned place-preference(Glimcher et al. 1984b). A discrepancy between the effects of the two peptides was also found for NT-induced excitation of VTA DA neurons. For instance, NT receptor activation was found to induce two excitatory responses in DA neurons: a fast and short duration excitation, and a late and prolong excitation. The fast component involves non-selective cationic conductance, Gαq and Gα11 (G protein subtypes) and inositol-3-phosphate (IP3). The slow component is comprised of a decrease in an inwardly rectifying K^+ channel conductance (I_h) and involves PKC activation. Both components were induced by NT1-13 but only the fast one could be induced by NT8-13. Moreover, the fast component was blocked by SR142948A, the non-selective NT antagonist, while the slow one was blocked by SR48692 (NTS1 antagonist). This shows that different NT receptors, with differential sensitivity to NT peptides, mediate excitatory responses in DA neurons that differ by latency and coupling to downstream intracellular cascades (Chien et al. 1996;Farkas et al. 1996;Nalivaiko et al. 1998a).

The present results show that NT8-13, over a wide range of concentrations, was as effective as NT1-13 at enhancing glutamatergic EPSCs, and this, independently of the VTA cell type recorded. The isolation of glutamatergic EPSCs in our preparation was achieved by the superfusion of BMI in the bathing solution. BMI is known to block SK channels and modulate the cell conductance and afterhyperpolarisation (AHP) by interfering with the calcium activated K^+ channel(Khawaled et al. 1999). However, in the present studies we did not find any change in the cell conductance upon addition of BMI to the superfusing medium (data not shown). The effect of BMI on AHP is not particularly relevant to our experiments.

Additionally, in the presence of BMI, we did observe no residual current in the presence of both CNQX and AP5, glutamatergic antagonists for iontotropic AMPA and NMDA receptors as shown in our previous studies (Yang et al. 2008;Zhang et al. 2014;Zhang and Warren 2002) suggesting that a role for BMI in modulating cell conductance or producing any other relevant effect apart from enabling isolation of glutamatergic EPSCs can be negated.

The excitatory effects induced by NT in our studies, is consistent with microdialysis studies showing that NT can increase glutamatergic transmission in the striatum (Ferraro et al. 1995;Ferraro et al. 1998;Ferraro et al. 2000) (Ferraro et al., 1995; Ferraro et al., 1998) and cortex (Ferraro et al. 2000). . In several other brain regions for instance the cortex, striatum , globus pallidus and the hippocampus, NT has been reported to augment glutamate release via activation of NTS1 receptor subtype(Antonelli et al. 2007;St-Gelais et al. 2006a;Zhang et al. 2015). Electrophysiological evidence suggest that NT augments the excitability and the firing rate of DA neurons suggesting a post synaptic effect. This is attributed to a reduction in K^+ conductance which consequentially reduce D2 receptor mediated inhibition (Farkas et al. 1997). At the cortico striatal glutamatergic terminal, a functional interaction between NTS1 and D_2 receptors mediated increase in extracellular glutamate levels was reported in a microdialysis study in awake rats(Ferraro et al. 1995). High concentrations of K^+ in the Ringer's solution resulted in an increase in extracellular glutamate levels and that this effect was blocked by the D_2 receptor agonist quinpirole. This inhibitory effect of quinpirole was counteracted by the addition of NT8-13 or NT1-13 thus indicating an NTS1 receptor modulation. It is thus possible that NT enhances glutamatergic

neurotransmission in VTA neurons by a similar mode of action. However, further investigations are required to delineate a modulatory role for D2 receptors in this effect.

Although, NTS1, has been the only well characterised receptor known to modulate D2 function, the potency of NT analogs in decreasing D2 receptor agonist binding affinity is incongruent with their binding affinities for NTS1. NT1-13 has a greater potency in decreasing the agonist binding affinity at D2 receptors than NT8-13, however the binding affinity of NT8-13 is higher than NT-13 at NTS1 receptors. This indicates the possible involvement of another NT receptor subtype, in addition to NTS1 in mediating this effect (Kitabgi et al. 1980a;Li et al. 1993a;Li et al. 1993b). At this point we can only speculate about the possibility of an involvement of NTS2 receptors on I_h^-neurons that mediate this action as we observe that there is a significant cell type by dose interaction in I_h^- neurons at the highest concentration. This is congruent with the fact that NTS2 receptors require a higher concentration of NT agonist (K_d=3-10nM) to be activated (Binder et al. 2001a;Gully et al. 1997a;Kempadoo et al. 2013a).

Nearly all dopaminergic neurons, and the great majority of GABA neurons, express an I_h current (Johnson and North 1992b;Lacey et al. 1989;Margolis et al. 2012b;Wanat et al. 2008b). According to Margolis et al.,(Margolis et al. 2006b) I_h^- neurons represent another subset of non-dopaminergic neurons. While both peptides equally enhanced glutamatergic EPSCs in I_h^- and I_h^+ at low and moderate concentrations, the enhancement was larger in I_h^- than in I_h^+ neurons at the highest concentration in our studies. Previous studies have reported inhibition a reduction of glutamatergic EPSC amplitude in DA neurons (Kempadoo et al. 2013a;Kortleven et al. 2012a). However, these studies suggest activation of different NT receptor subtypes in mediating the inhibition. While one, suggests an NTS1 mediated

reduction in glutamatergic EPSCs which also involves a role of endocannabinoid signaling (Kortleven et al. 2012a), the other, suggests a reduction in glutamatergic EPSCs which is NTS1 receptor independent. This latter study suggests, that NT8-13 induced an NTS1 dependent excitatory effect at lower concentrations on NMDA EPSCs, while it depressed AMPA EPSCs at equimolar concentrations via activation of another NT receptor subtype but not NTS1. Furthermore, they also found a consistent NTS1 independent depression of isolated AMPA EPSCs at all concentrations. This suggests that the actions of NT8-13 on VTA DA neurons are complex and controversial. The reason why we did not observe an inhibition when the concentration was increased is not clear. One possible explanation is that most of the cells that we recorded from were located below the surface of the slice. Peptides are highly sensitive to enzymatic degradation (Checler et al. 1983b;Kitabgi et al. 1980b) and it could be that in our study, the concentration of intact peptide that reached the cell or its afferent was smaller than the concentration in the superfusing medium. Whether the site of electrical stimulation has a role to play in the effect produced by NT is still open to investigation, in our studies the stimulation electrode was placed 0.5-1mm rostral to the recording site in the medial VTA whereas the stimulation site in the study by Kortleven is unclear (Kortleven et al. 2012a). Therefore it is possible that in our studies the afferents are stimulated although within the scope of our experiments, identification of the origins of these afferents were not possible. An evidence in support of our observation is that stimulation of hypothalamic afferents lead to potentiation of the NMDA component of glutamatergic EPSCs, when NT is applied at a concentration of 0.01μM suggesting that NT does induce NTS1 dependent excitatory effects (Kempadoo et al. 2013a). Several other factors for instance, the age of the animal, the slicing procedure that might differentially influence the

survival of different cell types of neurons and interspecies difference should also be taken into account in evaluating the variedness of the effects produced. It is particularly interesting to note that non-dopamine neurons with I_h (possibly glutamatergic neurons) are more commonly found in brain slices from guinea pigs (Cameron et al. 1997) and rats (Margolis et al. 2006a) in comparison to brain slices from mice (Margolis et al. 2010b;Ungless 2004;Zhang et al. 2010). Additionally, the ontogeny of NT receptor expression is dependent on the age of the animal and thus might be another contributing factor to the differences in the observed effects induced by NT agonists (Lepee-Lorgeoux et al. 1999;Nicot et al. 1992;Nicot et al. 1994;Walker et al. 1998).

Both NTS1 and NTS2 receptors are found in the VTA, and therefore we used the non-selective NTS1 and NTS2 antagonist, SR142948A, and the preferred NTS1 antagonist, SR48692 to determine which receptor subtype(s) is or are involved in the enhancement effect of NT8-13(Gully et al. 1993;Gully et al. 1997b). In I_h^+ neurons, the increase in glutamatergic EPSCs amplitude was prevented by the application of a low concentration of SR48692 and SR142948A, hence suggesting that it is mediated by NTS1 receptors. This result is consistent with Kempadoo et al.,(Kempadoo et al. 2013b) result showing that the SR48692 blocks the enhancement of glutamatergic EPSCs induced by a low concentration of NT8-13 in I_h^+ dopaminergic neurons, and that this enhancement is absent in NTS1 knockout mice. Kortleven et al., (Kortleven et al. 2012b) however, reported that the reduction in glutamatergic EPSCs they recorded in VTA dopaminergic neurons following application of NT8-13 was also blocked by SR48692 and SR142948A suggesting that the NTS1 receptor also mediates the inhibition. In this study, however, the authors used a concentration of SR48692 that was five times higher (0.5 μM) than that used in our study and that most likely

blocks both NTS1 and NTS2 receptors (Chalon et al. 1996;Gully et al. 1993). Moreover, a reduction of both AMPA and NMDA EPSCs following application of high concentrations of NT8-13 is still observed in NTS1 knockout mice suggesting that the inhibition possibly involves NTS2 receptor subtype (Kempadoo et al. 2013b).

Although it would be highly relevant to identify the locus of NT receptor modulation, the present studies do not allow us to do so. Since, NT augments the firing rate in DA neurons by reducing D2 receptor mediated autoinhibition it is likely that the effects of NT are a result of direct activation of post synaptic receptors (Farkas et al. 1996;Farkas et al. 1997;Johnson and North 1992a). A presynaptic locus of receptor modulation in either I_h^+ or I_h^- neurons cannot be ruled out, however it is unlikely as paired pulse experiments using NT8-13 by Kempadoo et al did not yield significant results (Kempadoo et al. 2013a). Additionally there is little information about the precise location of NT receptors in the VTA and therefore whether NT agonists have a preferential locus of action for one population of cells or the other remain undetermined. Nonetheless, it is important to note that in our studies we find NT8-13 has a preferential mode of activation of NTS1 receptors over NTS2 receptors in I_h^+ and I_h^- cells respectively. This raises the possibility of a preferential distribution of NTS2 receptors on I_h^- cells or terminals that innervate them. However further studies are required to support this hypothesis and at this point we can only speculate about such a possibility.

In the present studies ,the potentiation effect of NT8-13 on I_h^+ neurons likely stems from a NTS1 receptor mediated mobilization and accumulation of intracellular calcium in addition to glutamatergic excitation(Kempadoo et al. 2013b;St-Gelais et al. 2006b); the NTS1 receptor mediates several effects of NT that is dependent upon activation of phospholipase C, activation of protein kinase C and increase of intracellular Ca^{2+} release(Rostene et al.

1997). In I_h^- neurons, the increase in glutamatergic EPSCs amplitude was prevented by SR142948A but not SR48692. To our knowledge this provides the first evidence of a modulation of glutamatergic input to VTA non-dopamine neurons by NT. The ineffectiveness of SR48692 at blocking the enhancement of EPSCs by NT8-13 in I_h^- neurons, suggest that it is likely mediated by NTS2 receptors. The implication of a non-NTS2 receptor, sensitive to SR142948 but insensitive to SR48692 cannot be excluded. Previous studies have shown, for instance, that the enhancement of DA cell firing and ventral striatal DA release initiated by VTA NT is sensitive to SR142948A but insensitive to SR48692 and to the selective NTS2 partial agonist, levocabastine; it could be that the enhancement of glutamatergic EPSCs by NT8-13 in I_h^- neurons is mediated by a receptor that display a similar pharmacological profile. It is unlikely that this receptor is the NTS3. Although present in the VTA, this SR48692- and levocabastine-insensitive receptor is not coupled to a G-protein and there is no evidence to date supporting a direct modulatory role of the NTS3 in neurotransmission (Mazella and Vincent 2006a;Sarret et al. 2003a). Studies rather suggest that the NTS3 interacts with other receptors being involved in intracellular trafficking and in translocation of receptors to the plasma membrane(Mazella and Vincent 2006a).

The present findings shed additional light on the role of NT in the development of neural plastic changes that sub-serve sensitization to the behavioral effects of psychostimulant drugs. Indeed, previous studies have shown that VTA glutamate is necessary for the initiation of amphetamine sensitization (Cador et al. 1999b;Vezina and Queen 2000a). Moreover, repeated central NT injections sensitize to amphetamine, and sensitization is blocked by either a VTA microinjection of SR142948 or a systemic injection of SR48692 (Panayi et al. 2005a;Rompre and Perron 2000b;Rompre 1997b). These findings suggest that

NT may act upstream and initiate the development of sensitization by enhancing glutamatergic neurotransmission in VTA I_h^+ neurons through activation of NTS1 receptors. A role for the NTS2, or for the SR48692-insensitive, receptor in psychostimulant sensitization cannot be ruled out. The development of cocaine sensitization is also dependent upon VTA glutamate neurotransmission(Vanderschuren and Kalivas 2000) and Luo et al.,(Luo et al. 2010b) have shown that sensitization to cocaine is abolished by selective deletion of NMDA receptors on VTA non-dopamine neurons. Moreover, cocaine sensitization is initiated by repeated central NT injections but is not blocked by SR48692 (Horger et al. 1994;Rompre and Bauco 2006b). This suggests that the enhancement of glutamatergic EPSCs in VTA non-dopamine I_h^- neurons by NT may play a role in the initiation of sensitization to cocaine.

Acknowledgement:

This work was supported by a grant from the Canadian Institutes of Health Research (#102572) to PPR and RAW, le Fonds Recherche-Santé Québec to PPR and the Natural Sciences and Engineering Research Council of Canada to RAW. Authors express their special thanks to Claude Bouchard his support and sound advice.

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Figure 1

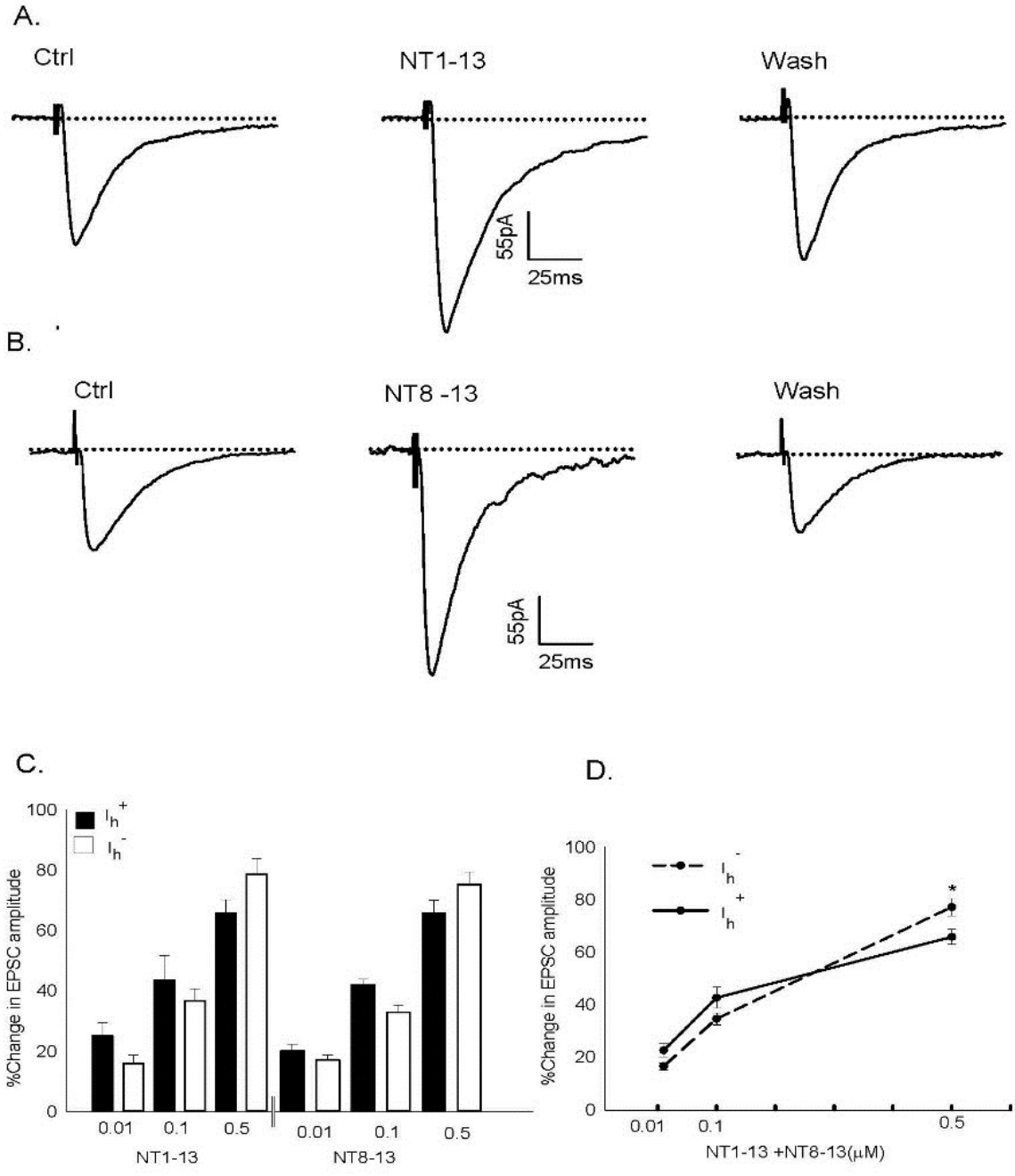
A.
Ctrl
NT1-13
Wash
55pA
25ms
B.
Ctrl
NT8 -13
Wash
55pA
25ms
C.
%Change in EPSC amplitude
100
80
60
40
20
0
I_h^+
I_h^-
0.01
0.1
0.5
NT1-13
0.01
0.1
0.5
NT8-13
D.
%Change in EPSC amplitude
100
80
60
40
20
0
I_h^-
I_h^+
*
0.01
0.1
0.5
NT1-13 +NT8-13(μM)

Figure 1. Effect of NT1-13 and NT8-13 on glutamatergic EPSCs. Evoked glutamatergic EPSCs recorded from I_h^- neurons before (left traces), during (middle traces) and after (right traces) application of 0.5 μM of NT1-13 (A) or NT8-13 (B). The bargraph in panel C represents the mean percent change in EPSC amplitude produced by different concentrations of NT1-13 and NT8-13 in I_h^+ and I_h^- neurons. NT 1-13 at 0.01μM (I_h^+ n=5, I_h^- n=4); 0.1 μM (I_h+ n=6, I_h^- n=4) and 0.5μM (I_h^+ n=6, I_h^- n= 6). For NT 8-13 at 0.01μM (I_h^+ n=4, I_h^- n=4); 0.1 μM (I_h^+ n=5, I_h^- n=5) and 0.5μM (I_h^+ n=8, I_h^- n= 5). Panel D shows the mean percent change in EPSC amplitude produced by NT1-13 and NT8-13 (pooled together) as a function of the peptide concentration in I_h^+ and I_h^- neurons. The star indicates a significant different between I_h^+ and I_h^- neurons ($p < 0.05$). See text for details.

Figure2

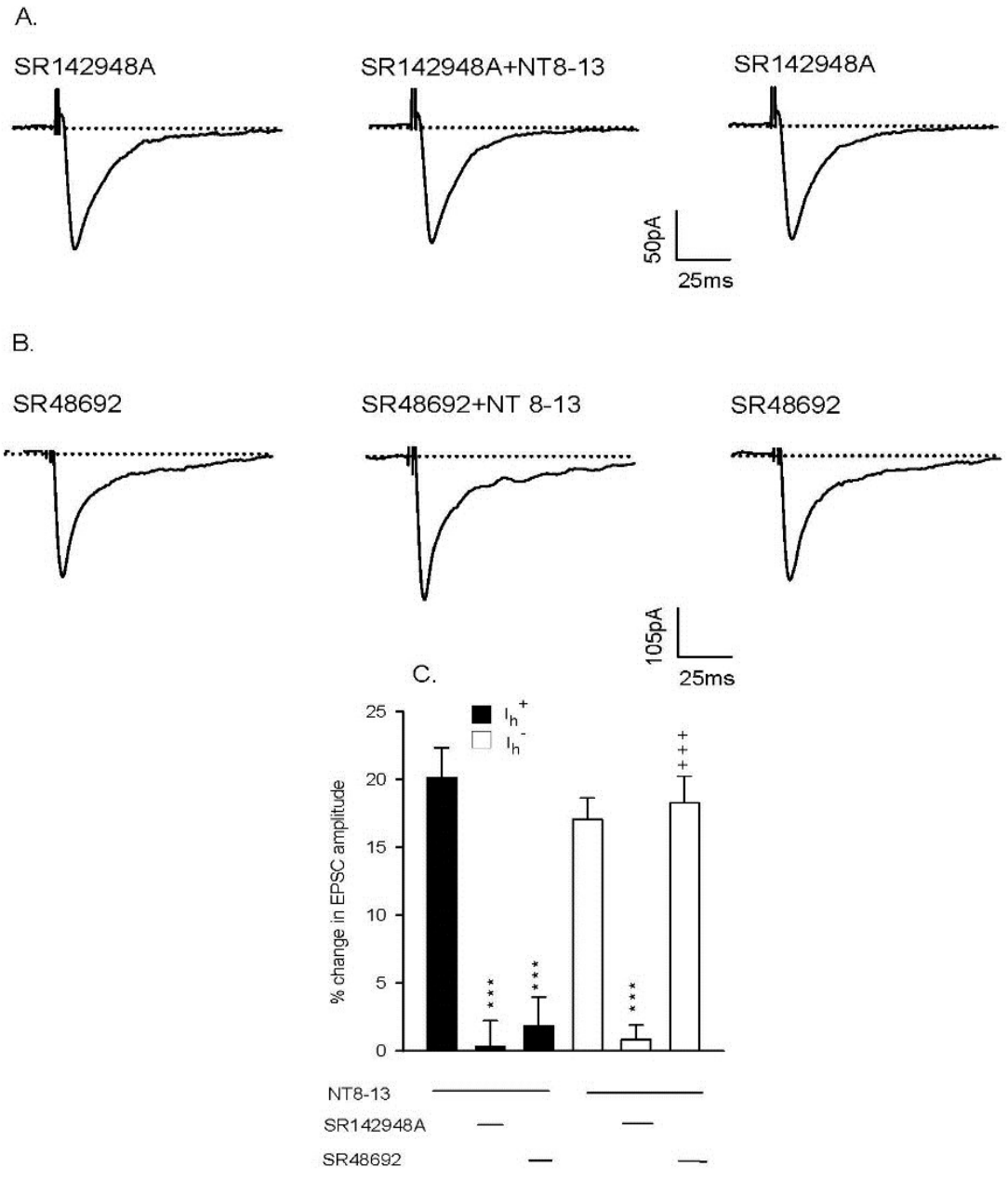
A.
SR142948A
SR142948A+NT8-13
SR142948A
50pA
25ms
B.
SR48692
SR48692+NT 8-13
SR48692
105pA
25ms
C.
% change in EPSC amplitude
25
20
15
10
5
0
I_h^+
I_h^-
***
***
***
+++
NT8-13
SR142948A
SR48692

Figure 2: Effect of NT receptor antagonists on glutamatergic EPSCs. Evoked glutamatergic EPSCs recorded from I_h^- neurons during application of 0.1 μM of SR142948A (A) or 0.1 μM of SR48692 (B) alone (left traces), in combination with 0.01 μM of NT8-13 (middle traces) and after the washout of NT8-13 (right traces). The bargraph in panel C represents the mean percent change in EPSC amplitude produced by NT8-13 in the absence, or in the presence, of SR142498A or SR48692 in I_h^+ (n=5) and I_h^- (n=6) neurons. Stars and crosses indicate a significant difference with NT8-13 alone and NT8-13+SR142948A respectively ($p < 0.001$). See text for details.

Article 2

Spotlight on the role of ventral midbrain neurotensin receptors in reward behavior

[1,3]Khalil Rouibi K., [2]Poulomee Bose, [*1,3]Pierre-Paul Rompré, [2]Richard A. Warren

[1]Department of Neurosciences, [2]Department of Psychiatry, Faculty of Medicine, Université de Montréal; [3]FRSQ Research Group in Behavioral Neurobiology, Department of Psychology, Concordia University, Montréal, Québec, Canada.

Running title: Neurotensin-glutamate interaction and reward

*Correspondence:

Pierre-Paul Rompré
Department of Neurosciences
Faculty of Medicine
Université de Montréal
Montréal, Québec, Canada
H3C 3J7

Status of the manuscript: Article published in Frontiers in Neuroscience (Neuropharmacology section).

Abstract

The present study was aimed at characterizing the mechanisms by which neurotensin (NT) is acting within the ventral midbrain to induce a psychostimulant-like effect. In a first experiment, we determine which subtype(s) of NT receptors is involved in the reward-inducing effect of ventral midbrain microinjection of NT using the conditioned place-preference (CPP) paradigm. In a second study, we used *in vitro* patch clamp recording technique to characterize the NT receptor subtype(s) involved in the modulation of glutamatergic neurotransmission (excitatory post-synaptic current, EPSC) in ventral tegmental neurons that expressed (I_h^+), or do not express (I_h^-), a hyperpolarization-activated cationic current. Behavioral studies were performed with adult male Long-Evans rats while electrophysiological recordings were obtained from brain slices of rat pups aged between 14 and 21 days. Results show that bilateral ventral midbrain microinjections of 1.5 and 3 nmol of D-Tyr[^{11}]NT induced a CPP that was respectively attenuated or blocked by co-injection with 1.2 nmol of the NTS1/NTS2 antagonist, SR142948, and the preferred NTS1 antagonist, SR48692. In electrophysiological experiments, D-Tyr[^{11}]NT (0.01-0.5 μM) attenuated glutamatergic EPSC in I_h^+ but enhanced it in I_h^- neurons. The attenuation effect (I_h^+ neurons) was blocked by SR142948 (0.1 μM) while the enhancement effect (I_h^- neurons) was blocked by both antagonists (0.1 μM). These findings suggest that i) NT is acting on ventral midbrain NTS1 receptors to induce a rewarding effect and ii) that this psychostimulant-like effect could be due to a direct action of NT on dopamine neurons and/or an enhancement of glutamatergic inputs to non-dopamine (I_h^-) neurons.

Keywords: Conditioned reward, Glutamate, Neurotensin, Ventral midbrain.

Introduction

Neurotensin (NT), a tridecapeptide (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) isolated from the hypothalamus more than four decades ago (Carraway and Leeman 1973), acts as a potent modulator of limbic neurotransmission. Cell bodies and terminals that express NT-like immunoreactivity are found in several limbic brain regions including the amygdala, the nucleus accumbens, the prefrontal cortex, the septum, and the ventral midbrain (Delle Donne et al. 1996;Hokfelt et al. 1984;Jennes et al. 1982a;Woulfe and Beaudet 1989). When released from nerve terminals, NT can activate three receptor sub-types, NTS1, NTS2 and NTS3 (see Vincent et al. 1999). The NTS1 and NTS2 are metabotropic receptors that are coupled to G-proteins linked to different signaling pathways such as cyclic guanosine-monophosphate, phospholipase C and mitogen-activated protein kinase. The NTS3 is a non G- protein coupled receptor that possesses a single transmembrane domain; this receptor appears to be non-selective for NT as its binds several other endogenous ligands (see Mazella and Vincent, 2006). The great majority of the central effects of NT have been attributed to its action on either NTS1 or NTS2 receptors. When administered into the lateral ventricle, for instance, NT produces a dose-dependent hypothermia and analgesia that are prevented by the NTS1/NTS2 antagonist, SR142948, but not the preferred NTS1 antagonist, SR48692, suggesting that they are mediated by the NTS2 receptor (Gully et al. 1997a). Central NT injections also attenuate spontaneous and methamphetamine-induced locomotor activity, effects that are prevented by SR48692 suggesting that they are mediated by the NTS1 receptor (Gully et al. 1995;Wagstaff et al. 1994). The behavioral effects of NT are not only dependent upon the receptor sub-type that is activated but also upon the site of action of the peptide within the limbic system. In the

ventral midbrain, for instance, NT stimulates dopamine impulse flow and dopamine-dependent behaviors (Holmes and Wise 1985;Kalivas et al. 1981;Rompre et al. 1992) while in the ventral striatum and the prefrontal cortex, it reduces the post-synaptic effect of dopamine and attenuates dopamine-dependent behaviors (Beauregard et al. 1992;Ervin et al. 1981;Jennes et al. 1982b;Kalivas and Miller 1984;Stowe et al. 2005). These findings led to the hypothesis that NT may act as either an endogenous antipsychotic- or psychostimulant-like neuromodulator (Berod and Rostene 2002;Kinkead and Nemeroff 2002).

The mechanisms by which NT produces psychostimulant-like effects remain imprecise. The main hypothesis is that NT enhances dopamine release and dopamine-dependent behaviors by stimulating dopamine impulse flow through activation of NTS1 receptors expressed on dopamine cell bodies and dendrites (see Berode and Rostene, 2002). Consistently, NT induces an increase in dopamine inward current and firing rate that is prevented by SR48692 (St-Gelais et al. 2006a). Activation of NTS1 receptors expressed on dopamine neurons also inactivates the dopamine auto-receptor which contributes to enhance dopamine impulse flow (Thibault et al. 2011). We, and others, also reported that NT and its C-terminal fragment, NT-(8-13), enhance excitatory post-synaptic currents (EPSCs) in presumed dopamine neurons, an effect that is blocked by SR48692 (Bose et al. 2015; Kempadoo et al. 2013). Unexpectedly, the increase in ventral striatal dopamine release induced by ventral midbrain application of NT is blocked by SR142948 but not by SR48692 (Leonetti et al. 2002;Steinberg et al. 1995) suggesting that NT is rather stimulating dopamine impulse flow through activation of NTS2 receptors. This latter finding, however, was not supported by another study showing that application of ventral midbrain NT enhances

ventral striatal dopamine release in NTS2 but not NTS1 knock-out mice (Leonetti et al. 2004).

Neurotensin structure-activity studies have also generated conflicting results regarding the role of NTS1 receptors in the psychostimulant-like effect of NT. For example, the enhancement effect of NT on locomotor activity and on brain stimulation reward is mimicked by NT-(8-13) and neuromedin N (Kalivas et al. 1986;Kalivas and Taylor 1985a;Rompre et al. 1992;Rompre and Gratton 1993b), two peptides that bind and activate the NTS1 receptor (Kitabgi et al. 1980a;Tanaka et al. 1990b). But the induction of a conditioned place-preference (CPP) by repeated ventral midbrain NT microinjections is not mimicked by an equimolar concentration of NT-(8-13); in fact it is mimicked by a NT fragment, NT-(1-11), that failed to interact with the NTS1 receptor (Glimcher et al. 1984a;Kitabgi et al. 1980a); these results suggest that the conditioned rewarding effect of NT may be mediated by a NT receptor other than NTS1 receptor. In order to clarifying this issue, we attempted to determine which ventral midbrain NT receptor is involved in the induction of a CPP using the NT analog, [D-Tyr^{11}]NT. Contrary to NT and NT-(8-13) which stimulate mesoaccumbens and mesoprefrontal dopamine projections, two pathways respectively involved in reward and aversion (Lammel et al. 2014), [D-Tyr^{11}]NT can selectively enhance the activity of the mesoaccumbens projection (Sotty et al. 2000a). Because NT and NT-(8-13) also enhance ventral midbrain glutamatergic neurotransmission, we characterized the effect of [D-Tyr^{11}]NT on glutamatergic EPSCs in putative ventral midbrain dopamine and non-dopamine neurons; the two populations were distinguished by the presence, or the absence, of a hyperpolarization-activated cationic current (Margolis et al. 2006a) using the patch clamp recording technique. Results of the behavioral experiments

show that [D-Tyr^{11}]NT induced a dose-dependent CPP that was blocked by SR48692 and attenuated by SR142948, suggesting that it is mediated by NTS1 receptors. Electrophysiological results show that [D-Tyr^{11}]NT dose-dependently attenuates glutamatergic EPSCs in putative dopamine neurons while it enhances the EPSCs amplitude in non-dopamine neurons; these effects are likely mediated by a respective activation of NTS2 and NTS1 receptors.

Materials and methods

Behavioral experiments

Animals

Male Long-Evans rats (Charles River, St-Constant, Qc, Canada) weighing 280-320g at the time of surgery were used. They were housed 1 (after surgery) or 2 per cage in a temperature (22±1 °C) and humidity (40-50%) controlled room with a 12h light/dark cycle (lights on 06:00); standard rat chow and water were available ad libitum. All testing was performed during the light phase of the light–dark cycle. All animal experimental procedures were approved by the Institutional Animal Ethics Committee (Comité de déontologie de l'expérimentation sur les animaux de l'Université de Montréal), in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (n°: 85-23, revised 1996). All efforts were made to minimize the suffering and number of animals used.

Surgery

Following one week habituation period to the colony room, each rat was anesthetized with isoflurane (2.5-3.5%, 0.75 L/min O_2); solutions of 0.1 ml of Anafen (5 mg/kg, s.c.) and 0.05 ml (i.m.) of duplocillin LA containing 15,000 I.U. of penicillin were administered to prevent inflammation and infection. The animals was then mounted on a stereotaxic apparatus, the surface of the skull was exposed and a guide cannula (Model C315G, Plastic One, VA, USA,) was implanted in each hemisphere, above the ventral tegmental area (VTA), using the following stereotaxic coordinates: 5.5 mm posterior to bregma, 1.7 mm lateral and 6.3 mm below the surface of the cranium (Paxinos and Watson, 1986); cannulae were inserted into the brain with a mediolateral angle of 8° and were closed with an obturator of the same length. Four stainless-steel screws were threaded into the bone and the cannulae were anchored to the skull with dental acrylic. Behavioral tests started one week after the surgery.

Conditioned place preference (CPP) paradigm

The CPP apparatus (Med Associates, St. Albans, VT, USA) consisted of a rectangular Plexiglas box divided into two large compartments (26x21x21 cm) separated by a smaller central compartment (21x12x21 cm). Two sliding doors separated the central grey compartment from the two others which have distinct wall colors (white or black) and floors (grid or bar). Locomotor activity and times spent in each chamber were measured by computer-interfaced infrared photobeams (Med Associates, St. Albans, VT, USA). The CPP experiment lasted 10 days and consisted of a habituation phase, a conditioning phase and a test phase. On the first day of the habituation phase, rats were allowed to explore the entire

CPP apparatus for 20-min to reduce neophobia. On day 2, all animals received a first intra-VTA injection of 0.5 µl/side of saline and were allowed to explore freely the entire CPP apparatus for 20-min. On the third day, animals were allowed to explore the entire CPP apparatus for 20-min and time spent in each of the two large compartments was measured; rats exhibiting higher or lower time interval than 20-80% of time in a compartment were excluded (unbiased procedure). Conditioning began the next day. Conditioning trials lasted 30-min and were conducted daily for 6 days. Control and drug treatment groups were conditioned in either the black or the white compartment of the apparatus. On the first day of the conditioning phase, the drug-conditioned animals were injected in the VTA with vehicle and were immediately placed into one compartment of the apparatus for 30 min. The next day, animals were injected with [D-Tyr^{11}]NT (1.5 or 3 nmol/0.5µl/side), SR142948 (1.2 nmol/0.5µl/side), SR48692 (1.2 nmol/0.5µl/side), [D-Tyr^{11}]NT (3 nmol/0.5µl/side) + SR142948 (1.2 nmol/0.5µl/side) or [D-Tyr^{11}]NT (3 nmol/0.5µl/side) + SR48692 (1.2 nmol/0.5µl/side) and were immediately placed into the other compartment of the apparatus for 30 min. This procedure was repeated three times so that rats received three vehicle (Day 4, 6 and 8) and three drug (Day 5, 7, 9) injections. Animals in the control group were injected with the vehicle on each day and were similarly conditioned for 6 days. Twenty four hours after the last day of the conditioning phase, on day 10, animals were allowed to explore the apparatus for 20 min and the time spent in each compartment was measured. Animals were tested between 11:00 and 17:00 under an ambient light intensity of 5 lux and were habituated to the experimental room for 1-hr prior to the behavioral testing.

Microinjection procedure

Bilateral microinjections were made by inserting into each guide cannula an injection cannula (model C315I) that extended 2 mm beyond the tip of the guide. Each cannula was connected with polyethylene tubing to a 2-µl microsyringe and a volume of 0.5 µl of solution was injected into each hemisphere simultaneously with a micro-infusion pump over a period of 60 sec; cannulae were left in place for an additional 60 sec to allow diffusion into the surrounding brain tissue.

Histology

At the end of the experiment, animals were deeply anesthetized with urethane (2 g/kg, i.p.) and transcardially perfused with 0.9% saline followed by 10% formalin. Brains were removed, stored in 10% formalin and subsequently sliced in serial 40-µm sections that were stained with formal-thionin solution. Locations of the injection sites were determined under light microscopic examination. Only animals that had both injection sites within the VTA, including the rostral and caudal linear nuclei, the paranigral, parabrachial and the interfascicular nuclei between 5.0 and 6.0 mm behind bregma (Paxinos and Watson, 1986) were included in the analyses.

Drugs

[D-Tyr^{11}]neurotensin-(1-13) was purchased from Bachem (Sunnydale, CA, USA) and dissolved in sterile 0.9% saline at a concentration of 3 or 6 nmol/µl. The neurotensin antagonist, SR-142948 and SR-48692 were purchased from Tocris Bioscience (Burlington, ON, Canada) and were dissolved at a concentration of 2.4 nmol/µl in a sterile 0.9% sodium

chloride solution that contained 20% dimethylsufoxyde (DMSO). All solutions were stored at -20°C in 50 µl aliquots in silicone-coated tubes; they were thawed just before testing and were used only once.

Statistical analysis

Preference score was determined by subtracting the time spent in the drug-paired compartment measured before the conditioning phase (Pre) to the time spent in the same compartment measured on the conditioning test day (Post). Preference score and locomotor activity (horizontal and stereotypic-like movements) measured during the conditioning test day were analyzed with a one-way analysis of variance (ANOVA). The Duncan's multiple range post-hoc tests was used for individual group comparisons. The accepted value for significance was set at 0.05 (Statistica V5.0, StatSoft).

Electrophysiological experiments

Animals and slice preparation

Fourteen to 21-day-old (P14-P21) Long Evans pups of either sex obtained from Charles River (St-Constant, QC) were used. Pups were anaesthetized by methoxyflurane vapor inhalation in a closed chamber, decapitated and their brain quickly removed and transferred to chilled, oxygenated artificial cerebrospinal fluid (ACSF) in which NaCl had been replaced by equivalent osmolarity of sucrose and containing (in mM) sucrose 252 (NaCl 126 in standard ACSF); KCl, 3; NaH_2PO_4, 1.25; $MgSO_4$ 7 H_2O, 1.3; $CaCl_2$, 2.5; $NaHCO_3$, 26; and glucose, 10, and saturated with a gas mixture of 95% O_2 and 5% CO_2.

Two hundred and fifty micrometer thick horizontal slices preserving the VTA afferents (Margolis et al. 2006) were cut using a vibrating microtome (DSK Microslicer). Slices were transferred to a submerged recording chamber maintained between 32 to 34°C and superfused with standard ACSF at a rate of 2 ml/min; slices were incubated for at least one hour before recording began.

Electrophysiological recordings

Whole-cell configuration was achieved using the 'blind' patch-clamp technique (Blanton et al. 1989). Pipettes were pulled from thin wall borosilicate capillary glass on a P-87 micropipette puller (Sutter Instrument, Novato, CA, USA). Recording pipettes had a resistance of 3-5MΩ when filled with a solution containing (in mM) potassium gluconate, 140; $MgCl_2$, 2; $CaCl_2$, 0.1; EGTA, 1.1; HEPES, 10; K_2-adenosine trisphosphate (ATP), 2; guanosine trisphosphate (GTP), 0.5 and biocytin (5%). The pH was adjusted to 7.3 with KOH solution, and final osmolarity was 280 ±5 mosmol/kg. Biocytin (5%) was added in the recording pipette and all recorded cells were processed after recording to confirm their location in the medial VTA.

Whole-cell recordings were made with an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, USA) in continuous single-electrode voltage-clamp mode. The output of the amplifier was fed into a LPF 200A DC amplifier/filter (Warner Instruments Corp., Hamden, CT, USA) and digitized at 5 to 10 kHz with a real-time acquisition system

(CED 1401 Power). Data acquisition was achieved using the Signal 4.0 software (Cambridge Electronic Design, Cambridge, England). Recording pipette's capacitance was optimally adjusted before whole-cell configuration was achieved. The resting membrane potential was measured just after rupturing the cell membrane and the offset potential, measured upon withdrawal of the electrode from the cell, was accounted for assuming that it drifted in a linear fashion with time from the start of the recording session. We did not correct for liquid junction potential which for a pipette containing 140 mM potassium gluconate amounts for an additional potential shift of around -10mV (Spigelman et al. 1992).

Synaptic activation and drug application

The presence of I_h current was determined by voltage clamping cells at -60mV and stepping to −40, −50, −70, −80, −90, −100, −110 and −120 mV. Input resistance was monitored with hyperpolarizing pulses in current clamp mode. A monopolar tungsten stimulating microelectrode was placed rostral to the recording site in the medial VTA, on the slice superficial layer, 0.5-1.0 mm from the recording electrode. Excitatory postsynaptic currents were evoked by 0.1ms, 3 to 6V cathodal pulses delivered at 15 sec intervals. In order to isolate glutamate receptor-mediated EPSCs, all experiments were performed in the presence of (-) bicuculline methiodide (BMI, 10μM) in bath solution to block $GABA_A$ receptor-mediated synaptic currents. BMI was applied 30 min before obtaining whole-cell configuration to ensure a complete diffusion in the slice tissue. In all experiments the EPSCs were recorded from an online voltage-clamped potential of -70mV.

The effects of D-Tyr [11] NT on glutamatergic EPSCs were assessed at a holding membrane potential of -70mV.Three concentrations of the peptide (0.01μM, 0.1 μM and 0.5 μM) were tested, one concentration per cell. Upon agonist application, the change in amplitude of the glutamatergic EPSC was measured. Five minutes of baseline EPSC activity was recorded before superfusion with the peptide. The EPSC amplitudes were recorded during 7 min after the onset of the peptide application and averaged over the last 5 min. A washout period of 15 min was allowed before the amplitude of the recovered EPSC was measured. In some experiments, the control EPSC amplitude was measured for 4 min before a NT antagonist was added to the superfusion medium. SR142948 nor SR48692 produced any change in EPSC amplitude (n=12; data not shown); therefore in further experiments where NTS receptor antagonists were used, SR142948 or SR48692 was added to the superfusing medium for 7 min and a control response was measured in the presence of the antagonist.

Drugs and peptides

The following pharmacological agents were applied through the superfusing ACSF: (-) bicuculline methiodide obtained from Sigma Aldrich (Oakville, Ontario, Canada); D-Tyr [11] NT from Bachem (Sunnyvale, CA, USA); SR-48692 and SR1429482 obtained from Tocris Biosciences (Burlington, ON, Canada). All drugs were made up as 10 mM stock solutions in distilled water and diluted with ACSF solution to final concentration just before addition to the perfusion medium with the exception of SR48692 which was dissolved in DMSO (final concentration 0.1%) and distilled water.

Data analysis

Data analysis was performed using Signal software (Cambridge Electronic Design, Cambridge, England). The magnitude of EPSC recorded after application of the peptide was expressed as percent of baseline and group means were calculated for drug condition. A one-way ANOVA was performed and Duncan post-hoc test used to determine significant differences between concentration or drug and peptide condition when justified; level of significance was set at 0.05 (Statistica V5.0, StatSoft).

Results

Behavioral experiment

From the 86 rats that completed the experiment, 10 were excluded from the analysis because the injection sites were dorsal or anterior to the VTA, or because the sites (left and right hemisphere) overlapped on the midline; an additional rat was excluded because the injection sites could not be located.

Ventral midbrain microinjection of [D-Tyr^{11}]NT induced a conditioned place preference.

Figure 1 illustrates the preference score (top panel) and locomotor activity (middle and bottom panels) measured during the conditioning test in different groups of rats that were conditioned with the vehicle and one of two doses of [D-Tyr^{11}]NT. As can be seen, animals that were conditioned with VTA [D-Tyr^{11}]NT microinjections spent more time in the peptide associated compartment than the animal conditioned with VTA microinjections of the vehicle. The ANOVA yielded a significant effect of treatment ($F_{2,31} = 13.1$, $p < 0.001$) and post-hoc test showed that preference score of each [D-Tyr^{11}]NT group was significantly different than vehicle; although the preference score for the group treated with the highest dose was superior to that of the lower dose there was no significant difference between the two doses ($p > 0.05$). In order to determine whether the preference for the [D-Tyr^{11}]NT-paired compartment was in part related to a conditioned aversion to the unpaired compartment, we compared the preference score for this compartment and the neutral compartment among the three groups. Animals that were injected with [D-Tyr^{11}]NT spent less time in the unpaired compartment on the conditioned test day than those injected with the vehicle (Figure 2, top panel) but the ANOVA yielded no significant effect of treatment ($F_{2,31} = 2.4$, $p > 0.05$). Moreover the animals conditioned with the high dose of [D-Tyr^{11}]NT spent slightly less time in the neutral compartment (Figure 2, bottom panel) compared to the other groups but the difference was not significant ($F_{2,31} = 2.33$, $p > 0.05$). Altogether, these results suggest that the reduction in the time spent in the compartment non-associated with VTA [D-Tyr^{11}]NT in the conditioned groups was due to an increase in the amount of time spent in the conditioned but not the neutral compartment, hence confirming the occurrence of a conditioned preference effect.

The overall locomotor activity (in the entire apparatus) measured during the conditioned test did not differ between groups suggesting that repeated exposure to VTA [D-Tyr^{11}]NT did not induce conditioned locomotor activity (Figure 1, middle and bottom panels). The ANOVA performed on each measure of activity, horizontal and stereotypy-like, yielded no significant effect of treatment (horizontal activity, $F_{2, 31} = 0.63$ $p > 0.05$; stereotypy-like activity, $F_{2, 31} = 0.14$, $p > 0.05$).

[D-Tyr^{11}]NT-induced a conditioned place preference: Role of NTS1 receptors.

To determine which NT receptor is involved in the induction of a CPP by VTA [D-Tyr^{11}]NT, we compared the preference score obtained from animals that were conditioned with vehicle and [D-Tyr^{11}]NT alone to that of animals conditioned with either SR142948 with [D-Tyr^{11}]NT or SR48692 with [D-Tyr^{11}]NT, or each NT antagonist alone. Results presented in Figure 3 (top panel) shows that SR48692 blocked the induction of a CPP. The ANOVA yielded a significant effect of treatments ($F_{5,61} = 5.74$, $p < 0.001$) and post-hoc test showed that the preference score of the group injected with preferred NTS1 antagonist, SR48692, with [D-Tyr^{11}]NT is not significantly different than that of the vehicle injected animals but is significantly different than that of the [D-Tyr^{11}]NT alone injected animals. The NTS1/NTS2 antagonist, SR142948, attenuated the induction of a CPP. When administered alone during the conditioning phase, the antagonists induced no conditioned effect. Altogether, these results show that the conditioned preference is due to activation of

VTA NTS1 receptors. Locomotor activity measured in the entire apparatus during the conditioned test did not differ between groups (Figure 3, middle and bottom panels; horizontal activity, $F_{5, 61} = 1.14$, $p > 0.05$; stereotypy-like activity, $F_{5, 61} = 0.93$. $p > 0.05$).

Electrophysiological results

Whole-cell voltage-clamp recording was carried out on 96 physiologically identified VTA neurons. Neurons were designated as I_h^+ (n=54) or I_h^- (n=42) based on the presence or absence of the hyperpolarisation activated cationic current (I_h). The amplitude of I_h in I_h^+ positive neurons ranged from 67 pA to 419 pA with an average of 192.3 pA (n = 54; data not shown).

Effects of D-Tyr [11] NT on glutamatergic EPSCs in I_h^+ and I_h^- neurons.

The effects of [D-Tyr^{11}]NT on glutamatergic EPSCs in VTA neurons were measured at a holding membrane potential of -70mV upon application at three different concentrations (0.01μM, 0.1 μM and 0.5 μM). Desensitization of the response to [D-Tyr^{11}]NT application allowed only one concentration of the peptide to be tested per cell.

Representative traces of the evoked EPSCs from a single I_h^+ cell obtained before, during and after washout of 0.01 μM of [D-Tyr^{11}]NT are shown in Figure 4. It can be seen that [D-Tyr^{11}]NT attenuated the EPSC and that this effect was completely reversible. As shown in Figure 5 [D-Tyr^{11}]NT produced a dose dependent reduction in the amplitude of the

glutamatergic EPSCs in I_h^+ cells. At concentrations of 0.01 μM, 0.1 μM and 0.5 μM, the mean decrease in EPSC amplitude was 20±1.5 % (n=6), 29 %±3 % (n=6) and 47%±4% (n=7) respectively. A one way ANOVA yielded significant effect ($F_{2,16} = 20.95$, $p < 0.001$) and post-hoc test confirmed that the highest concentration produced a decrease that was significantly different than that produced by the lower concentration ($p < 0.001$).

In I_h^- cells, [D-Tyr^{11}]NT produced a dose-dependent increase in the amplitude of the evoked EPSC. Representative traces obtained from a single I_h^- cell illustrated in Figure 6 show that the enhancement effect of [D-Tyr^{11}]NT was also reversible. The mean increase at concentrations of 0.01 μM, 0.1 μM and 0.5 μM was 15±2.5 % (n=6), 28±2 %(n=6) and 56±3.5 % (n=9) respectively (Figure 5). A one way ANOVA yielded significant results ($F_{2, 18} = 51.7$, $p < 0.001$); post-hoc test showed that there a significant different between the highest concentration and the two others ($p < 0.001$), and between the medium and the lowest concentration ($p < 0.01$).

Effect of NTS antagonists in I_h^+ and I_h^- neurons

To identify the neurotensin receptor subtype(s) involved in the enhancement and attenuation effects of [D-Tyr^{11}]NT on the evoked EPSCs in each cell population, we measured the EPSCs in the presence of SR142948 or SR48692. We found that in I_h^+ cells, SR48692 (0.5μM) and SR142948 (0.5 μM) were both effective at blocking the decrease in EPSC amplitude produced by [D-Tyr^{11}]NT (Figure 7, top panel). It can be noted that in the

in the presence of this high concentration of the antagonists, [D-Tyr^{11}]NT produced an enhancement of the EPSC amplitude. A one way ANOVA yielded a significant effect ($F_{2,11}$, 89.3 = $p < 0.001$) and post-hoc test confirmed that the EPSCs measured in the presence of the antagonist were significantly different than the EPSCs measured in the presence of [D-Tyr^{11}]NT alone; there was also a significant difference between the effect of SR142948 and SR48692. Interestingly, different results were obtained when the concentration of the antagonist was reduced to 0.1 μM. At this concentration, SR142948 blocked the attenuation effect of [D-Tyr^{11}]NT while SR48692 had no effect (Figure 4 and 7). The ANOVA yielded significant effect ($F_{2,11} = 37.7$, $p < 0.001$) and post-hoc test confirmed that the mean EPSC measured in the presence of SR142948 was significantly different than that measured in the presence of [D-Tyr^{11}]NT alone or SR48692 + [D-Tyr^{11}]NT.

In I_h^- cells, at the highest concentration (0.5μM), SR48692 and SR142948 blocked the enhancement effect of [D-Tyr^{11}]NT on the EPSC (Figure 7, bottom panel); in the presence of SR142948, [D-Tyr^{11}]NT produced a large attenuation of the EPSC. The ANOVA yielded significant effect ($F_{2, 14} = 40.6$, $p < 0.001$) and post-hoc test showed that the EPSC measured in the presence of SR142948 and SR48692 was significantly different than that measured in the presence of [D-Tyr^{11}]NT alone. There was also a significant difference in EPSCs measured in the presence of SR142948 and SR48692, confirming that SR142948 led to a significant attenuation. Similarly to what we observed in I_h^+ cells, different results were obtained when the concentration of the antagonists was reduced to 0.1 μM. At this concentration, SR142948 and SR48692 similarly blocked the enhancement effect of [D-

Tyr^{11}]NT (Figure 6 and 7). The ANOVA yielded significant effect ($F_{2,13} = 18.5$, $p < 0.001$) and post-hoc test confirmed that the mean EPSC measured in the presence of SR142948 and SR48692 were not different but were both different than that measured in the presence of [D-Tyr^{11}]NT alone.

Discussion

The main finding of this study is that activation of ventral midbrain NTS1 receptor induces a CPP and that this effect can be mediated, at least in part, through an enhancement of glutamatergic synaptic input in non-dopamine neurons in the VTA. Our results also show that [D-Tyr^{11}]NT activates NTS2 receptors to reduce glutamatergic synaptic input to VTA dopamine and non-dopamine neurons. To our knowledge these findings constitute the first evidence that NT acts on two different NT receptor sub-types to modulate in an opposite manner glutamatergic neurotransmission in different populations of VTA neurons.

Previous studies have shown that ventral midbrain NT microinjection produces a rewarding effect as it sustains self-administration (Glimcher et al. 1987), enhances brain stimulation reward (Kalivas and Taylor 1985a;Rompre et al. 1992) and induces a CPP (Kitabgi et al. 1980a). Consistently, we found that ventral midbrain microinjections of [D-Tyr^{11}]NT dose-dependently induced a preference for the environment associated with the peptide. The fact that the time spent in the other two compartments, the neutral compartment and the compartment associated with the vehicle, was not significantly different between vehicle and [D-Tyr^{11}]NT microinjected animals confirms that the increase in the amount of

time spent in the peptide associated compartment was due to its reward inducing effect; it also suggests that no aversive effect developed during the day that follows the peptide injection. The induction of a CPP by [D-Tyr^{11}]NT is consistent with many other results showing that this NT analog mimics several behavioral, physiological and neurochemical effects of NT (al-Rodhan et al. 1991;Donoso et al. 1986;Ferraro et al. 2000;Jolicoeur et al. 1984;Steinberg et al. 1995). According to Glimcher et al. (Glimcher et al. 1984a), however, the induction of CPP by repeated ventral midbrain NT is not reproduced by an equimolar concentration of NT-(8-13); such a result was unexpected because NT-(8-13) displays a high affinity for the NTS1 (Kalivas et al. 1986;Rompre and Gratton 1993b) and has been shown to be as effective as NT at inducing locomotor activity (Steinberg et al. 1995), enhancing brain stimulation reward (Rompré and Gratton 1993), increasing dopamine cell firing (Seutin et al. 1989;Shi and Bunney 1991a) and inducing polydipsia (Hawkins et al. 1989). One possible explanation is that Glimcher et al. (1984) compared the effectiveness of bilateral VTA microinjections of NT to equimolar unilateral microinjections of NT-(8-13); it could be that at the concentration used unilateral microinjections were insufficient to induce a CPP.

In order to determine which sub-type(s) of NT receptor is involved in the conditioned rewarding effect of [D-Tyr^{11}]NT, we studied the effectiveness of SR142948 and SR48692 at preventing the induction of a CPP. Results show that SR142948 attenuated and SR48692 blocked the conditioned rewarding effect of [D-Tyr^{11}]NT, hence suggesting that it is mediated by NTS1 receptor. A large body of evidence shows that VTA dopamine neurons play a key role in reward. For instance, VTA microinjection of drugs that enhance dopamine impulse flow sustains self-administration, induces a CPP and enhances brain stimulation reward (see Ikemoto and Bonci, 2014; Wise 1996). More convincing are recent optogenetic

results showing that selective activation of VTA dopamine neurons induces a CPP and sustains operant responding (Tsai et al. 2009; Witten et al 2011). NTS1 receptors are expressed on dopamine cell bodies and dendrites and their activation stimulates dopamine cell firing and dopamine release in brain regions known to play a key role in reward (Luo et al. 2010a;Rompre and Bauco 2006b). Kempadoo et al (2013) also showed that activation of VTA NTS1 receptors by local NT release reinforces operant responding. Altogether this suggests that the induction of a CPP by [D-Tyr^{11}]NT is due to activation of NTS1 receptors expressed on VTA dopamine neurons.

Conditioned place-preference is a learning process that involves neural plasticity. Drugs that induce a CPP, such as cocaine and morphine, induce lasting changes in VTA glutamatergic neurotransmission (Zweifel et al. 2008) and blockade of VTA glutamatergic receptors prevents cocaine- (Harris and Aston-Jones 2003) and morphine-induced CPP (Harris et al. 2004). Kempadoo et al (Kempadoo et al. 2013a) have also shown that the rewarding effect of VTA NT release is associated with an enhancement of glutamatergic input to dopamine neurons. In order to determine whether the induction of a conditioned reward by [D-Tyr^{11}]NT was related to a modulation of glutamatergic inputs to VTA neurons, we investigated the effect of [D-Tyr^{11}]NT on glutamatergic EPSCs in different population of neurons distinguished with the presence or absence of an I_h current. Nearly all dopaminergic neurons express an I_h current while I_h^+ neurons represent a subset of non-dopaminergic neurons; some GABA and glutamatergic neurons also express an I_h current (Hnasko et al. 2012; Johnson and North, 1992; Lacey et al., 1989; Margolis et al., 2006; 2012).

In the present study, we observed that bath application of varying concentrations of [D-Tyr^{11}]NT generated a dose-dependent enhancement in the amplitude of glutamatergic EPSCs in I_h^- neurons (non-dopamine neurons). This enhancement was most likely mediated by activation of NTS1 receptors as it was blocked by SR142948 and SR48692. These findings are in parallel with the behavioral results and suggest the action of NT on glutamatergic inputs to non-dopamine neurons may also play a key role in conditioned reward. Since the VTA contains a high density of NT terminals, it is thus possible that the effect of NT is not limited to glutamatergic inputs to dopamine neurons in this region (Jennes et al. 1982a;Sarret et al. 2003b;Seroogy et al. 1987). Luo et al. (2010), for instance, showed that cocaine still induces a CPP in animals that had selective deletion of NMDA receptors onto dopamine neuron, and that this conditioned rewarding effect was NMDA-dependent.

In several limbic regions such as in the entorhinal cortex and the dentate gyrus of the hippocampus, activation of NTS1 receptors induces an excitatory effect and an increase in glutamate release (Antonelli et al. 2007;Antonelli et al. 2008); these effects are dependent on coupling to PLC, phosphokinase C (PKC) and Ca^{2+}influx through L-type Ca^{2+} channels and activation of myosin light chain kinases respectively (Xiao et al. 2014;Zhang et al. 2015). Additionally, evidence of a facilitatory NTS1-NMDA receptor interaction at cortico-striatal glutamate terminals strengthens the role of NT in modulating glutamate release (Antonelli et al. 2004). Although within the scope of our study, we were not able to identify the exact effector molecules mediating this action, association of NTS1 receptors to such downstream excitatory signaling cascades might have come into play.

When tested over the same range of concentrations, [D-Tyr^{11}]NT generated a dose-dependent attenuation in the amplitude of glutamatergic EPSCs in I_h^+ neurons. This attenuation was most likely mediated by activation of NTS2 receptors as it was blocked by a low concentration of SR142948 but not SR48692. As mentioned previously, all VTA dopamine neurons are I_h^+ and that strongly suggests that [D-Tyr^{11}]NT reduces glutamatergic inputs to at least a population of VTA dopamine neurons. In view of the evidence of a role for VTA dopamine in reward, and of the enhancement effect of NT on VTA dopamine impulse flow, these results were unexpected. They suggest that the action of [D-Tyr^{11}]NT on glutamatergic inputs to VTA dopamine neurons through activation of NTS2 receptors is unlikely involved in the induction of a conditioned reward. In fact, the action of [D-Tyr^{11}]NT on NTS2 should oppose its action on NTS1 and contribute to reduce its effectiveness at inducing a conditioned reward. This may explain why SR142948 which displays a similar affinity for NTS1 and NTS2 (Gully et al. 1997) was less effective than SR48692, a preferred NTS1 antagonist (Gully et al. 1995), at attenuating the induction of CPP.

There is also a large proportion of VTA GABA neurons that express an I_h current and it has been shown that GABA provides an inhibitory drive to dopamine neurons that is under the control of glutamate (Grace et al. 2007). An attenuation of glutamatergic input to these neurones is likely to enhance dopamine impulse flow resulting in reward and/or reward enhancement. If [D-Tyr^{11}]NT is acting on NTS2 receptors to reduce glutamatergic EPSCs to these neurons, SR142948 would have been more effective than SR48692 at attenuating the induction of CPP; but as mentioned above, we observed the opposite.

The attenuation effect of [D-Tyr^{11}]NT on glutamatergic EPSCs in I_h^+ neurons contrasts with the enhancement effect of NT and NT-(8-13) reported in previous studies (Bose et al. 2015; Kempadoo et al. 2013). Indeed both NT and NT-(8-13) enhance glutamatergic EPSC in I_h^+ neurons by activating NTS1 receptors. Kempadoo et al. (Kempadoo et al. 2013a), however, observed a biphasic effect with NT-(8-13), an enhancement of NMDA EPSCs at a low concentration and an attenuation at a high concentration; the former but not the latter was blocked by SR48692 suggesting that the attenuation is not mediated by the NTS1 receptor. It thus appears that both NTS1 and NTS2 modulate glutamatergic inputs to I_h^+ neurons and that [D-Tyr^{11}]NT has a predominant effect on the NTS2 receptor subtype. [D-Tyr^{11}]NT has a higher affinity for NTS2 than NTS1 (Kitabgi et al. 1980; Labbe-Jullie et al. 1994) and activation of NTS2 receptors do not induce excitatory effects. For instance, activation of human NTS2 receptors expressed on CHO cell lines lacks the potential to elevate intracellular Ca^{2+} levels by mobilizing internal calcium reserves or accumulation IP3; it was rather associated with activation mitogen activated protein kinases (MAPK) that led to inhibition (Sarret et al. 2002). It could be that activation of NTS2 receptors on putative dopamine neurons enhances MAPK signaling and produces a reduction in glutamatergic signaling.

The 11th position substitution in [D-Tyr^{11}]NT by a D-tyrosine residue makes it more resistant to cleavage by endopeptidases (Checler et al. 1983a). In fact, after an intracerebroventricular injection of NT, 98% of the NT was cleared and degraded in brain tissues during a 30 min period after the injection. Under the same conditions, 33% of [D-

Tyr[11]]NT was retained, suggesting a half-life 1.5 times greater than that of NT (Checler et al. 1983a). Owing to the relatively stable metabolic profile of [D-Tyr[11]]NT it is possible that the reduction in EPSC observed by Kempadoo et al (2013) with higher concentrations of NT8-13 reflects that produced by the lower concentrations of [D-Tyr[11]]NT used in the present study.

Interestingly, in the presence of a high concentration (0.5 μM) of SR142948, but not SR48692, [D-Tyr[11]]NT produced an opposite, significant inhibition, of glutamatergic EPSCs in I_h^- neurons. This could possibly arise because at this concentration, SR142948 interacts with an NT receptor subtype other than NTS1 and NTS2 (possibly NTS3). The NTS3 protein and its mRNA are present in VTA and are mainly expressed on cell bodies and dendrites. According to Mazella et al. (1998), the NTS3 receptor is nearly insensitive to SR48692. Others, however, reported that SR142948 is effective at blocking the NTS3-mediated growth response to NT in cancer cells (Dal Farra et al. 2001); that would rather exclude a role for this receptor in the opposite effect of [D-Tyr[11]]NT on EPSC in the presence of SR142948. In I_h^+ neurons, the presence of a high concentration of SR142948 and SR48692 had the same impact; [D-Tyr[11]]NT enhanced the EPSC, an effect opposite to what was observed when it was infused alone. As mentioned previously, it remains unclear why the antagonists produce such a reverse effect.

Conflict of interest

The authors declare no conflict of interest for this work.

Funding

Funding for this study was provided by Canadian Institutes of Health Research (Grant #102572 to PPR and RAW), Natural Sciences and Engineering Research Council of Canada (Grant # 184095-2009 to |RAW) and Fonds de Recherche Santé Québec.

Author and Contributors

PPR and KR designed the behavioral experiments; KR carried out the behavioral experiments and analyzed the data with PPR. RAW and PB designed the electrophysiological experiments; PB carried out the electrophysiological experiments and analyzed the data with RAW and PPR. All authors contributed and approved the final version of the manuscript.

Acknowledgements

The authors would like to thank Claude Bouchard for his support, excellent expertise and sound advice as well as all the staff of the Pavillon Paul-G Desmarais animal's facility.

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Figure 1

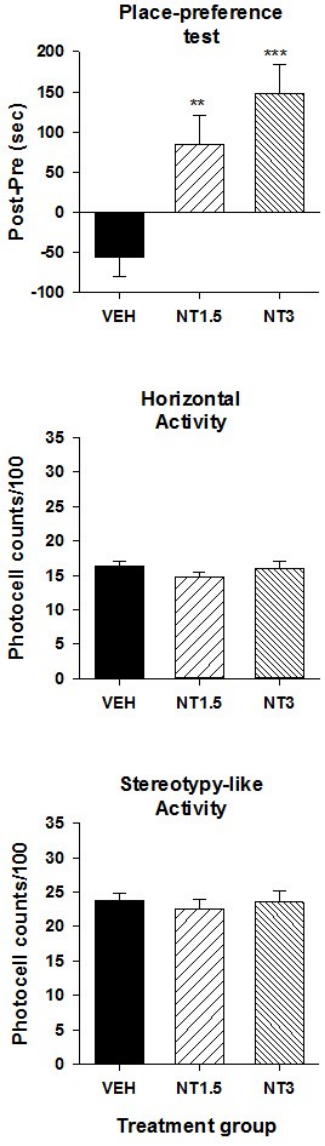

Place-preference test
Post-Pre (sec)
200
150
100
50
0
-50
-100
**
***
VEH
NT1.5
NT3
Horizontal Activity
Photocell counts/100
35
30
25
20
15
10
5
0
VEH
NT1.5
NT3
Stereotypy-like Activity
Photocell counts/100
35
30
25
20
15
10
5
0
VEH
NT1.5
NT3
Treatment group

Figure 1. Induction of a CPP by [D-Tyr^{11}]NT. Top panel illustrates the preference score measured on the test day for the animals that were injected with 1.5 nmol (NT1.5, n = 6), 3 nmol (NT3, n = 13) of [D-Tyr^{11}]NT or its vehicle (VEH, n = 15). Preference score corresponds to the amount of time (in sec) spent in the paired compartment on the test day minus the time spent at baseline in the same compartment. Measures of locomotor activity recorded during the preference test for the animals in each treatment group are presented in the middle panel (horizontal) and bottom panel (stereotypy-like). Asterisks indicate a statistical significant difference with VEH (**$p < 0.01$; ***$p < 0.001$).

Figure 2

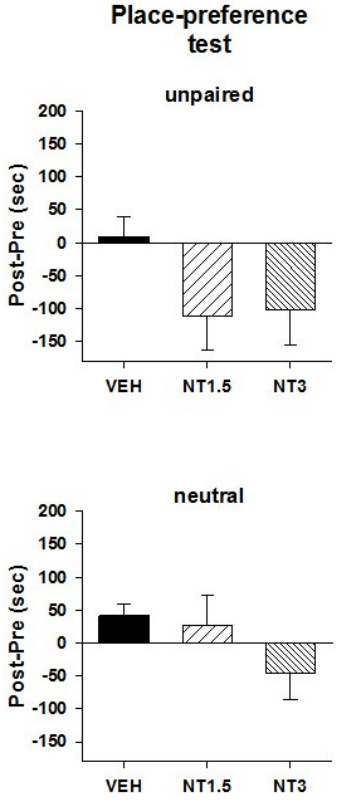

Place-preference test
unpaired
Post-Pre (sec)
200
150
100
50
0
-50
-100
-150
VEH
NT1.5
NT3
neutral
Post-Pre (sec)
200
150
100
50
0
-50
-100
-150
VEH
NT1.5
NT3

Figure 2. Mean preference score measured on the test day in the unpaired (top panel) and neutral (bottom panel) compartment for the animals that were injected with 1.5 nmol (NT1.5, n = 6), 3 nmol (NT3, n = 13) of [D-Tyr^{11}]NT or its vehicle (VEH, n = 15). Preference score corresponds to the amount of time (in sec) spent in the unpaired or neutral compartment on the test day minus the time spent at baseline in the same compartment. See text for details.

Figure 3

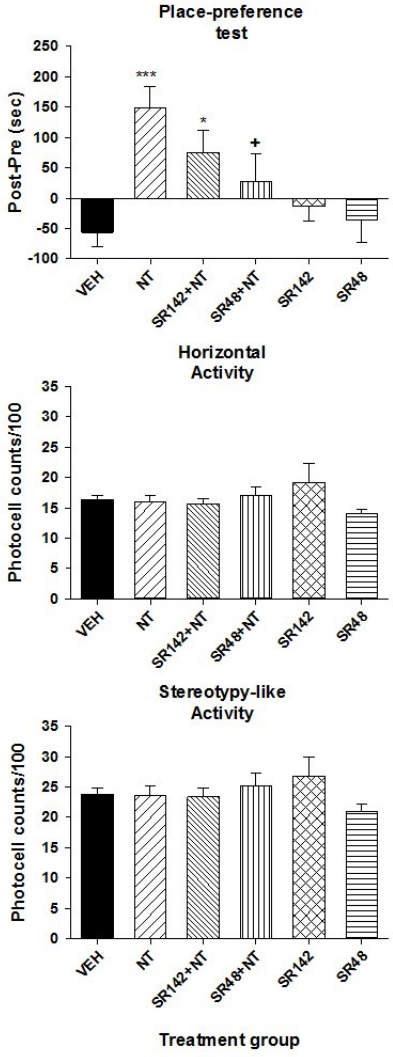

Place-preference test
Post-Pre (sec)
250
200
150
100
50
0
-50
-100
***
*
+
VEH
NT
SR142+NT
SR48+NT
SR142
SR48
Horizontal Activity
Photocell counts/100
35
30
25
20
15
10
5
0
VEH
NT
SR142+NT
SR48+NT
SR142
SR48
Stereotypy-like Activity
Photocell counts/100
35
30
25
20
15
10
5
0
VEH
NT
SR142+NT
SR48+NT
SR142
SR48
Treatment group

Figure 3. Effects of SR142948 and SR48692 on [D-Tyr^{11}]NT-induced CPP. Top panel illustrates the preference score measured on the test day for the animals that were injected with 3 nmol of [D-Tyr^{11}]NT (NT, n = 13), 1.2 nmol of SR142948 (SR142, n = 10), 1.2 nmol of SR48692 (SR48, n = 7), SR142948 + [D-Tyr^{11}]NT (SR142+NT, n = 12), SR48292 + [D-Tyr^{11}]NT (SR48+NT, n = 10) or the vehicle (VEH, n = 15). Preference score corresponds to the amount of time (in sec) spent in the paired compartment on the test day minus the time spent at baseline in the same compartment. Measures of locomotor activity recorded during the preference test for the animals in each treatment group are presented in the middle panel (horizontal) and bottom panel (stereotypy-like). The asterisks and the cross indicate a statistical significant difference with VEH (**$p < 0.01$; ***$p < 0.001$) and NT (+ $p < 0.05$) respectively.

Figure 4

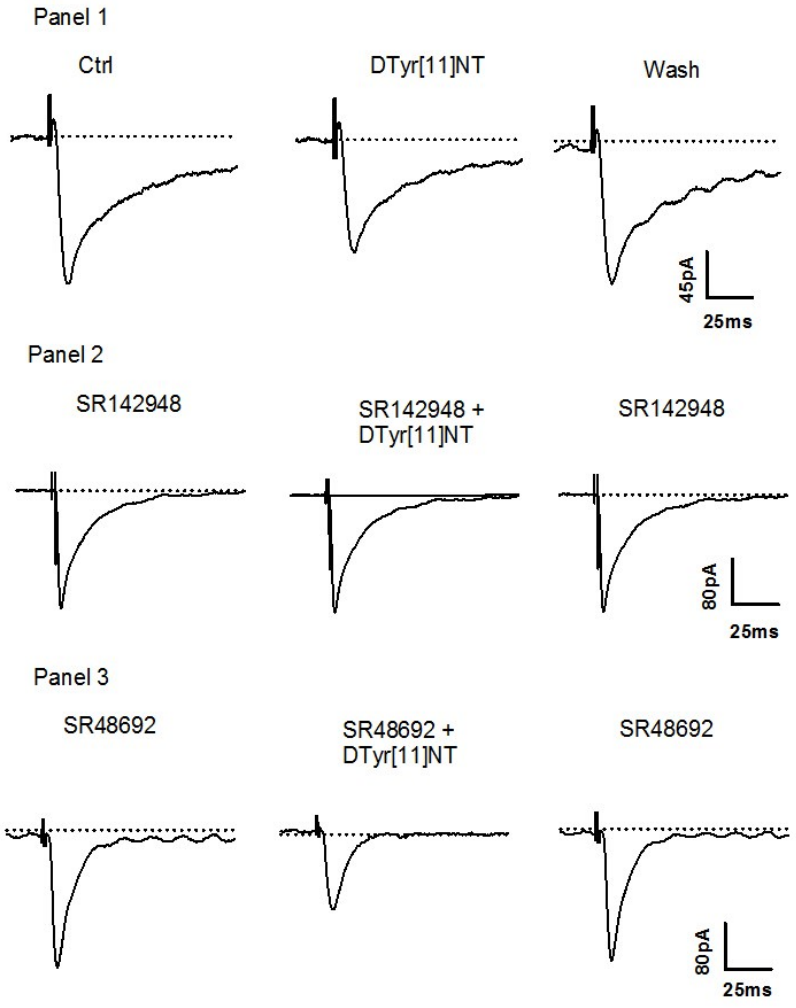

Panel 1
Ctrl
DTyr[11]NT
Wash
45pA
25ms
Panel 2
SR142948
SR142948 +
DTyr[11]NT
SR142948
80pA
25ms
Panel 3
SR48692
SR48692 +
DTyr[11]NT
SR48692
80pA
25ms

Figure 4. Effect of [D-Tyr^{11}]NT and antagonists on I_h^+ neurons. Panel 1: Current traces of glutamatergic EPSC recorded during superfusion of [D-Tyr^{11}]NT ; control(1), with [D-Tyr^{11}]NT (0.01μM)(2) and following the washout of [D-Tyr^{11}]NT (3) at a holding membrane potential of -70mV in I_h^+ neurons (n=6). Panel 2: Current traces of glutamatergic EPSC recorded during superfusion with SR142948 (0.1 μM) (1), with SR142948and [D-Tyr^{11}]NT (0.01μM) (2) and with SR142948 following the washout of (3) at a holding membrane potential of -70mVin I_h^+ neurons (n=5). Panel 3: Current traces of glutamatergic EPSC recorded during superfusion with SR48692 (0.1 μM) (1), with SR48692and [D-Tyr^{11}]NT (0.01μM) (2) and with SR48692 following the washout of [D-Tyr^{11}]NT (3) at a holding membrane potential of -70mVin I_h^+ neurons (n=4).

Figure 5

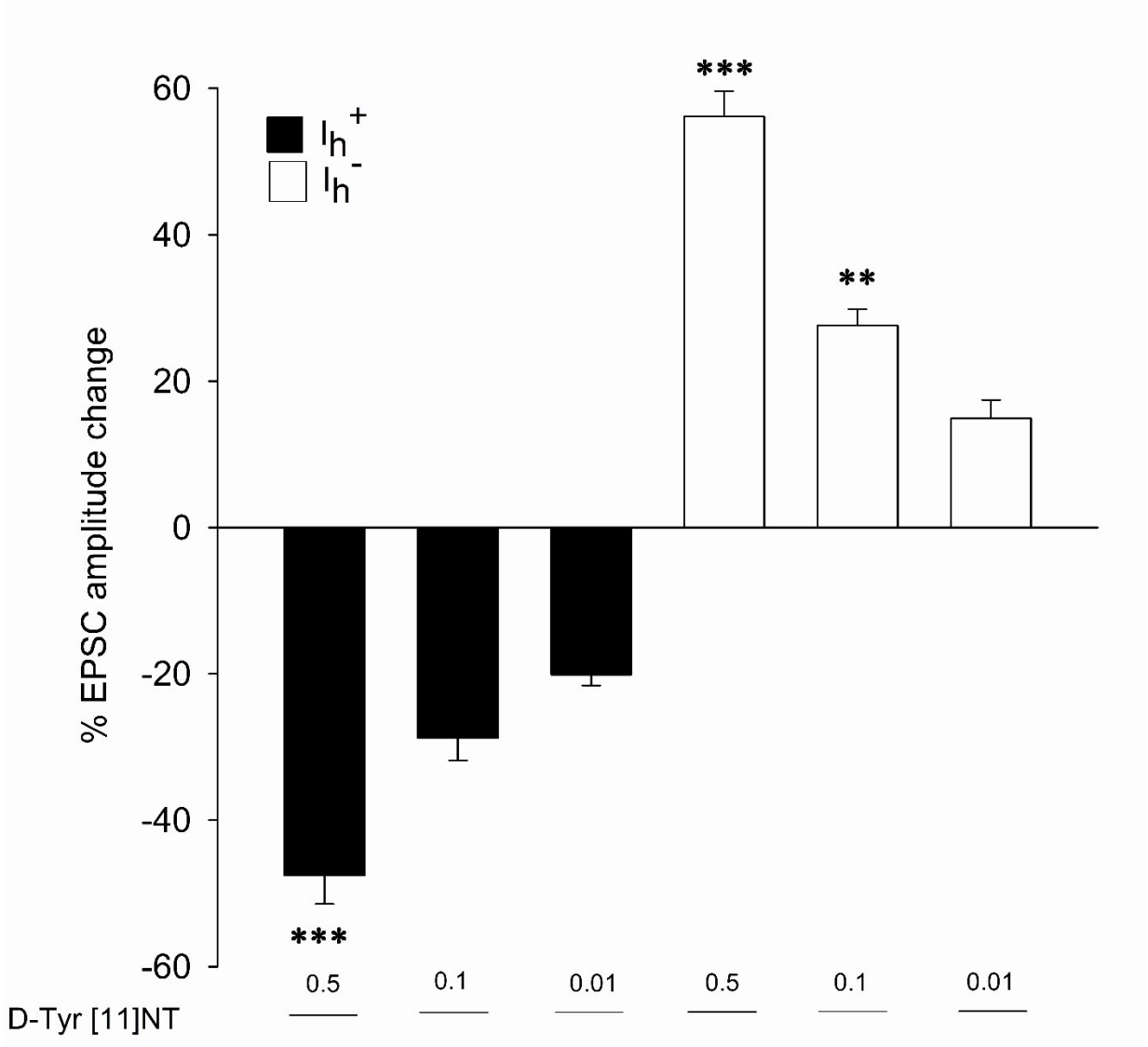

60
40
20
0
-20
-40
-60
% EPSC amplitude change
I_h^+
I_h^-
***
**
***
0.5
0.1
0.01
0.5
0.1
0.01
D-Tyr [11]NT

Figure 5. Effects of [D-Tyr^{11}]NT on glutamatergic EPSCs in I_h^+ and I_h^- neurons. Mean percent change in EPSC amplitude recorded in I_h^+ (black bar) and I_h^- (white bar) following application of different concentrations of [D-Tyr^{11}]NT. The number of neurons recorded at each concentration is as follow: 0.01μM, n=12 (I_h^+ n = 6, I_h^- n = 6); 0.1 μM, n = 12 (I_h^+ n = 6, I_h^- n = 6); 0.5μM, n = 16 (I_h^+ n = 7, I_h^- n = 9). All concentrations of D-Tyr[11]NT are reported in μM. Asterisks indicate a statistically significant difference with the lowest concentration (**$p < 0.001$; *** $p < 0.001$). See text for details.

Figure 6

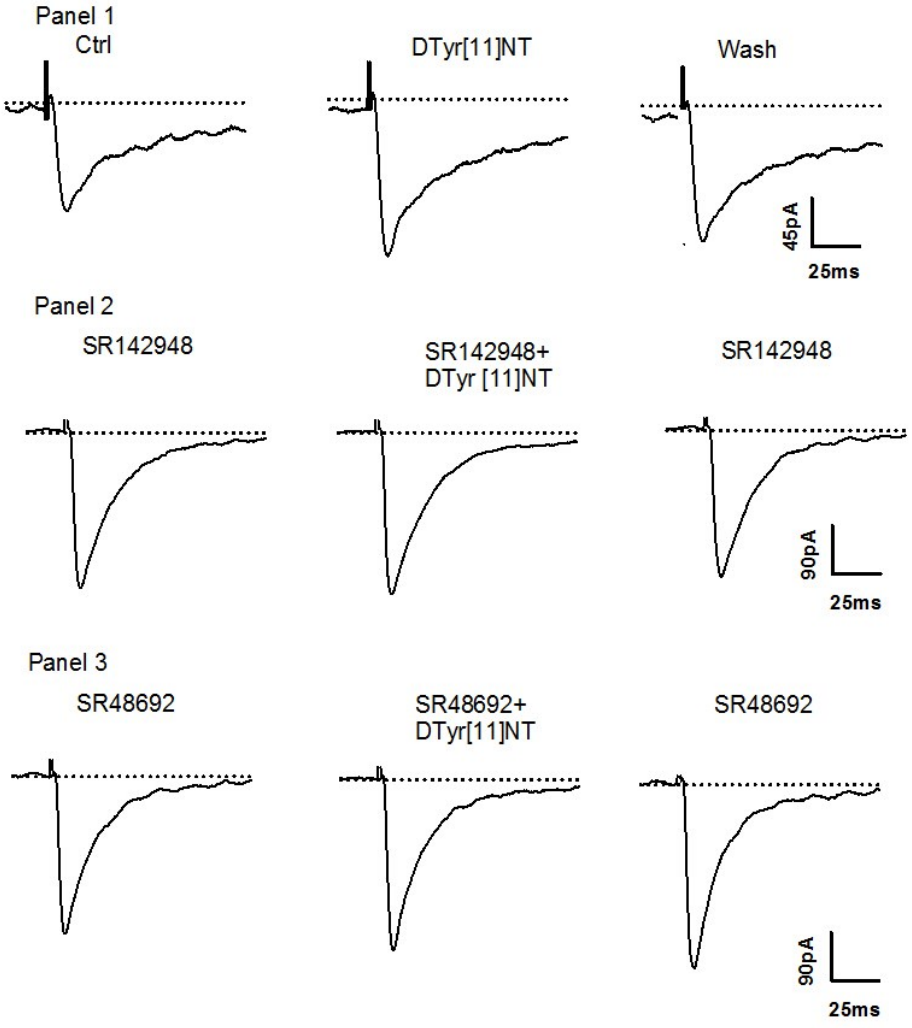
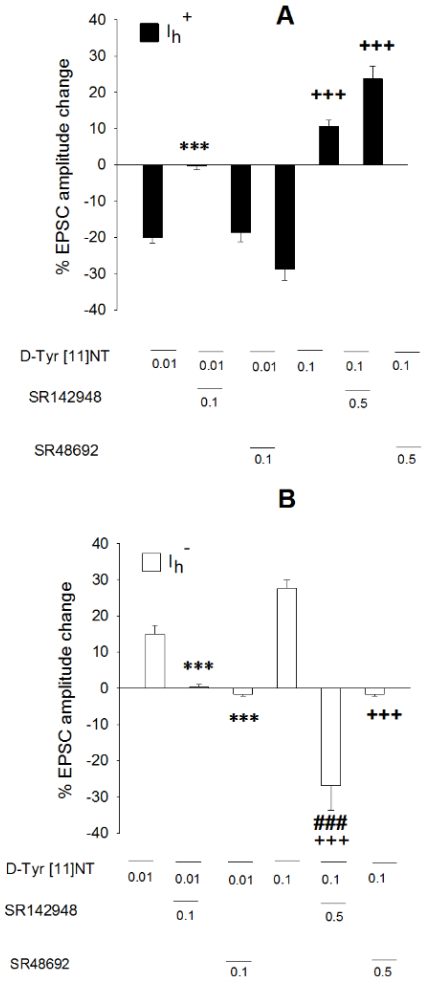

Panel 1
Ctrl
DTyr[11]NT
Wash
45pA
25ms
Panel 2
SR142948
SR142948+
DTyr [11]NT
SR142948
90pA
25ms
Panel 3
SR48692
SR48692+
DTyr[11]NT
SR48692
90pA
25ms

Figure 6. Effect of [D-Tyr^{11}]NT and antagonists on I_h^- neurons. Panel 1: Current traces of glutamatergic EPSC recorded during superfusion of [D-Tyr^{11}]NT; control (1), with [D-Tyr^{11}]NT(0.01μM)(2) and following the washout of [D-Tyr^{11}]NT(3) at a holding membrane potential of -70mV in I_h^- neurons (n=6). Panel 2: Current traces of glutamatergic EPSC recorded during superfusion with SR142948 (0.1 μM) (1), with SR142948 and [D-Tyr^{11}]NT (0.01μM) (2) and with SR142948 following the washout of [D-Tyr^{11}]NT(3) at a holding membrane potential of -70mVin I_h^- neurons (n=5). Panel 3: Current traces of glutamatergic EPSC recorded during superfusion with SR48692 (0.1 μM) (1), with SR48692and [D-Tyr^{11}]NT (0.01μM) (2) and with SR48692 following the washout of [D-Tyr^{11}]NT (3) at a holding membrane potential of -70mVin I_h^- neurons (n=5).

Figure 7


A
I_h^+
% EPSC amplitude change
40
30
20
10
0
-10
-20
-30
-40
***
+++
+++
D-Tyr [11]NT
0.01
0.01
0.01
0.1
0.1
0.1
SR142948
0.1
0.5
SR48692
0.1
0.5
B
I_h^-
% EPSC amplitude change
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D-Tyr [11]NT
0.01
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SR142948
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SR48692
0.1
0.5

Figure 7. Effect of SR142948 and SR48692 on glutamatergic EPSCs in I_h^+ and I_h^- neurons. Mean percent change in EPSC amplitude recorded in I_h^+ (panel A) and I_h^- neurons (panel B) following application of [D-Tyr^{11}]NT alone or in the presence of SR142948 or SR48692. The number of neurons recorded under each condition is as follow: I_h^+ neurons[D-Tyr^{11}]NT (0.01μM, n =6; 0.1μM, n = 6); SR142948 (0.1μM, n = 5; 0.5μM, n = 4); SR48692 (0.1μM, n = 4; 0.5μM, n=4); I_h^- neurons, [D-Tyr^{11}]NT (0.01μM, n =6; 0.1μM, n = 6); SR142948 (0.1μM, n = 5; 0.5μM, n = 5); SR48692 (0.1μM, n = 5; 0.5μM, n=4). All concentrations of D-Tyr[11]NT, SR142948 and SR48692 are reported in μM. Asterisks and crosses indicate a statistically significant difference with [D-Tyr^{11}]NT alone at 0.01μM and 0.1μM respectively (*** $p < 0.001$ with 0.01 μM; +++ $p < 0.001$ with 0.1 μM). The ### sign indicates a statistical significant difference between SR142948 (0.5μM) +D-Tyr[11]NT (0.1μM) and SR48692(0.5μM) +D-Tyr[11]NT(0.1μM). See text for details.

Article 3

D-Tyr [11] NT differentially modulates glutamatergic neurotransmission in VTA neurons.

Poulomee Bose[1] and Richard A. Warren[1]

[1]Department of Psychiatry University of Montreal, Montreal, Canada

Running title: Effects of D-Tyr [11] NT on glutamatergic EPSCs in VTA neurons.

Address for correspondence:

Richard A. Warren

Department of Psychiatry

Faculty of Medicine, University of Montreal

C.P. 6128, Succursale Centre-ville

Montreal, QC, Canada

H3C 3J7

Status of the manuscript: Manuscript submitted to PLoS One.

Abstract:

Neuroadaptation at glutamatergic synapses in the ventral tegmental area (VTA) appears to underlie the development of behavioural sensitization to drugs of abuse in animals. The VTA is heavily innervated by neurotensin (NT) terminals and receptors and NT antagonists have been shown to block the development of behavioural sensitization in rats. It is thus possible that NT modulates glutamatergic neurotransmission in VTA neurons and that this modulation may explain why NT antagonists block sensitization mechanisms. However, how NT modulates glutamatergic neurotransmission in VTA neurons remain largely unknown. In the present study, VTA neurons were classified as I_h^+ or I_h^- based on the presence or the absence of the hyperpolarisation activated cationic current (I_h) respectively. Using *in vitro* whole cell patch-clamp electrophysiology we measured the effect of D-Tyr [11] NT on pharmacologically isolated n-methyl-D-aspartate (NMDA) and AMPA excitatory post-synaptic currents (EPSCs) in I_h^+ and Ih^- VTA neurons. We found that in I_h^+ neurons both NMDA and AMPA receptor mediated EPSCs were attenuated by the neurotensinergic analog D-Tyr [11] NT. This attenuation was mediated by both NTS1 and NTS2. In contrast, both NMDA and AMPA receptor mediated EPSCs were enhanced by an NTS1 dependent mechanism in I_h^- neurons. This enhancement in EPSC amplitudes in I_h^- neurons appeared to be mediated by neurotensinergic receptor located on glutamatergic terminals. These results provide additional evidence that NT exerts a bidirectional modulation on glutamatergic neurotransmission in VTA neurons and highlights a novel

peptidergic modulation of glutamatergic inputs to non-DA neurons that might be implicated in drug seeking behaviours.

Keywords: VTA, Neurotensin, Excitatory post synaptic currents, NMDA receptors, AMPA receptors.

Introduction

The endogenous neuropeptide, neurotensin (NT), found in several limbic regions have been implicated in sensitization and reward mechanisms (Dobner et al. 2003;Rompre and Perron 2000a). Sensitization is a progressive augmentation of drug elicited responses that relates to the rewarding properties of abused drugs or psychostimulants and is a key feature of compulsive pattern of drug intake (Glimcher et al. 1984a;Robinson and Berridge 1993;Wise and Bozarth 1987). The ventral tegmental area (VTA), an anatomical substrate involved in the development of sensitization contains a significant number of NT cell bodies and receives dense NT innervation from brain regions such as the lateral hypothalamus, preoptic area and rostral lateral septum (Binder et al. 2001a;Fallon 1988;Sarret et al. 2003b;Uhl et al. 1979). It contains a high density of NT receptors which, when activated, stimulate dopamine cell firing mediated partly by inactivation of somatodendritic autoreceptors and an increase in non-selective cationic conductance (Farkas et al. 1997;St-Gelais et al. 2006a). Activation of NT receptors on DA neurons elicits a two component excitatory response; an NTS2 mediated fast, short component and another NTS1 mediated slow, long component (Binder et al. 2001a;Chien et al. 1996;Farkas et al. 1996). However, these responses are NT analog specific; only the hexapeptide NT analog (NT8-13) induces the fast response by activation of NTS2 whereas both the native peptide NT1-13 and NT8-13 elicit the slow response mediated by NTS1 (Farkas et al. 1996;Nalivaiko et al. 1998a). Another active NT analog, D-Tyr[11]NT has a substitution at the 11^{th} position by a d-tyrosine residue that renders it more resistant to cleavage by endopeptidases, has a higher affinity for NTS2 and a lower affinity for NTS1 receptor compared to NT1-13 and NT8-13 (Steinberg et al. 1995). However, VTA

injections of D-Tyr [11] NT induces dopamine dependent behaviours like NT or NT8-13(Bauco and Rompre 2003;Blackburn et al. 2004). It has also been shown that repeated central injections of NT or D-Tyr [11] NT induces sensitization to psychostimulants (such as cocaine and amphetamine) (Rompre 1997a;Rompre and Bauco 2006b). However, VTA injections of D-Tyr [11] NT produce different levels of dopamine efflux in the rostral and caudal nucleus accumbens (nAcb) and is not effective in the prefrontal cortex (PFC) in contrast to NT or NT8-13 (Sotty et al. 2000a).

Interestingly, a role for glutamate in the initiation of sensitization was suggested as blockade of VTA N-methyl D-aspartate receptors (NMDA) disrupted the induction of locomotor sensitization to amphetamine (Cador et al. 1999a). This is congruent with another study that showed that the long term effects of sensitization to cocaine by NT were blocked by an NMDA antagonist (Vezina and Queen 2000b). The involvement of glutamate is further supported by a recent optogenetic study in which the selective stimulation of hypothalamic neurotensinergic efferents to the VTA DA neurons and induced a rewarding effect that is NMDA dependent (Kempadoo et al. 2013a) . Thus NMDA receptor activation induced VTA neural plasticity tempts us to hypothesize that NT's role in initiating sensitization involves modulating glutamatergic inputs to VTA neurons. However, this modulation is complex and depends on the concentration of the neurotensin agonist used as well as the glutamatergic component being studied. For instance, Kempadoo et al., (Kempadoo et al. 2013a)suggested a bidirectional effect on the NMDA component dependent on the concentration of the NT agonist used and a dose-dependent decrease of the AMPA component both being NTS1 receptor independent; whereas another study reported an NTS1 mediated depression of glutamatergic excitatory

post synaptic currents (EPSCs) using a higher concentration of the agonist (Kempadoo et al. 2013a;Kortleven et al. 2012a).

We therefore sought to characterize the NT modulation of VTA glutamatergic neurotransmission, by using whole cell patch clamp recordings to measure the effects of D-Tyr [11] NT on isolated glutamatergic EPSC components measured in putative VTA DA and non-DA neurons. Putative VTA DA neurons were characterized by the presence of the hyperpolarisation activated cationic current (I_h) and the absence of it categorized the neuron as non-DA. The effects of NT antagonists were also evaluated to identify the receptors mediating the effects.

Methods

Animals and slice preparation

All experimental procedures were approved by the Institutional Animal Committee in accordance with guidelines of the Canadian Council on Animal Care. All efforts were made to minimize the suffering and number of animals used.

Fourteen to 21-day-old (P14-P21) Long Evans pups of either sex obtained from Charles River (St-Constant, QC) were used. Pups were anaesthetized by inhalation of methoxyfluran vapour in a closed chamber, decapitated and their brain quickly removed and transferred to chilled, oxygenated artificial cerebrospinal fluid (ACSF) in which NaCl had been replaced by equivalent osmolarity of sucrose and containing (in mM) sucrose 252 (NaCl 126 in standard ACSF); KCl, 3; NaH_2PO_4, 1.25; $MgSO_4$ 7 H_2O, 1.3; $CaCl_2$, 2.5; $NaHCO_3$, 26; and glucose, 10, and saturated with a gas mixture of 95% O_2 and 5% CO_2.

Two hundred and fifty micrometer thick horizontal slices preserving the VTA afferents (Margolis et al. 2006b) were cut using a vibrating microtome(DSK Microslicer). Slices were transferred to a submerged recording chamber maintained between 32 to 34°C and superfused with standard ACSF at a rate of 2 ml/min; slices were incubated for at least one hour before the commencement of recording.

Drugs and peptides

The following pharmacological agents were applied through the superfusing ACSF: (-) bicuculline methiodide (BMI), 6 cyano-7-nitroquinoxaline-2,3-dione (CNQX), (+)-2-amino-5-phosphonopentanoic acid (APV) obtained from Sigma Aldrich(Oakville, Ontario, Canada) ; D-Tyr[11]NT was obtained from Bachem (Sunnyvale, CA,USA); SR-48692 [2-(1-[7-chloro-4-quinolinyl]-5-[2, 6-dimethoxyphenyl]-1H-pyrazol-3-)carbonylamino-adamantane-2-carboxyl acid] ,SR142948A2-[[[5-(2,6-Dimethoxyphenyl)-1-[4-[[[3-(dimethylamino)propyl]methylamino]carbonyl]-2-(1-methylethyl)phenyl]-1*H*-pyrazol-3-yl]carbonyl]amino]-tricyclo[3.3.1.13,7]decane-2-carboxylic acid (Tocris Biosciences). All drugs were made up as 10 mM stock solutions in distilled water and diluted with ACSF solution to final concentration just before addition to the perfusion medium with the exception of SR48692 and CNQX which were dissolved in DMSO (final concentration 0.1%) and distilled water.

Electrophysiological recordings

Whole-cell configuration was achieved using the 'blind' patch-clamp technique(Blanton et al. 1989a). Pipettes were pulled from thin wall borosilicate capillary glass on a P-87 micropipette puller (Sutter Instrument, Novato, CA, USA). Recording pipettes had a

resistance of 3-5MΩ when filled with a solution containing (in mM) potassium gluconate, 140; $MgCl_2$, 2; $CaCl_2$, 0.1; EGTA, 1.1; HEPES, 10; K_2-adenosine triphosphate (ATP), 2; guanosine triphosphate (GTP), 0.5 ,biocytin (5%) and QX314 ,2. The pH was adjusted to 7.3 with KOH solution, and final osmolarity was 280 ±5 mosmol/kg. All recorded neurons were labelled to confirm their location in the medial VTA.

Whole-cell recordings were made with an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, USA) in continuous single-electrode voltage-clamp mode. The output of the amplifier was fed into a LPF 200A DC amplifier/filter (Warner Instruments Corp., Hamden, CT, USA) and digitized at 5 to 10 kHz with a real-time acquisition system (CED 1401 Power). Data acquisition was achieved using the Signal 4.0 software (Cambridge Electronic Design, Cambridge, England). Recording pipette's capacitance was optimally adjusted before whole-cell configuration was achieved. The resting membrane potential was measured just after rupturing the cell membrane and the offset potential, measured upon withdrawal of the electrode from the cell, was accounted for assuming that it drifted in a linear fashion with time from the start of the recording session. We did not correct for liquid junction potential, which for a pipette containing 140 mM potassium gluconate amounts for an additional potential shift of around -10mV(Spigelman et al. 1992).

Synaptic activation and drug application

The presence of I_h was first determined by voltage clamping cells at -60mV and stepping to −40, −50, −70, −80, −90, −100, −110 and −120 mV. Input resistance was monitored with hyperpolarizing pulses in current clamp mode. A monopolar tungsten stimulating microelectrode was placed rostral to the recording site in the medial VTA, on the slice

superficial layer, 0.5-1.0 mm from the recording electrode. Excitatory postsynaptic currents were evoked by 0.1ms, 3 to 6V cathodal pulses delivered at 15 sec intervals. In order to isolate glutamate receptor-mediated EPSCs, all experiments were performed in the presence of (-) bicuculline methiodide (BMI, 10μM) in bath solution to block $GABA_A$ receptor-mediated synaptic currents. BMI was applied 30 min before obtaining whole-cell configuration to ensure a complete diffusion in the slice tissue. In experiments that studied AMPA or NMDA EPSCs, APV or CNQX (respectively) was added to the superfusion medium 30min before the commencement of recording. The composite glutamatergic EPSCs were recorded from an online voltage-clamped potential of -70mV, while the NMDA EPSCs were voltage clamped at-20mV in the presence of CNQX (20μM) and the AMPA EPSCs were voltage clamped at -70mV in the presence of APV (50μM). The effects of D-Tyr[11]NT(0.01μM) on glutamatergic EPSCs were assessed at a holding membrane potential of -70mV in case of AMPA EPSCs and -20MV in the case of NMDA EPSCs. D-Tyr [11] NT (0.01μM) was tested only once per cell. Upon agonist application, the change in amplitude of the glutamatergic EPSC was measured. Five minutes of baseline EPSC activity was recorded before superfusion with the peptide. The EPSC amplitudes were recorded during 7 min after the onset of the peptide application and averaged over the last 5 min. A washout period of 15 min was allowed before the amplitude of the recovered EPSC was measured. In some experiments, the control EPSC amplitude was measured for 4 min before a NT antagonist was added to the superfusion medium. However, SR142948A or SR48692 produced no effect on the control EPSC amplitude (n=9; data not shown). Therefore in further experiments where NTS receptor

antagonists were used, SR142948 or SR48692 was added to the superfusing medium for 7 min and control response was measured in the presence of the antagonist.

Data analysis

Data analysis was performed using Signal software (Cambridge Electronic Design, Cambridge, England). Statistical analysis was performed using Sigmaplot 12 software (Systat, San Jose, CA, USA). The magnitude of EPSC recorded after application of the peptide was expressed as % of baseline and group means were calculated for drug condition. Student's t test was performed to compare between groups of cells (NMDA vs non-NMDA). A one-way ANOVA was performed in comparing the effects of the antagonists and the agonist for a cell group and post hoc Bonferroni's test was used to determine significant differences between concentration and peptide condition when justified; level of significance was set at 0.05 (Statistica V5.0, StatSoft).

RESULTS

Whole-cell voltage-clamp recording was obtained from 102 VTA neurons in slices from rats between P14 and P21. Based on the presence or the absence of the hyperpolarisation activated cationic current (I_h), neurons were categorized as I_h^+ (n=49) or I_h^-(n=53). The amplitude of I_h in I_h^+ neurons ranged between 92 pA and 437 pA with an average of 206.72 pA (±10.87) (data not shown). All neurons filled with biocytin (5%) were examined under light microscopy and were confirmed to be located in the VTA.

Characteristics of glutamatergic EPSCs

Typically, postsynaptic currents evoked by electrical stimulation in the presence of the $GABA_A$ receptor antagonist BMI consisted of a compound glutamatergic EPSC comprising an early and a late component mediated, respectively, by the activation of AMPA/KA and NMDA receptors respectively (Fig. 1). We characterized composite postsynaptic glutamatergic EPSCs in 11 neurons; the late component of the EPSC was isolated and recorded in 35 neurons and the early component was isolated and recorded in 33 neurons. The remaining 23 neurons were tested for paired pulse protocols for early (n=12) and late components (n=11).

The early EPSC peaked between 3.4 and 20 msec. after stimulus onset at a holding membrane potential of –70 mV, had a linear relationship with the membrane potential and reversed around 0 mV (n=44). In contrast, the maximal amplitude of the late EPSC occurred much later, was usually observed at holding membrane potentials of –20 or –40 mV, displayed a non-linear relationship with voltage and also reversed around 0 mV (n=36).

Figure 1 shows a representative example of an EPSC recorded in an I_h^+ VTA neuron from a P20 animal on which specific glutamatergic antagonists were tested. During the control period (Fig. 1A panel 1), the early EPSC peaked 9 msec. after the stimulus onset at a holding membrane potential of –70 mV and the response decayed to baseline within 25 msec. The current voltage relationship (I_R-V_m) of the early EPSC was almost linear at membrane potentials between –120 mV and 20 mV (Fig. 1B panel 1). Bath application of the AMPA/KA receptor antagonist CNQX completely abolished the early component of the

EPSC and there was virtually no residual postsynaptic current at all membrane potentials at the latency the early response was measured (Fig. 1A panel 2 and Fig. 1B panel one).

The late component, measured after the early component had decayed, increased at membrane potentials between –100 and –40 mV and reached its maximum usually at–20 mV. At more depolarized membrane potentials, it decreased and reversed polarity around 0 mV (Fig. 1A and Fig. 1B panel 2), a current-voltage relationship typical of NMDA receptor-mediated current. The further addition of the NMDA receptor antagonist APV to the superfusing medium completely abolished the late EPSC (Fig. 1A panel 3), demonstrating that it was mediated by NMDA-type receptors. In the presence of CNQX alone, the NMDA receptor-mediated EPSC was recorded in isolation showing that measurements of the late component of the EPSC made on the compound EPSC were close to the peak of the NMDA-mediated EPSC and represented mostly NMDA receptor-mediated current (Fig. 1A panel 2). Also, note there was no residual postsynaptic current in the presence of CNQX and APV, showing that glutamatergic EPSCs were effectively isolated by the addition of BMI to the superfusing medium. CNQX and APV were tested together in 11(I_h^+ n=5; I_h- n=6) other neurons producing similar results. There were no differences in the characteristics of glutamatergic EPSCs in I_h^+ or I_h^- neurons (data not shown). In addition, CNQX and APV were tested individually in 35 and 33 neurons respectively.

[Figure 1]

In most neurons, the effects of neurotensinergic agonist and antagonists were assessed at holding membrane potentials usually between -100 and 20 mV in steps of 20 mV. The AMPA/KA-mediated EPSC was measured at the peak of the early component of

the EPSC at a holding membrane potential of –70 mV, when the amplitude of the late component was minimal (left vertical dotted lines in Fig. 1A) whereas the effects on NMDA receptor-mediated currents were measured at a latency at which the early component recorded at a holding membrane potential of –70 mV had decayed (see right vertical dotted lines in Fig. 1A).

Effects of D-Tyr [11] NT on NMDA EPSCs.

The addition of the neurotensin agonist, D-Tyr [11] NT to the superfusing medium in the presence of BMI and CNQX typically produced a decrease of the late NMDA component of the EPSC in I_h^+ neurons. The average decrease in the amplitude of the pharmacologically isolated NMDA EPSC produced was found to be 30±3.0% (n=6; see Figure2 E). A representative example is shown in Figure 2A. In this case, the amplitude of the NMDA component of the EPSC recorded at–20 mV, was reduced by 33% during the application of D-Tyr [11] NT.

To identify the type of receptors mediating the inhibitory action of D-Tyr [11] NT, it was administered in the presence of the broad spectrum NT antagonist SR142948 or NTS1 antagonist, SR48692. When administered alone, SR142948A (0.1μM) produced no change in the amplitude of the NMDA EPSC (data not shown), however when of D-Tyr [11] NT is administered in the presence of SR142948A (n=7), the inhibition of the NMDA EPSC was successfully blocked (see Figure 2E). A representative example of this effect is shown in Figure 2B. However, in the presence of D-Tyr [11] NT, SR48692 (0.1μM) (n=5) significantly attenuated the NMDA EPSC inhibition by 15±1.5%, suggesting the involvement of NTS1 and NTS2 in mediating the effect. A one way ANOVA yielded a

significant effect ($F_{2,11}$=57.95,$p<0.001$) and post-hoc test confirmed that the mean change in EPSC measured in the presence of SR142948 was significantly different than that measured in the presence of [D-Tyr^{11}]NT alone or SR48692 + [D-Tyr^{11}]NT ($p<0.001$).

In contrast the addition of D-Tyr [11] NT in in I_h^- neurons produced an increase in the amplitude of the pharmacologically isolated NMDA EPSCs. The average change in the amplitude of excitation produced was found to be 35±5.5% (n=7; see figure 2E). A representative example of this effect is shown in Figure 2C. In this case, the amplitude of the late component recorded at -20mV was enhanced by 31% during the application of D-Tyr [11] NT.

We next sought to characterize the neurotensin receptors responsible for mediating the excitatory effect of D-Tyr [11] NT in I_h^- neurons. SR142948A when applied alone produced no effect on the late component of the EPSC (data not shown). In the presence of SR142948A (0.1μM), D-Tyr [11] NT failed to produce the enhancement in the NMDA EPSC amplitude (n=6; see figure 2E). A representative example of this effect is shown in Figure 2D. SR48692, the NTS1 receptor antagonist also blocked the enhancement effect, thus confirming the role of NTS1 in mediating this effect (n=5). A one way ANOVA yielded a significant effect ($F_{2,12}$=36.22,$p<0.001$) and post-hoc test confirmed that the mean change in EPSC measured in the presence of SR142948 was significantly different than that measured in the presence of [D-Tyr^{11}]NT alone or SR48692 + [D-Tyr^{11}]NT ($p<0.001$).

[Figure 2]

Effects of D-Tyr [11] NT on non-NMDA EPSCs.

Similar to the observation in NMDA EPSCs, the addition of D-Tyr [11] NT to the superfusing medium in the presence of BMI and APV also resulted in a decrease of the early AMPA component of the EPSC in I_h^+ neurons. The average inhibitory effect was found to be 10±1.0% (n=7; see Figure 3E). Figure 3 A shows a representative example of this effect. In this case the, pharmacologically isolated AMPA EPSC recorded at -70mV and it was inhibited by 10%. When compared to the inhibition produced in the amplitude of NMDA component, the decrease in the amplitude of the AMPA EPSC was significantly lower (student's t test $p<0.001$).

To characterize the receptor subtypes responsible in mediating this effect, when D-Tyr [11] NT was added to the superfusing medium in the presence of SR142948A (0.1μM), the inhibitory effect in AMPA EPSCs was completely abolished (n=6; see figure 3E). A representative example of this effect is shown in Figure 3 B. However, when SR48692 (0.1μM) was tested (n=6; see Figure3E) the inhibitory effect of D-Tyr [11] NT persisted, thus ruling out the possibility of a role for NTS1 receptor. A one way ANOVA yielded a significant effect ($F_{2,12}=34.85, p<0.001$) and post-hoc test confirmed that the mean change in EPSC measured in the presence of SR142948 was significantly different than that measured in the presence of [D-Tyr^{11}]NT alone or SR48692 + [D-Tyr^{11}]NT ($p<0.001$).

In I_h^- neurons, addition of D-Tyr [11] NT to the superfusing medium produced an increase in the AMPA component of the EPSC. The average enhancement of the EPSC was found to be 13±1.5 %(n=7; see figure3E). Figure 3C shows a representative example of this effect in

the AMPA EPSC in the presence of APV and recorded at-70mV in an I_h^-neuron. The enhancement effect in this case was 12.65%.

In comparison to the enhancement of the NMDA component in I_h^- neurons (13±1.5 %) produced by D-Tyr [11] NT, the AMPA EPSC was significantly less enhanced (6±0.5 %) (Student's t test $p<0.001$).

To identify the receptor subtype mediating the increase in the AMPA EPSC amplitude, D-Tyr [11] NT was tested in the presence of SR142948A (0.1μM). SR142948A failed to completely block the enhancement effect; instead it significantly attenuated to about 50% of the increase produced. The average decrease in the enhancement of the AMPA EPSCs was found to be 6±0.5 %(n=6; see figure3E). However, when SR48692 (0.1μM) was tested, the excitation effect was significantly blocked (n=5). A representative example of this effect is shown in Figure 3D. A one way ANOVA yielded a significant effect ($F_{2,13}=25.89, p<0.001$) and post-hoc test confirmed that the mean change in EPSC measured in the presence of SR142948 was significantly different than that measured in the presence of [D-Tyr^{11}]NT alone or SR48692 + [D-Tyr^{11}]NT ($p<0.001$).

[Figure 3]

Locus of neurotensinergic modulation of evoked EPSCs.

Our evidence suggests that D-Tyr[11]NT modulates AMPA glutamatergic responses in I_h^- neurons exclusively by presynaptic mechanisms. The rationale behind using a paired pulse protocol in discerning pre-synaptic mechanisms resides in the fact that synaptic facilitation is associated with augmentation in presynaptic Ca^{2+} levels and neurotransmitter release i.e

the residual calcium hypothesis. The use of two consecutive stimuli with a 50msec interval generates a larger 2nd EPSC than the 1st, reflecting an enhanced neurotransmitter release from the residual calcium [23, 28, 29]. In our experiments, we found that the ratio (PPR; 2nd EPSC amplitude/1st EPSC amplitude) significantly changed during the application of D-Tyr[11]NT in I_h^- neurons, thus suggesting presynaptic mechanisms. In the presence of D-Tyr[11]NT (n=7), the amplitude of both the first and second evoked EPSCs increased, but the second response increased to a larger extent, resulting in an increase in the PPR (Fig. 4A). In contrast, no significant changes in PPR was observed in I_h^+ neurons (Fig.4A, panel 3).

To identify the locus (pre- or postsynaptic) of action of neurotensin agonist D-Tyr[11]NT, we compared two features of our recordings in the presence and absence of the neurotensinergic agonist. Since NMDA receptors are voltage dependent and activated at greater positive potentials (-20mV) than AMPA receptors, the depolarized membrane potentials are more susceptible to generate population spikes and consequently affecting paired pulse ratios. Therefore we next investigated the locus of modulation of AMPA responses by D-Tyr[11]NT.

This suggests that under the present experimental conditions neurotensin receptors agonists produced no detectable postsynaptic effects in VTA I_h^- neurons and that the present results reflect an action on presynaptic receptors.

Second, we found that under the present experimental conditions D-Tyr [11] NT produced a direct effect on the membrane and/or firing properties of I_h^- neurons when QX314 was omitted from the pipette solution. Figure 4B shows an example of the effects produced by D-Tyr [11] NT (0.01μM) under these conditions. In this case, D-Tyr [11] NT, produced a membrane depolarization of 13 mV. Intracellular depolarizing current pulse that was subthreshold during control readily increased spiking when D-Tyr [11] NT was added to the superfusing medium and the number of action potentials increased in response to suprathreshold current injection. These results suggest that the presence of QX314 into the recording pipette occluded the postsynaptic effects mediated by neurotensinergic receptors.

[Figure4]

Discussion

We found that D-Tyr[11]NT, an active analog of NT, reduced and enhanced glutamatergic neurotransmission respectively in VTA I_h^+ and I_h^- neurons respectively and this modulation was mediated by activation of different NT receptor subtypes. While the decrease in glutamatergic neurotransmission was mediated by NTS2 in I_h^+ neurons, the enhancement in I_h^- neurons involved NTS1 seemingly located on glutamatergic terminals. To our knowledge this is the first study that highlights a neurotensinergic modulation of

glutamatergic neurotransmission in non-DA neurons (I_h^-) in the VTA that might be relevant to sensitization and reward mechanisms.

The substitution of a d-tyrosine residue at the 11th position in the native NT peptide (NT1-13), renders D-Tyr[11]NT more resistant to cleavage by endopeptidases when compared to NT1-13 itself or to the carboxy terminal hexapeptide, NT8-13 (Checler et al. 1983a). Both NT1-13 and NT8-13 have a higher binding affinity for NTS1 than NTS2(Binder et al. 2001a). On the contrary, D-Tyr [11] NT has a higher binding affinity for NTS2 than NTS1 (Sotty et al. 2000a). However, despite differences in binding affinities for NT receptor subtypes in comparison to either NT1-13 or NT8-13, D-Tyr [11] NT injections in the VTA induced DA release both *in vitro* and *in vivo* and produced dopamine dependent behaviours like NT1-13or NT8-13 (Rompre 1997a;Steinberg et al. 1995). Nonetheless, in comparison to NT, it has greater potency in inducing these effects. This can be accounted for by the stability of D-Tyr [11] NT. It was found that in the presence of thiorphan, a peptidase inhibitor, it induced larger locomotor effects caused by increased levels of DA concentrations (Steinberg et al. 1995). Since D-Tyr [11] NT has differential binding affinities for NT receptor subtypes we used D-Tyr [11] NT to delineate the role of NT receptors in modulating glutamatergic neurotransmission in VTA neurons (Binder et al. 2001a;Gully et al. 1997a;Steinberg et al. 1995).

Effect of D-Tyr [11] NT on I_h^+ neurons

The disruption in the development of cocaine sensitization could be induced by blockade of NMDA receptors in the VTA(Rompre and Bauco 2006b). Concomitantly, sensitization to amphetamine could also be blocked by administration of NT antagonists(Panayi et al. 2005b). This is suggestive of an interaction between NMDA receptors and NT, which could contribute to the disruption of the development of sensitization. We have previously shown that both NT1-13 and NT8-13 enhance glutamatergic neurotransmission in I_h^+ and I_h^- neurons by recruiting NTS1 and 2 receptors respectively(Bose et al. 2015) . However, we obtained different results when D-Tyr [11] NT was tested in VTA neurons. D-Tyr [11] NT induced a reduction in glutamatergic neurotransmission in I_h^+ neurons and that this involved NTS2 (unpublished data). The present results show that D-Tyr [11] NT reduces the amplitude of isolated NMDA EPSCs by activating NTS2receptors. An NTS1 independent form of reduction in NMDA EPSCs induced by NT8-13 in VTA DA neurons has been reported by Kemapdoo et al., (Kempadoo et al. 2013a). However, the authors also reported a bidirectional modulation rather than a dose-dependent effect. At lower concentrations of 0.01μM, NT8-13 induced an enhancement effect which was NTS1 receptor dependent, whereas higher concentrations of NT8-13 attenuated the EPSC amplitude, an effect which was not NTS1 dependent. This is partly consistent with our results as we observed that D-Tyr [11] NT attenuated NMDA receptor mediated EPSCs in I_h^+ neurons dose-dependently. Moreover, we found that the selective NTS1 antagonist, SR48692 attenuated the inhibitory effect whereas SR142948A the non-selective antagonist blocks the inhibitory effect in I_h^+ neurons thereby suggesting an NTS2 receptor involvement. Additionally, activation of NTS2 is associated with the lack of mobilization of intracellular calcium reserves and increase in Ca^{2+} levels. Also accumulation of inositol-

3-phosphate (IP3) or inhibition of IP3 production is associated with the induction of inhibitory effects to depolarise the cell (Sarret et al. 2002). Kortleven et al. (Kortleven et al. 2012a) suggested a reduction in glutamatergic EPSCs through an NTS1 dependent mechanism. Thus in I_h^+ neurons, D-Tyr [11] NT induces a reduction in glutamatergic neurotransmission. Within the population of I_h^+ neurons in our studies it is possible that there might be some glutamatergic neurons in addition to the majority of them being DA neurons. Additionally, glutamatergic neurons with I_h current have been mostly found in the medial VTA, the site of recording in our studies (Hnasko et al. 2012). It is important to note that Margolis et al., (Margolis et al. 2012a) reported that GABA VTA neurons are also characterized by the presence of I_h current and are almost equal in number compared to I_h^+ DA neurons. However many other studies have considered I_h to be a reliable marker for putative DA neurons (Binder et al. 2001a;Kempadoo et al. 2013a;Mao et al. 2011;Margolis et al. 2006c;Ungless and Grace 2012;Zweifel et al. 2008). The distribution of GABA I_h^+ neurons have been reported to be rather uniform throughout the VTA according to the study by Margolis and are reconciled to as being I_h^+ TH^- neurons. However, in a previous study by the same group (Margolis et al. 2006c) these neurons were noticeably less found in the medial VTA. Considering the reported heterogeneity of I_h^+ neurons, a more heterogeneous pattern of response to an NT agonist is logically expected. However, we observe a uniform response in all I_h^+ neurons tested with NT agonists. Moreover, categorizing VTA neurons as I_h^+ or I_h^- allowed us to demonstrate profound physiological and pharmacological differences in synaptic properties that are modulated by D-Tyr[11]NT.

Previous studies have reported a reduction in glutamatergic EPSCs recorded at -70mV in DA cells of the VTA through endocannabinoid release. Additionally this depression also involved NTS1 (Kortleven et al. 2012a). However, Kempadoo et al., reports a similar decrease in pharmacologically isolated AMPA EPSCs that was not mediated by NTS1 (Kempadoo et al. 2013a). Our results are congruent with this latter study as we observe a reduction in isolated AMPA EPSC amplitude in I_h^+ neurons in the presence of D-Tyr[11]NT and this decrease was not mediated by NTS1 as SR49692 failed to block the depression. Additionally, the broad spectrum NT antagonist SR142948A significantly blocked the depression of the EPSCs thus suggesting a role for NTS2. Since D-Tyr [11] NT has a higher affinity for NTS2, it is possible that this NT agonist preferentially binds to NTS2 and causes a reduction in AMPA receptors mediated glutamatergic neurotransmission. An NTS1 mediated glutamate induced excitotoxic effect is reported in the mPFC which lacks NTS2 (Antonelli et al. 2008). This suggests that NTS2 receptor activation likely produce an inhibitory effect. Indeed, when expressed in CHO cell lines, NTS2 receptor activation was not associated with excitatory effects (Sarret et al. 2002). Given the fact that D-Tyr[11]NT has a higher binding affinity at NTS2 than NTS1 and the metabolically stable profile of D-Tyr[11]NT, we suggest that NTS2 are activated on putative DA cells in the presence of relatively higher concentrations of NT. However, whether the site of action is presynaptic or postsynaptic in I_h^+ neurons is still unclear as we obtained non-significant results with paired pulse experiments. It should be noted that the inclusion of QX314 in the recording pipette could have blocked the post synaptic effects, and in such a situation we should have observed a presynaptic effect if there was one.

Additionally Kempadoo et al., (Kempadoo et al. 2013a) also failed to observe a significant effect in paired pulse experiments with NT8-13.

Effect of D-Tyr [11] NT on I_h^- neurons

The possible role of VTA non-DA neurons in reward and sensitization mechanisms have received less attention compared to VTA DA neurons. In a study by Luo et al., (Luo et al. 2010a)selective ablation of NMDA receptors on VTA DA neurons did not disrupt the development of sensitization to cocaine. The sensitization was only blocked upon administration of an NMDA antagonist, suggesting that NMDA receptors on non-DA neurons might play a role in sensitization mechanisms. However, NTergic modulation of glutamatergic neurotransmission on VTA non-DA neurons has not received much attention compared to its DA counterpart. In other brain areas for instance the cortex, activation of NTS1 are reported to enhance NMDA receptor functions thereby augmenting the glutamatergic neurotransmission, an effect that is blocked by SR48692 (Ferraro et al. 2011). This suggests an NT induced excitatory effect. We observe a similar augmentation in the NMDA EPSC in I_h^- neurons, by D-Tyr [11] NT at equimolar concentrations at which it reduces NMDA glutamatergic transmission in I_h^+ neurons. This augmentation in I_h^- is NTS1 dependent and reflective of an NTS1 mediated excitatory effect of neurotensin at lower concentrations. Both SR142948A and SR48692 blocked the excitatory effect of D-Tyr [11] NT in I_h^- neurons thereby asserting the role for NTS1. Although D-Tyr [11] NT has a greater affinity for NTS2 than NTS1 the difference in the binding affinities do not vary greatly, suggesting the possibility of NTS1 binding (Kitabgi et al. 1980a;Labbe-Jullie

et al. 1994). An alternative explanation for this observation can be that NTS1 on I_h^- neurons are differently sensitive than in I_h^+ neurons and are activated at lower concentrations. The possibility of existence of a single NT receptor in multiple active states in which each agonist is able to select its own receptor state has been suggested. These receptor states can be coupled to different G proteins and transduce varied effects (Sotty et al. 2000a).

Similar to the augmentation of NMDA receptor mediated EPSC amplitudes; we found an augmentation of AMPA mediated EPSCs in I_h^- neurons in our studies. Albeit, the AMPA mediated enhancement effect is lower compared to the NMDA mediated enhancement given that glutamate has a greater binding affinity for NMDA receptors than AMPA receptors (Isaacson 1999;Kullmann and Asztely 1998). When tested with a paired pulse protocol, the NTS1 mediated enhancement of the AMPA component was found to be presynaptic. We examined the changes in paired pulse ratio (PPR) produced during D-Tyr [11] NT application as pre- and or post synaptic mechanisms. We found that bath application of D-Tyr [11] NT enhanced the EPCSs but the enhancement resulted in an increase of PPR, suggesting that NT acted presynaptically to increase the probability of glutamate release from presynaptic terminals. A similar presynaptic mode of glutamate release is suggested in a study that characterized the effect of NT8-13 on glutamatergic EPSCs in medium spiny neurons of the nucleus accumbens (Yin et al. 2008). As we routinely added QX314 to the recording pipette solution to block action potential generation, we found that by omitting QX314 from the recording pipette solution, D-Tyr[11]NT modulated the membrane and firing properties of non-DA neurons. In addition to blocking voltage gated Na^+ channels, QX314 is also known to block G-protein gated K^+

conductances (Gabel and Nisenbaum 1999;Galarraga et al. 1999). The reduction of such conductance produces membrane depolarisation, an effect that is observed on postsynaptic NT receptor activation (Binder et al. 2001a;Farkas et al. 1996). These observations further strengthens the notion that activation of NTS1 on terminals close to non-DA cells, leads to increased glutamate release which then activates non-DA cells.

Conclusions

Glutamatergic neurotransmission in the VTA has been ascribed to be crucial for the development of sensitization and reward mechanisms (Britt and Bonci 2013;Nestler 2013). However blockade of AMPA receptors induced a time dependent increase in reward threshold whereas injection of NMDA antagonists in the same VTA sites resulted in a time dependent decrease in reward threshold. This suggests opposite modulatory roles for NMDA and AMPA receptors in brain stimulation reward mechanisms involving the VTA(Ducrot et al. 2013). Therefore it is tempting to hypothesize that these opposite modulation of glutamate receptors can be ascribed to two different populations of cells in the VTA –the putatively dopaminergic (I_h^+) and non-dopaminergic (I_h^-). The rationale for such a hypothesis lies in the observation that while in putative dopaminergic neurons D-Tyr [11] NT depress both NMDA and AMPA components of glutamatergic EPSCs, it produced an enhancement in putative non-dopaminergic neurons(Khalil Rouibi K. 2015) (In press).

D-Tyr[11] NT possess differential binding affinities for NTS1 and 2 receptors(Sotty et al. 2000a) and that activation of either receptor subtype respectively generates a depression or augmentation in I_h^+ or I_h^- neurons. This suggests that the observed effects might be

concentration dependent. The likelihood of the idea, that NT activates two separate populations of cells at high and low concentrations, is supported by the already reported concentration dependent actions of NT on NMDA and AMPA mediated neurotransmission in VTA DA neurons (Kempadoo et al. 2013a). This leads us to propose that when NT concentrations are high, NT activates NTS2 on glutamatergic terminals or putative DA neurons and decrease glutamatergic neurotransmission. On the contrary when endogenous NT concentrations are low, NT activates presynaptic NTS1 that increase glutamatergic neurotransmission mediated by AMPA receptors. At these concentrations, NT can also bind to pre or post synaptic NTS1 that increase glutamatergic neurotransmission through NMDA receptors. Depending on the neurotransmitter content of the non-dopaminergic neuron, two possibilities arise. If the non-dopaminergic neuron is GABAergic, then enhanced excitatory input to GABA neurons, may increase the brake like effect that GABA neurons are known to exert on DA neurons and prevent them from firing (Clark et al. 1992). Recent studies that selectively activated GABA neurons using optogenetic techniques suggest that activation of GABA neurons inhibited the spontaneous firing rate of DA neurons (Tan et al. 2012). In contrast, inhibiting GABA neurons resulted in disinhibition of DA neurons (Bocklisch et al. 2013). Activity of GABA neurons is high in the presence of an aversive stimulus and is relevant in reward prediction error and reward consumption (Ungless 2004;van et al. 2012). However, if the non-DA neuron is glutamatergic, then enhanced glutamatergic input to DA neurons may promote enhanced firing and DA release in terminal fields of projection leading to dopamine dependent behaviours similar to those that are observed upon administration of psychostimulants (Sotty et al. 2000a). Although we can only speculate on the outcomes of increased

glutamatergic neurotransmission by NT on non-DA neurons at this point, further behavioural insights are required to characterise the implications of enhanced glutamate signalling in eliciting neuroadaptive changes associated with sensitization and reward mechanisms involving VTA non-DA neurons.

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Figure 1

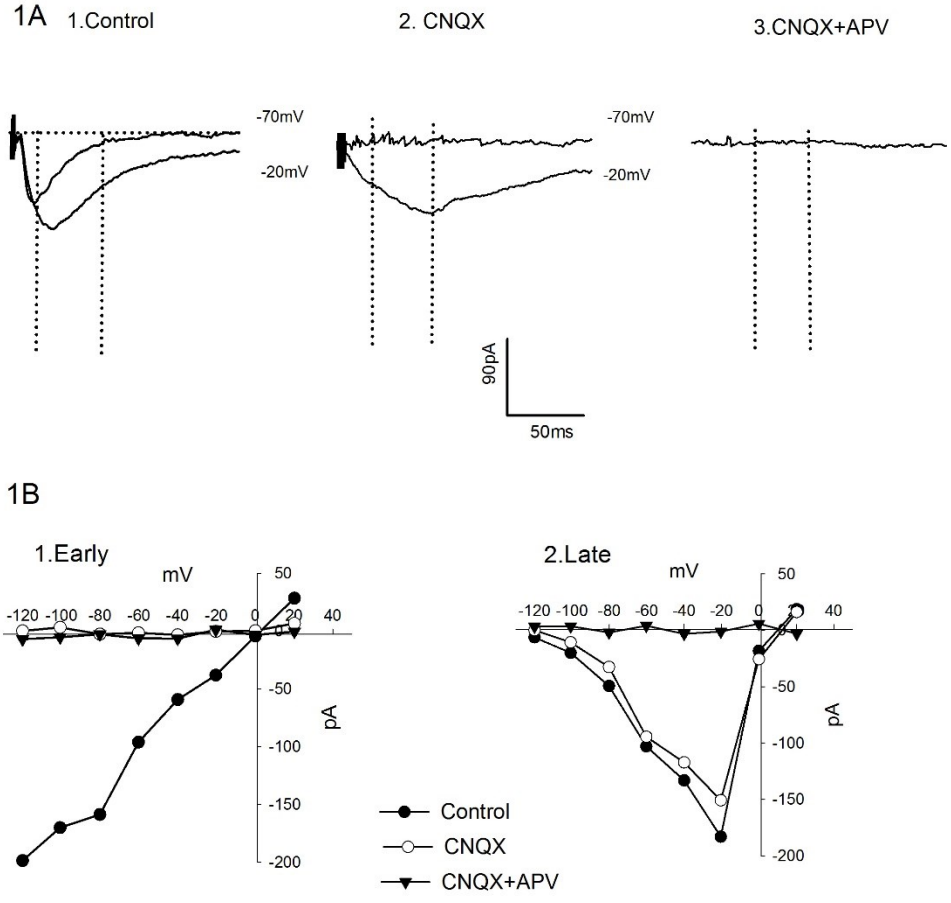
1A
1.Control
2. CNQX
3.CNQX+APV
-70mV
-20mV
-70mV
-20mV
90pA
50ms
1B
1.Early
mV
50
-120 -100 -80 -60 -40 -20 0 20 40
-50
-100
-150
-200
pA
2.Late
mV
50
-120 -100 -80 -60 -40 -20 0 20 40
-50
-100
-150
-200
pA
Control
CNQX
CNQX+APV

Figure 1.Characteristics of the glutamatergic EPSCs.

Nature of the EPSC evoked by local electrical stimulus in the presence of BMI (10μM). **A**:Current traces of the response evoked by single electrical stimulus and recorded at holding membrane potentials of –20 and –70 mV before glutamatergic antagonists application (1.Control) and during superfusion with CNQX (20μM; 2.CNQX) and CNQX and APV (50μM; 3.CNQX+APV). Recordings were obtained in an I_h^+ neuron from a P20 animal. Current traces represent the average of 6 sweeps. **B**: Current-voltage relationship of the response (I_R-V_m) between –120 and 20 mV. The early component was measured 9 ms, after the stimulus as indicated by the left vertical dotted line in A. The late component was measured 43 msec. after the stimulus as indicated by the right vertical dotted line in A.

Figure2

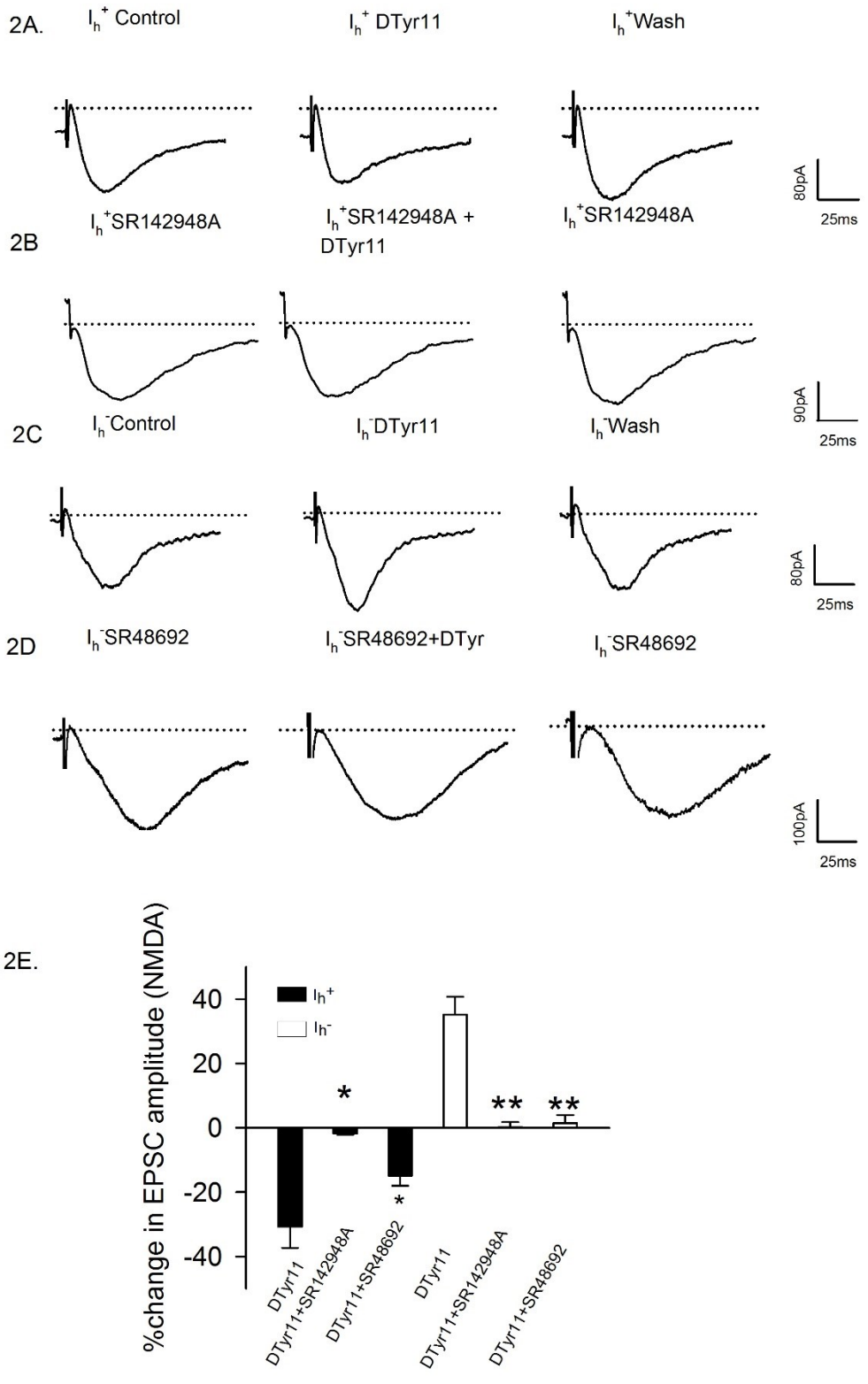
2A.
I_h^+ Control
I_h^+ DTyr11
I_h^+Wash
I_h^+SR142948A
I_h^+SR142948A + DTyr11
I_h^+SR142948A
80pA
25ms
2B
2C
I_h^-Control
I_h^-DTyr11
I_h^-Wash
90pA
25ms
2D
I_h^-SR48692
I_h^-SR48692+DTyr
I_h^-SR48692
80pA
25ms
100pA
25ms
2E.
%change in EPSC amplitude (NMDA)
I_h^+
I_h^-
40
20
0
-20
-40
*
*
**
**
DTyr11
DTyr11+SR142948A
DTyr11+SR48692
DTyr11
DTyr11+SR142948A
DTyr11+SR48692

Figure 2: Effects of D-Tyr [11] NT on NMDA EPSCs. A: Current traces of NMDA EPSC recorded before (1), during (2) and after (3) superfusion with D-Tyr [11] NT (0.01µM) at a holding membrane potential of -20mV in I_h^+ neurons (n=6). **B:** Current traces of NMDA EPSC recorded during superfusion with SR142948A (1), with SR142948A and D-Tyr [11] NT (0.01µM) (2) and with SR142948A following the washout of D-Tyr [11] NT (3) at a holding membrane potential of -20mV in I_h^+ neurons (n=7). **C:** Current traces of NMDA EPSC recorded before (1), during (2) and after (3) superfusion with D-Tyr [11] NT (0.01µM) at a holding membrane potential of -20mV in I_h^- neurons (n=5) **D:** Current traces of NMDA EPSC recorded during superfusion with SR48692 (1), with SR48692and D-Tyr [11] NT (2) and with SR48692 following the washout of D-Tyr [11] NT (3) at a holding membrane potential of -20mVin I_h^- neurons (n=6). **E:** Summary of the effect of D-Tyr [11] NT (0.01µM), SR142948A (0.1µM) and SR48692 (0.1µM) on I_h^+ and I_h^- neurons at a holding membrane potential of -20mV. One way ANOVA in I_h^+ neurons; $p<0.001$. * indicates statistical significance ($p<0.05$). ** indicates statistical significance ($p<0.001$).

Figure3

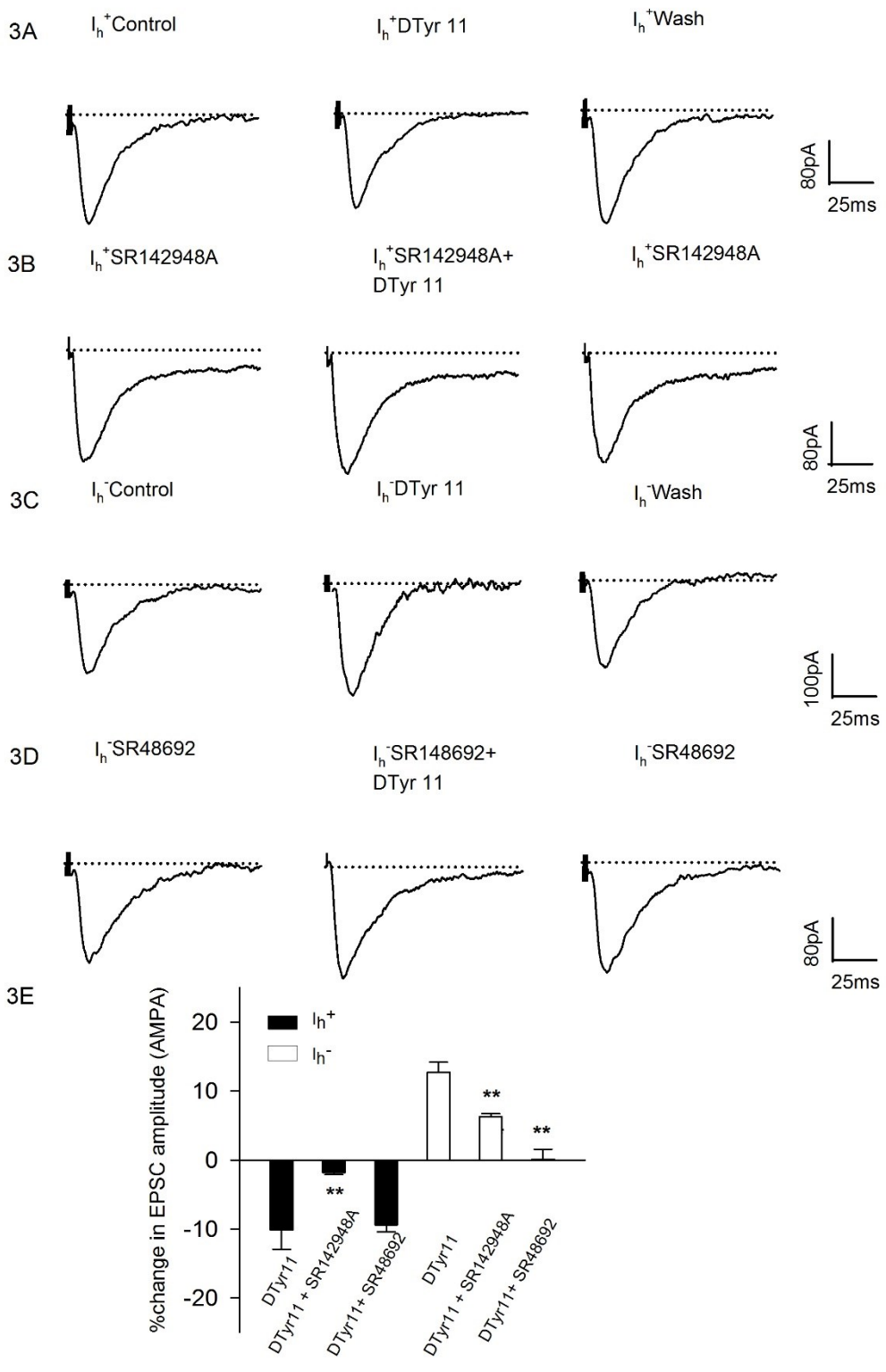
3A
Ih+Control
Ih+DTyr 11
Ih+Wash
80pA
25ms
3B
Ih+SR142948A
Ih+SR142948A+
DTyr 11
Ih+SR142948A
80pA
25ms
3C
Ih-Control
Ih-DTyr 11
Ih-Wash
100pA
25ms
3D
Ih-SR48692
Ih-SR148692+
DTyr 11
Ih-SR48692
80pA
25ms
3E
%change in EPSC amplitude (AMPA)
20
10
0
-10
-20
Ih+
Ih-
**
**
**
DTyr11
DTyr11 + SR142948A
DTyr11+ SR48692
DTyr11
DTyr11 + SR142948A
DTyr11+ SR48692

Figure 3: Effects of D-Tyr [11] NT on non-NMDA EPSCs. A: Current traces of AMPA EPSC recorded before (1), during (2) and after (3) superfusion with D-Tyr [11] NT (0.01µM) at a holding membrane potential of -70mV in I_h^+ neurons (n=7).**B:** Current traces of AMPA EPSC recorded during superfusion with SR142948A (1), with SR142948A and D-Tyr [11] NT (0.01µM) (2) and with SR142948A following the washout of D-Tyr [11] NT (3) at a holding membrane potential of -70mV in I_h^+ neurons (n=7). **C:** Current traces of AMPA EPSC recorded before (1), during (2) and after (3) superfusion with D-Tyr [11] NT (0.01µM) at a holding membrane potential of -70mV in I_h^- neurons (n=6) **D:** Current traces of AMPA EPSC recorded during superfusion with SR48692 (1), with SR48692and D-Tyr [11] NT (2) and with SR48692 following the washout of D-Tyr [11] NT (3) at a holding membrane potential of -70mVin I_h^- neurons (n=6). **E:** Summary of the effect of D-Tyr [11] NT (0.01µM), SR142948A (0.1µM) and SR48692 (0.1µM) on I_h^+ and I_h^- neurons at a holding membrane potential of -70mV.One way ANOVA in I_h^+ neurons; $p<0.001$.* indicates statistical significance ($p<0.05$). ** indicates statistical significance ($p<0.001$).

Figure 4

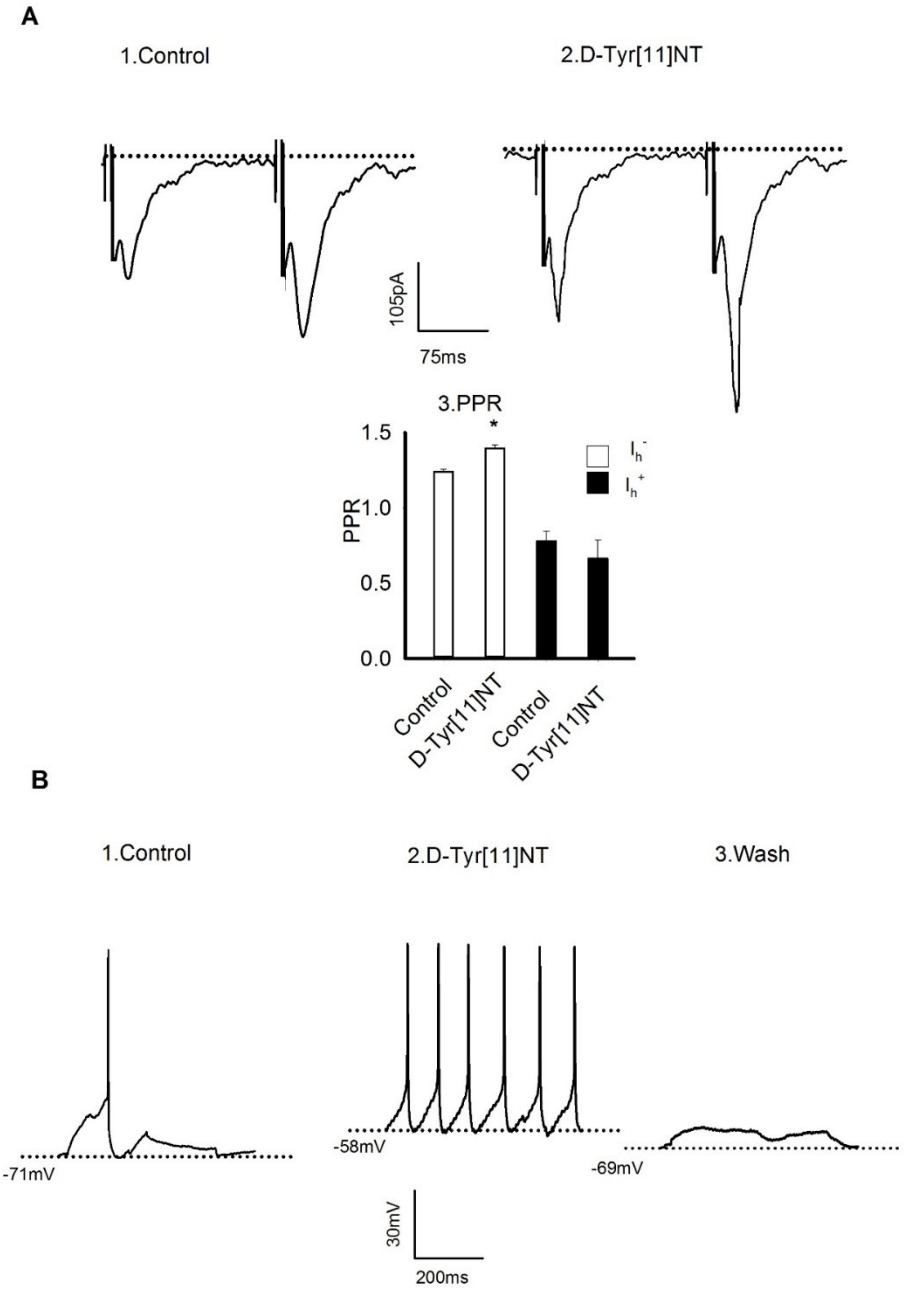
A
1.Control
2.D-Tyr[11]NT
105pA
75ms
3.PPR
PPR
1.5
1.0
0.5
0.0
*
I_h^-
I_h^+
Control
D-Tyr[11]NT
Control
D-Tyr[11]NT
B
1.Control
2.D-Tyr[11]NT
3.Wash
-71mV
-58mV
-69mV
30mV
200ms

Figure 4: Locus of neurotensinergic modulation of evoked AMPA EPSCs in I_h^-neurons.

A: Current traces of the responses evoked by a pair of single local electrical stimuli 50 ms apart at a holding membrane potential of -100 mV before (1) and during (2) superfusion with D-Tyr [11] NT1-13 (0.01μM).3 : the average amplitude of PPR from 6 neurons before and during superfusion with D-Tyr [11] NT 1-13. PPR was statistically larger in the presence of D-Tyr [11] NT 1-13 than during control conditions (student's t test; $p<0.001$) in I_h^- neurons (n=6). No significant effect was found in I_h^+ neurons (student's t test; $p> 0.05$; n=7). **B:** Effect of D-Tyr [11] NT 1-13 on the membrane and firing properties of I_h^- neurons. Voltage responses evoked with intracellular current pulses of 10pA from resting membrane potential before (1) , during (2) and after (3) addition of D-Tyr [11] NT 1-13(0.01μM) to the superfusing medium. In this case the addition of the neurotensin agonist produced a depolarisation of 13mV of the membrane potential. Recordings were obtained under the same experimental conditions with the exception that QX314 was omitted from the internal recording solution in the pipette

4. Discussion

This section of the thesis aims to provide a short summary of the results obtained in the studies followed by a global discussion and interpretation of the results obtained in the three articles. Lastly I would like to draw the attention of the readers to some technical considerations encountered while performing the experiments and conclude my thesis with the implications of the results in understanding behavioural disorders like addiction.

4.1 Summary of the results

In the work undertaken in this thesis, we sought to characterise the neurotensinergic modulation of glutamatergic neurotransmission in VTA neurons. To this end we first tested the effects of NT1-13 and its analogs on glutamatergic EPSCs in VTA DA and non-DA neurons and identify the receptors involved in mediating the effects. The first study is aimed at comparing the effects of NT-(1-13), to that of the active C terminal fragment NT-(8-13) and test the ability of the NT antagonists at blocking the effects of these NT peptides. The results of the experiments conducted to characterize these effects are presented in the first article. We found that both NT-(1-13) and NT-(8-13) dose dependently excite both I_h^+ (putative DA neurons) and I_h^- neurons (non-DA neurons) in the VTA. While the enhancement of glutamatergic EPSCs in putative DA neurons involves activation of NTS1

receptors, the enhancement of glutamatergic EPSCs in non-DA neurons is mediated by NTS2 receptors.

Since NT8-13 activated two different NT receptor subtypes in different population of VTA neurons despite having a higher binding affinity for NTS1 than NTS2 receptors, we sought to test the effects of another NT analog, D-Tyr[11]NT which has a higher binding affinity at NTS2 receptors than NTS1 on glutamatergic EPSCs of VTA neurons. In the second article, we describe the work undertaken to characterise the effects of D-Tyr[11]NT on glutamatergic neurotransmission in VTA neurons. D-Tyr[11]NT was found to decrease glutamatergic neurotransmission in putative DA neurons in a dose dependent manner, whereas in non-DA neurons it was found to enhance glutamatergic neurotransmission dose dependently. The reduction in glutamatergic EPSCs in putative DA neurons was mediated by the activation of NTS2 receptors whereas in non-DA neurons, the enhancement involved activation of NTS1 receptors. CPP was found to be dose-dependently enhanced by D-Tyr [11] NT and at the highest concentration, the effect was blocked by SR48692 suggesting the involvement of NTS1. Since both behavioural and electrophysiological paradigms suggested the involvement of NTS1, it is likely that the development of sensitization induced by D-Tyr [11] NT involves the activation of NTS1 on non-DA neurons.

The existence of NT receptors on both cell bodies and axon terminals (Rostene et al., 1992), suggests that in addition to exerting its effect on VTA neurons, NT could also modulate glutamate release from terminals. Bath application of NT may directly modulate glutamate release through the activation of NT receptors on terminals or indirectly activate DA neurons, stimulate DA release that acts on D1/ D2 receptors on terminals to modulate glutamatergic neurotransmission. Since D-Tyr[11]NT is known to act on different receptors

and we found bidirectional effects on putative DA and non-DA neurons, we sought to characterize the effects of D-Tyr[11]NT on isolated NMDA and AMPA components of the glutamatergic EPSCs and identify the locus of the receptors involved in mediating these effects. In the third article, we describe the work undertaken to characterize the effects of D-Tyr[11]NT on isolated NMDA and AMPA EPSCs and the locus of receptors involved in mediating these effects. D-Tyr[11]NT reduced the amplitude of both NMDA and AMPA EPSCs dose dependently in I_h^+ neurons by an NTS2 dependent mechanism. In I_h^- neurons, D-Tyr[11]NT produced an NTS1 dependent enhancement of glutamatergic EPSCs. Paired pulse experiments on I_h^- neurons suggest that the enhancement effect involves activation of presynaptic NTS1 receptors. A summary of all the results obtained (categorized by type of neuronal population has been presented in Table 2.

Table 2

I_h^+ neurons	I_h^- neurons
NT1-13 and NT8-13 increase glutamatergic neurotransmission. Receptor involved : NTS1	NT1-13 and NT8-13 increase glutamatergic neurotransmission. Receptor involved : NTS2
D-Tyr [11] NT1-13 decreases glutamatergic neurotransmission. Receptor involved : NTS2	D-Tyr [11] NT increases glutamatergic neurotransmission. Receptor involved: NTS1.
	D-Tyr [11] NT increases CPP. Receptor involved : NTS1
D-Tyr [11] NT1-13 decreases both AMPA and NMDA EPSC components. Receptor involved: NTS2. Locus of receptor modulation: post synaptic.	D-Tyr [11] NT increases both AMPA and NMDA EPSC components. Receptor involved: NTS1. Locus of receptor modulation: presynaptic.

4.2 NT exerts a complex effect on glutamatergic neurotransmission in VTA neurons

4.2.1 Bidirectional effects of NT analogs on glutamatergic EPSCs in I_h^+ neurons

The present study entails the use of three NT analogs: NT1-13, NT 8-13 and D-Tyr[11]NT. These analogs have been previously shown to elicit different cellular and behavioural responses in the same anatomical substrate. NT1-13 is the 13 amino acid, native neurotensin peptide which is endogenous in the brain, mostly found in the dopamine rich regions (for example the VTA) and most effects mediated by this peptide are through its C terminal region fragment, NT8-13. At the rat NTS1 receptor, NT1-13 has a K_d of 1.97nM while NT8-13 has a K_d of 1.607nM. NT 1-13 has a lower binding affinity than NT8-13 at NTS1 receptors whereas at the NTS2 receptors, NT 1-13 has a similar binding affinity as that of NT 8-13(Kitabgi et al. 1980a;Labbe-Jullie et al. 1994). Local application of NT1-13 increased dopamine cell firing in the VTA (Seutin et al. 1989;Shi and Bunney 1991b), and consequentially resulted in dopamine release in the terminal fields of DAergic projections (Cador et al. 1995;Kalivas and Taylor 1985a) and that these effects were similarly mimicked by NT 8-13. NT8-13 produces most of the known effects of NT and therefore most peptide agonists for NT receptors are analogs of this hexapeptide. For example, a study by Rompre et al., (Rompre and Boye 1993) suggests, both NT1-13 and NT8-13 are equally effective in operant responding for brain stimulation reward paradigms. The similarity of both these peptides in terms of binding affinities and receptor activation explains the mimicking of the effects (Kitabgi et al. 1980a).

In our studies, we find an enhancement in glutamatergic neurotransmission in both I_h^+ and I_h^- neurons that is induces by either NT1-13 or NT8-13. Several previous studies have shown a similar NT induced increase in glutamate release in the striatum (Ferraro et al. 1995;Ferraro et al. 1998), the SN (Ferraro et al. 2001) and the cerebral cortex (Ferraro et al. 2000). This increase in glutamatergic release is suggested to involve the interference of NT in inhibitory D2 autoreceptor signalling by formation of an NTS1-D2autoreceptor complex (Antonelli et al. 2007). Such complexes might exist on the terminals and cell bodies/dendrites of VTA DA neurons. Activation of such complexes on DA cell bodies by NT could antagonize the inhibitory D2 receptor mediated signalling, lead to an increased firing of DA cells and consequentially release dopamine. The released dopamine could then activate post junctional D2 receptors on glutamate terminals and increase glutamate release which activate glutamatergic receptors on DA neurons and increase glutamatergic neurotransmission. Alternatively, NT could directly increase glutamate release from the terminals by the antagonizing effect of NT on D2 receptors located on glutamatergic terminals. The NTS1-D2 interaction in DA neurons also involves calcium dependent and protein kinase C mechanisms (Antonelli et al. 2004).

Prior activation of D2 receptors inhibit NTS1 signalling indicating the possibility of the NTS1-D2 interactions being oppositely regulated (Jomphe et al. 2006). Nevertheless, evidences of facilitatory NTS1-NMDA receptor interactions at cortico-striatal glutamate terminals strengthen the role of NT in inducing glutamate release (Antonelli et al. 2004). Since our population of I_h^+ neurons could also include a subset of glutamatergic neurons (about 10 %) (Yamaguchi et al. 2007;Yamaguchi et al. 2013) , we think that the observed increase in glutamatergic neurotransmission is mediated not only by mechanisms involving

reduced D2 receptor autoinhibition through putative DA neurons but also direct activation of glutamate receptors on glutamatergic neurons.

Interestingly, there are studies that report an NT induced reduction in glutamatergic neurotransmission in VTA DA neurons (Kempadoo et al. 2013a;Kortleven et al. 2012a). However, while Kortleven et al., reported an attenuation of glutamatergic neurotransmission involving NTS1 receptors and endocannabinoid mediated long term depression (LTD), Kempadoo et al, suggested an NTS1 independent form of reduction. Although a small biphasic concentration dependent effect on NMDA EPSCs has been suggested by Kempadoo et al., we did not observe an inhibition of the glutamatergic EPSCs with NT1-13 or NT8-13 ay any of the concentrations used (0.01 μM, 0.1μM or 1μM). This is possibly due to the technique used in recording from the cells or other factors for example interspecies difference, age of the animals or the slicing procedure itself might be a source of the difference (Ungless and Grace 2012). Since we used the blind patch technique to record from VTA neurons, it is possible that the effective concentration of NT8-13 reaching the receptors on the cell bodies and the terminals in the deeper layers of the slice are less than what they receive on the surface of the slice (as in the other two studies cited). Therefore, we think that had we used an even higher concentration of the NT peptides, we would have observed a decrease in the glutamatergic neurotransmission. This hypothesis is supported by the decrease in glutamatergic EPSC amplitudes in I_h^+ neurons by D-Tyr[11]NT. We have found a dose dependent decrease in glutamatergic EPSCs by this NT analog in I_h^+ neurons and this reduction is mediated by the activation of NTS2 receptors as SR142948A blocks the inhibition which SR48692 fails to. D-Tyr[11]NT is reported to have a half -life 1.5 times greater than that of NT8-13

(Checler et al. 1983a). Therefore we suspect that NT8-13 at the highest concentrations used in our studies resemble the effects of D-Tyr[11]NT at lower concentrations. Since NT8-13 has a greater affinity for NTS1 than NTS2 receptors and NTS1 receptors have a lower binding affinity than NTS2, we propose that at the concentrations used NTS1 receptors are activated. NTS1 receptors have been associated with inducing excitatory effects as it is coupled to an increase in IP3 , PLC and elevation in intracellular levels of Ca^{2+} (Binder et al. 2001a;St-Gelais et al. 2004). On the contrary, D-Tyr[11]NT has a higher affinity for NTS2 receptors than NTS1 and activation of these receptors do not induce excitatory effects. For example, activation of human NTS2 receptors expressed on CHO cell lines lacked the potential to elevate intracellular Ca^{2+} levels by mobilizing internal calcium reserves or accumulate IP3 but were associated with MAPKs to induce inhibitory effects (Sarret et al. 2002). This suggests, that when NT is present in higher concentrations, NTS2 receptors may be preferentially activated thereby inducing inhibitory effects.

On evaluating the effects of D-Tyr[11]NT on isolated NMDA and AMPA components, we observed reduction in both components mediated by activation of NTS2 receptors; albeit the reduction being larger in NMDA components than AMPA owing to the higher affinity of glutamate for NMDA than AMPA receptors. A similar NTS1 independent reduction in both AMPA and NMDA components of glutamatergic EPSCs by higher concentrations of NT8-13 (0.1μM, 0.3μM and 0.5μM) further supports our hypothesis. Surprisingly Kortleven et al., reports a decrease in glutamatergic EPSCs in VTA DA neurons by NT8-13 (0.05μM) that is mediated by NTS1 receptors (Kortleven et al. 2012a).

This leads us to suggest that NT modulates glutamatergic neurotransmission is concentration specific and that higher concentrations of this peptide, by activation of NTS2

receptors decrease glutamatergic neurotransmission in I_h^+ neurons. Our studies highlight a novel mechanism for synaptic glutamatergic neurotransmission modulation in DA neurons of the VTA in response to a neuropeptide, which is capable of modulating DA neurons and subsequently DA release.

4.2.2 NT analogs increase glutamatergic neurotransmission in I_h^- neurons

Most studies till now have focussed on the modulation of glutamatergic neurotransmission on DA neurons of the VTA and characterize plasticity mechanisms that are relevant to sensitization and addiction (Britt and Bonci 2013;Kauer and Malenka 2007;Nestler 2013). However, the VTA is home to non-DA neurons which may be glutamatergic or GABAergic and these establish local synapses on DA cells (Dobi et al. 2010). The inhibitory control exerted by GABA neurons on the VTA shape the DA neuron activity (Britt and Bonci 2013;Nestler 2013).

Although, the perisomatic GABAergic innervation to DA and non-DA cells are not significantly different, there exists a higher degree of dendritic innervation on GABA neurons than DA neurons. This is indicative of a possible indirect modulatory effect that involves disinhibition of the GABAergic inputs to GABA neurons, thereby releasing the DA neurons from the tonic inhibition (Ciccarelli et al. 2012). Alternatively, enhanced glutamatergic neurotransmission at these GABA neurons could also result in the same effect. Since NT terminals densely innervate the VTA, it is possible that NTergic modulation could enhance or attenuate glutamatergic neurotransmission on non-DA cells.

In the present studies, we found that NT1-13 and NT8-13 dose dependently enhance glutamatergic neurotransmission in I_h^- cells. The similarity in the effects of both the peptides suggests that these peptides share similarities in their binding affinities for NTS receptors. Surprisingly, we observe that the increase in glutamatergic neurotransmission in I_h^- neurons is mediated by NTS2 receptors as SR142948A blocks the increase in the EPSC amplitudes produced by NT1-13or NT8-13 whereas SR48692 did not block it. Given the fact that NTS2 receptors are activated in the presence of high concentrations of NT, it would be logical to suggest that increased NT levels activate NTS2 receptors to mediate the increase in glutamatergic neurotransmission. However, NTS2 receptors are associated with inhibitory actions. This leads to two hypotheses. One, the formation of NTS1-NTS2 heterodimers that leave the NT induced internalization of NTS1 receptors intact but modulates the trafficking of these receptors by making it more similar to NTS2 receptors and decrease the NTS1 receptor density on the surface (Perron et al. 2007). Two, NTS2 receptors on non-DA neurons are differently sensitive to NT agonists and may be activated at a lower concentration of the agonist than that required to activate them on DA neurons. The possibility of the existence of a single receptor in multiple activation states has been suggested before (Sotty et al. 2000a). Moreover, there is evidence that NT may act as a partial or weak agonist or a neutral antagonist at NTS2 receptors (Richard et al. 2001). It is possible that at the concentration of NT used in the study cited above, NT acts as a neutral antagonist and blocks NTS2 receptors and activate NTS1 receptors which mediate the increase in glutamatergic neurotransmission. In fact, we observe an NTS1 mediated dose dependent increase in the amplitude of glutamatergic EPSCs in non-DA neurons by D-Tyr[11]NT. Additionally, both the isolated AMPA and NMDA components of the

glutamatergic EPSCs were also enhanced by D-Tyr[11]NT and that NTS1 receptors mediated this enhancement. The increase in AMPA EPSCs is mediated by presynaptic NTS1 receptors suggesting that D-Tyr[11]NT acts on NTS1 receptors on glutamatergic terminals and triggers intracellular signalling cascades that result in enhanced glutamate release. A similar mode of presynaptic glutamate release at the synapse between the perforant path and the granule cells of the hippocampus has been reported (Zhang et al. 2015). The authors suggest that NT induced an increase in the release probability of readily releasable vesicles at the presynaptic terminals that required influx of Ca^{2+} ions through L –type calcium channels and functional calmodulin and myosin light chain kinase. A previous study in cortical neurons has also implicated a Ca^{2+} dependent mechanism that is associated with the functional coupling of NTS1 receptors with the IP3 signalling cascade to mediate glutamate release (Pigozzi et al., 2004; Antonelli et al., 2004). Although we have not been able to identify the precise intracellular effector molecules involved in this effect, we suggest a similar mechanism of NT induced glutamate release.

There are evidences for NTS1-NMDA interactions that enhance glutamate release and often lead to excitotoxic glutamate effects (Ferraro et al., 2007). In our studies we found an enhancement of NMDA EPSCs in non-DA neurons mediated by NTS1 receptors. It is thus possible that presynaptic activation of NTS1 receptors that lead to enhanced glutamate release acts on postsynaptic NMDA receptors on non-DA cells and increase glutamatergic neurotransmission. Additionally, NTS1 receptor induced PKC activation is known to cause a sixfold increase in NMDA receptor surface expression involving phosphorylation of the NMDA receptor subunit itself or a protein associated with the receptor (Lan et al. 2001). Alternatively, direct facilitatory NTS1-NMDA interactions have been reported in rat

cortical cells as application of NMDA resulted in increase of endogenous extracellular glutamate levels that was blocked by the NTS1 antagonist SR48692 (Antonelli et al. 2004). From these evidences we hypothesize that NT acts on presynaptic and /or postsynaptic NTS1 receptors and increase glutamatergic signalling in non-DA neurons. This potentiation of glutamatergic signal in the VTA may trigger neuroadaptive changes that are pertinent in addiction mechanisms.

4.3 Conclusion

Taken altogether, within the scope of our studies, we could not identify the neurotransmitter content of the non-DA neuron and thus these population of neurons may be either glutamatergic or GABAergic. Since we found that NT increases glutamatergic neurotransmission in non-DA neurons, two possibilities arise. One, if the non-DA neuron is GABAergic, then enhanced excitatory input to these neurons might release GABA and exert a brake like effect on DA neurons and prevent them from firing. Synaptic GABA inputs to the VTA DA neurons alter reward related behaviours. Recent studies show that optogenetic activation of GABA neurons that directly synapse on DA neurons suppress the activity and excitability of neighbouring DA neurons and decrease the release of DA in the nAcb (van et al. 2012).

The second possibility is that if excitatory input to glutamatergic neurons are increased by NT, it could result in more glutamate release to DA neurons and thus increase DA cell firing and consequentially DA release and DA dependent behaviours. In parallel to the

electrophysiological studies, behavioural studies show an increased conditioned place preference in animals that receive an intra-VTA injection of D-Tyr[11]NT. This effect was blocked by the NTS1 antagonist suggesting that D-Tyr[11]NT acted on NTS1 receptors to induce this effect. Since we found a role for NTS1 receptors in increasing glutamatergic neurotransmission in non-DA neurons, we think that we think that this second possibility is more likely as increased activation of glutamatergic neurons result in a larger glutamate signal that induce neuroadaptive changes that lead to a sensitized state in the animal. In fact, selective inactivation of NMDA receptors on DA neurons leaves behavioural measures of cocaine sensitization like locomotor activity and conditioned place preference intact highlighting the role of glutamatergic neurotransmission on non-DA neurons in sensitization mechanisms (Luo et al. 2010a). The possibility of regulating non-DA neuron activity in the VTA through modification of its excitatory inputs by NT thus represents a potential area of investigation in addiction mechanisms.

4.4. Technical considerations:

4.4.1 Identification of DA neurons in the VTA

In the present studies, VTA neurons have been categorized as DA or non-DA neurons based on the presence or the absence of the hyperpolarisation activated cationic current (I_h) respectively. Around 80-90% of DA neurons of the VTA possess this characteristic current and has therefore been used routinely in many studies as the identification criteria (Bonci and Malenka 1999;Grace and Onn 1989;Johnson and North 1992a;Kempadoo et al.

2013a;Korotkova et al. 2002;Korotkova et al. 2004;Mao et al. 2011). The remaining 10-20% of DA neurons that are without I_h, have undergone considerations and reconsiderations owing to the heterogeneity of VTA DA neurons and the variety of criteria that have been used, to classify these neurons over the course of VTA DA neuron literature(Ungless and Grace 2012). For example, although Margolis et al., (Margolis et al. 2006c)reports a subset of DA neurons that are characterized as $I_h^+TH^-$, neurons that are I_h^- have been reported to be non-DAergic, suggesting that DA neurons might be I_h^+ but TH^-. The absence of I_h reliably predicts the non-DAergic entity of a VTA neuron and therefore the identification criteria used in the present studies can be considered reliable. Moreover, classifying cells with this approach enabled us to demonstrate profound physiological and pharmacological differences in synaptic properties that are modulated by NT and thus validates our approach. It is important to note that we have routinely processed the recorded slices for biocytin labelling and consistently confirmed their location in the medial VTA. This region is reported to contain some glutamatergic neurons with the I_h current (Hnasko et al. 2010;Li et al. 2013). Therefore within our population of I_h^+ neurons, it is possible that some cells are glutamatergic. The medial VTA sends projections to the mPFC and nACb (Margolis et al. 2008). Interestingly, the efferent projections from PFC that synapse on DA neurons of this area project back to the PFC and release DA when neuronal activity of these DA neurons are stimulated (Carr and Sesack 2000;Dobi et al. 2010;Ungless and Grace 2012). These two regions are known to undergo neuroadaptive changes that are crucial for sensitization mechanisms. Nevertheless, the absence of I_h identifies a non-dopaminergic cell type and we have used this criteria to define our population of non-DA neurons (Margolis et al. 2006a). However, it is important to note

that the identification criteria for classifying DA neurons have undergone controversies owing to the heterogeneity of this neuronal population.

The classical identification criteria proposed by Johnson and North(Johnson and North 1992a) using *in vitro* patch clamp recording in slices, discerned on a convention for classifying VTA neurons as principal (mostly DAergic) that stained positive for TH ,were hyperpolarised by dopamine and fired spontaneously (1-3Hz). Later, the presence of I_h (hyperpolarisation activated cationic current), dopamine transporter (DAT), D_2 autoreceptors and G protein regulated inward rectifier potassium channel subtype 2 (GIRK2) was added to the list and these have since, been considered a conventional criteria for identifying a DA neuron (Bellone and Luscher 2006;Saal et al. 2003;Wanat et al. 2008a). Nonetheless, different studies have used combinations of different parameters from the list of conventional criteria. Recent studies question these criteria and suggest that although the phenotype of the cell may be DAergic as evidenced from positive TH staining or DAT expression, it might not have I_h(Margolis et al. 2006a;Zhang et al. 2010). Additionally not all DA neurons may be sensitive to DA induced hyperpolarisation (Lammel et al. 2008) and differ from non-DA neurons in their action potential width(which in case of a putative DA neuron is broader than that of a non-DA neuron) (Ungless and Grace 2012). It was also reported that prolonged duration of intracellular recordings (more than 10 mins) can lead to TH washout leading to false negatives (Zhang et al. 2010). However, since the present studies do not entail the use of TH immunohistochemistry for identifying DA neurons, the possibility that longer recording duration might have incorporated false negatives in the scientific data can be negated.

Use of alternative techniques, such as optogenetic techniques, to selectively activate or inhibit DA neurons in TH Cre rats could be useful. Virus mediated expression of channel rhodopsin on DA neurons followed by selectively activating or inhibiting them could enable achieving precision on the identification criteria (Witten et al. 2011). Alternatively, singe cell RT-PCR of the contents of the recorded cell would enable neurotransmitter content identification of the recorded cells.

4.4.2 Recording technique:

The use of blind patch technique (Blanton et al. 1989b), although uncommon has allowed us to record from neurons that are located in the deeper layers of the slice and generate stable recordings of up to 1 hr. or more. The blind patch technique is uncommon as it does not allow the visualization of the cell being recorded, before or immediately after the recording. However, this technique is useful for obtaining stable recordings of long duration. Post hoc labelling with biocytin enabled us to visualize the cells within the deeper layers of the slice and confirm the neuron's location in the medial VTA. While this technique is excellent for recording the electrophysiological activity of the cells within the scope of our studies, post hoc immunochemical labelling results for TH to identify DA neurons was not consistent. This may be due to the inefficiency of the antibodies to permeabilize into the cells of the deeper layers of the slice and thus did not always generate double labelled cells (TH and biocytin) on the same focal plane. However, we could always visualize the biocytin filled cell as biocytin was added in the patch pipette.

4.5 Implications in behavioural disorders and future directions

A peculiar aspect of the neurotensin literature is that both NT agonists and antagonists have been showed to be effective in animal models of addiction. For example the NT agonist, NT69L is effective in blocking nicotine induced hyperactivity (Boules et al. 2013), initiation and expression of sensitization to nicotine, nicotine self-administration in animal models of addiction (Fredrickson et al. 2003). NT69L when given intraperitoneally , also blocks the acute locomotor effects of cocaine and d-amphetamine (Boules et al. 2001). This is one line of evidence suggesting the potential efficacy of NT agonists in treatment of nicotine, cocaine and amphetamine addiction. In fact, Acamprosate, which is one of the FDA approved drugs for treating alcohol use disorder acts by modulating extracellular concentrations of dopamine and glutamate in the striatum. Interestingly, NT69L also induces similar modulations in dopamine and glutamate concentrations in the striatum (Dahchour and De 2000;Prus et al. 2007). The efficacy of NT69L in attenuating alcohol preference and consumption in mice and nicotine self-administration in alcohol dependent rats, indicate that there are common substrates of cross-sensitization that are modulated by this NT agonist (Boules et al. 2011;Li et al. 2011).

In contrast, the non-specific NT antagonist, SR142948A was found to block initiation of amphetamine induced sensitization to NT (Panayi et al. 2005b). In another study, locomotor sensitization and conditioned place preference to cocaine was attenuated by NTS1 antagonist. SR48692 (Felszeghy et al. 2007).

The intriguing question that surfaces is, how then can NT agonists and antagonists induce similar effects in animal models of addiction? Or, do NT agonists have abuse potential?

It should be noted that in the studies cited above, NT69L was shown to be effective in nicotine and d-amphetamine sensitization mechanisms whereas SR48692 was shown to be effective in cocaine and d-amphetamine induced sensitization. This would predict that mice null for NTS1 receptors would not sensitize to psychostimulants. Strikingly, an acute injection of d-amphetamine had an enhanced response to the sensitizing effects in null mice than wild type mice (Boules et al. 2006;Boules et al. 2007). This observation argues in favour of using NT agonists for treating psychostimulant induced drug abuse.

Although, intra-VTA NT injections in animals, elicit effects that mimic those induced by psychostimulants, NT injections in the nAcb block psychostimulant induced effects (Caceda et al. 2006). Additionally, extracranial administration of NT analogs attenuate nicotine self-administration in rats (Boules et al., 2006). Another evidence that argues against an abuse potential of NT agonists comes from a study in rhesus monkeys, in which animals failed to self-administer NT69L (Fantegrossi et al. 2005). Therefore the prevalence of evidences argues against NT agonists possessing an abuse potential.

Nonetheless, the development of highly selective NT antagonists and agonists are required to ameliorate further investigations involving the deciphering of common substrates of addiction that NT exerts an effect upon. Although no NT antagonists or agonists have reached the stage of clinical trials, the potential of NT in modulating glutamatergic processes that are crucial for addiction mechanisms hold a promise for development of future therapeutics.

In part the glutamate homeostasis in nAcb and VTA is maintained by proteins localized to astrocytes such as GLT1 and xCT (Hazell et al. 2001;Kalivas et al. 2009). Psychostimulant drug exposure lead to downregulation of these proteins that result in enhanced glutamate signalling and spillover. Such glutamatergic events have been implicated in cue-induced drug seeking and relapse to drug seeking behaviour. Recent pharmacotherapies target to induce mGluR2/3 agonism in the nACb and enhancing xCT and GLT1 expression to modulate the glutamate tone (Scofield and Kalivas 2014). Although there has been no compelling evidence of a drug that rescues the increased glutamate tone in drug exposed animals, Ceftriaxone, a third-generation cephalosporin β-lactam antibiotic, has been evaluated in attenuating glutamate induced excitotoxicity and neuronal damage by enhancing GLT1 and xCT levels in drug seeking animals (Fischer et al. 2013).

Given, that NT modulates glutamatergic neurotransmission in VTA neurons that project to the nAcb and a subset of glutamatergic neurons in the VTA project to the accumbens where DA and glutamate might be co released (Hnasko et al. 2012), the findings from our studies open up the question of whether these astrocytic proteins in addition to glutamatergic neurotransmission in VTA neurons might be regulated by NT.

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