Neurotensin inhibits both dopamine and GABA mediated inhibition of ventral tegmental area dopamine neurons

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Running title: Neurotensin inhibits D2R and GABA_B IPSCs in dopamine neurons

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Abstract:

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

Keywords: dopamine, neurotensin, D2R, GABA_B, GIRK, calcium, TrpC
Introduction

Dopamine (DA) is an essential neurotransmitter involved in many different behaviors including motor behavior, incentive motivation, reward and reinforcement, learning, memory, drug intake, and habit formation (Wise 2004), and disruptions in DA signaling have been implicated in many disorders such as drug addiction, obesity, Parkinson’s disease, and schizophrenia (Grace et al. 2007; Howes et al. 2015; Kenny 2011a; b; Lewis and Barker 2009). Most DA producing neurons are found in the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc) of the midbrain (Wise 2004). At rest VTA/SNc DA neurons fire tonically (2-10 Hz) causing a baseline low level of DA at efferent target sites, but in response to a reward DA neurons fire in bursts causing phasic increases in DA release (Grace et al. 2007; Morikawa and Paladini 2011; Paladini and Roeper 2014). Phasic increases in DA release at efferent target sites are thought to be a salient signal and learning cue (Grace et al. 2007; Morikawa and Paladini 2011; Paladini and Roeper 2014). DA burst firing is primarily controlled by glutamatergic afferent inputs, but DA neuron activity can also be modulated by other neurotransmitters and neuropeptides acting either directly on DA neurons or indirectly through regulation of GABAergic or glutamatergic inputs to DA neurons (Grace et al. 2007; Morikawa and Paladini 2011; Paladini and Roeper 2014). Characterizing how DA neuron activity is regulated is important for understanding the function of DA under normal and pathological conditions.

In addition to releasing DA from their axon terminals, DA neurons release DA locally within the VTA/SNc from their soma and dendrites (Beckstead et al. 2004; Bjorklund and Lindvall 1975; Geffen et al. 1976; Kalivas and Duffy 1991; Rice et al. 1997). This somatodendritic DA release inhibits neighboring DA neurons through dopamine D2 receptor
(D2R) mediated activation of G-coupled inward rectifying potassium (GIRK) channels 
(Aghajanian and Bunney 1977; Beckstead et al. 2004; Lacey et al. 1987; Mercuri et al. 1997). 
D2Rs in VTA DA neurons regulate DA neuron activity and also regulate DA mediated 
behaviors. For example, injection of quinpirole, a D2R agonist, directly into the VTA causes 
conditioned place aversion, blocks food induced conditioned place preference, decreases food 
intake, and decreases cocaine-induced reinstatement (Liu et al. 2008; Xue et al. 2011).  
Furthermore selective knockout of autoreceptor D2Rs within midbrain DA neurons causes 
increased motor activity to a novel environment, increased food self-administration, and 
increased responses to cocaine such as increased locomotor activity and conditioned place 
preference compared to wild type mice (Anzalone et al. 2012; Bello et al. 2011).  In addition, 
DA neuron burst firing is followed by a pause, and it has been proposed that D2R mediated 
inhibition terminates bursts of action potentials and is responsible for the pause following burst 
firing (Beckstead et al. 2004).  Thus, this autoinhibitory D2R signaling in the VTA plays an 
important role in the regulation of DA activity and DA mediated behaviors. 
Neurotensin is a tricapeptide that was first isolated and characterized from bovine 
hypothalamus (Carraway and Leeman 1973), and is widely expressed in both the central and 
peripheral nervous systems.  The actions of neurotensin are mediated by three known 
neurotensin receptors: NTS1, NTS2 and NTS3 (for review see (Vincent et al. 1999)).  There is 
abundant evidence that neurotensin interacts with the DA system (for review see (Binder et al. 
2001)) and dysregulation of these interactions have been proposed to be involved in pathologies 
such as schizophrenia, drug abuse, and Parkinson’s disease (Binder et al. 2001; Mustain et al. 
2011; St-Gelais et al. 2006; Tanganelli et al. 2012).  Fibers containing neurotensin heavily 
nervate midbrain DA neurons (Hokfelt et al. 1984; Woulfe and Beaudet 1989) and DA neurons
of the VTA and SNc express neurotensin receptors, primarily the NTS1 receptor (Binder et al. 2001; Fassio et al. 2000; Lepee-Lorgeoux et al. 1999; Nicot et al. 1995; Palacios and Kuhar 1981; Szigethy and Beaudet 1989). Furthermore, D2Rs and NTS1 receptors have been shown to form heteromers in heterologous expression systems, which resulted in a decrease in D2R agonist binding and decreases in D2R signaling after treatment with neurotensin (Borrot-Escuela et al. 2013; Koschatzky et al. 2011). Previous research has shown that neurotensin modifies midbrain DA neuron activity through two NTS1 receptor dependent mechanisms: increased DA neuron firing through activation of a non-selective cation channel (Farkas et al. 1996; Jiang et al. 1994; Jomphe et al. 2006; Shi and Bunney 1991; St-Gelais et al. 2004; Werkman et al. 2000) and a reduction in the inhibition of firing caused by D2R activation (Jomphe et al. 2006; Nimitvilai et al. 2012; Shi and Bunney 1990; Shi and Bunney 1991; 1992; Werkman et al. 2000). The majority of evidence suggests that the effects of neurotensin on DA neurons occur through activation of signaling pathways downstream of Gq-proteins, specifically through PKC, IP3, and calcium (Jomphe et al. 2006; Nimitvilai et al. 2013; St-Gelais et al. 2004; Thibault et al. 2011; Wu et al. 1995), although neurotensin has also been reported to affect DA neuron activity through a PKA dependent mechanism (Shi and Bunney 1992). In these studies we sought to examine the mechanisms by which neurotensin reduces the D2R-mediated inhibition of DA neuron activity by testing the hypothesis that neurotensin inhibits the D2R-mediated inhibitory post-synaptic current (D2R IPSC) (Beckstead et al. 2004) that occurs in response to the local, somatodendritic release of DA within the VTA.

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

Slice preparation and Electrophysiology: Acute brain slices were prepared as previously described (Roseberry et al. 2007). Briefly, adult male mice were anesthetized with isofluorane and decapitated. The brain was then removed and placed in carbogen (95% O₂ and 5% CO₂) saturated ice-cold artificial cerebral spinal fluid (aCSF), containing (in mM) 126 NaCl, 2.5 KCl, 2.4 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, 11.1 glucose and 21.4 NaHCO₃. A brain block containing the VTA was made and pseudo-horizontal sections (220 μM) were cut with a vibrating blade microtome. Slices were then incubated in aCSF (~35°C) containing 10 μM MK-801 [(+)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] for at least 30 minutes before recording. Slices were placed in a recording chamber and perfused with carbogen-saturated aCSF at a flow rate of ~1-2 ml/min. Whole-cell recordings were made using an Axon multiclamp 700B microelectrode amplifier and Axograph software. Putative DA neurons were identified by their location relative to the medial terminal nucleus of the accessory optic tract, the presence of hyperpolarization-activated cation currents (H-current), the presence of spontaneous pacemaker firing, and the sensitivity to DA (Johnson and North 1992). Although recent studies have raised questions on the utility of using these measures to identify VTA DA neurons (Margolis et al. 2006), the characteristics described above have been widely used in electrophysiology studies to identify DA neurons within the VTA (Beckstead et al. 2004; Nimitvilai et al. 2012; Nimitvilai et al. 2013; Perra et al. 2011; Roseberry et al. 2007).
Electrodes (2.0-3.0 MΩ) were filled with a potassium gluconate (KGluc) based internal solution containing (in mM) 128 KGluc, 10 NaCl, 1 MgCl₂, 10 HEPES, 10 BAPTA, 2 ATP, 0.3 GTP, and 10 creatine phosphate. For the experiments testing the effects of neurotensin under reduced calcium buffering conditions, low-calcium buffering potassium methyl sulfate (KMeSO₄) or K-Gluconate based internal solutions were used containing: (in mM) 115 KMeSO₄, 20 NaCl, 1 MgCl₂, 10 HEPES, 0.1 EGTA, 2 ATP, 0.3 GTP, and 10 creatine phosphate; or (in mM) 128 KGluc, 10 NaCl, 1 MgCl₂, 10 HEPES, 0.1 EGTA, 2 ATP, 0.3 GTP, and 10 creatine phosphate. No differences were observed between these two low calcium buffering internal solutions, so experiments using these two different internal solutions were pooled. Series resistance values were approximately ~3-15 MΩ. If the series resistance increased by more than 20% or if the IPSC or holding current were unstable in any of the experiments, the experiment was terminated and excluded from analysis. Neurons were voltage clamped at -60mV for all experiments, and D2R IPSCs and GABA_B IPSCs were evoked using a bipolar stimulating electrode placed 100-300 μM posterior to the recorded cell. D2R IPSCs were evoked with 5-stimuli (0.5ms) at 40 Hz, and GABA_B IPSCs were evoked with 6-stimuli (0.3ms) at 50 Hz. D2R-mediated currents were also evoked using the iontophoretic application of DA. DA was applied iontophoretically through a ~70-100 MΩ glass pipette filled with 1 M DA and ejected as a cation with a single pulse (10 nA, 25 ms). Leak of DA from the pipette was prevented with a constant negative back current (2 nA). To isolate D2R IPSCs, picrotoxin (100 μM), CGP 55845 (0.5 μM), and DNQX (10 μM) were included in the perfusion solution to block GABA_A, GABA_B, and AMPA receptors, respectively. GABA_B IPSCs were isolated by including sulpiride (200 nM), picrotoxin (100 μM), and DNQX (10 μM) to block D2R, GABA_A, and AMPA receptors respectively. The peak amplitude of all IPSCs was measured from baseline and
calculated as the mean current 30 ms before and after the peak IPSC amplitude. For all experiments cells were held for 10 minutes prior to drug application to allow for diffusion of the internal solution into the cell. For the experiments examining muscarinic acetylcholine receptor induced currents, the nicotinic receptor antagonist, mecamylamine (30 μM), was included both before and during application of acetylcholine. To determine the voltage current relationship and reversal potential of the neurotensin current, voltage ramps were applied (-120mV to +40mV at 160mV s\(^{-1}\) or -120mV to +20mV at 140mV s\(^{-1}\)) in the presence of TTX.

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

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

**Results**
We initially examined how neurotensin affects DA neuron activity by assessing both the inward current activated by neurotensin and its ability to affect the inhibitory current generated by somatodendritic DA release in the VTA (D2R IPSC) (Beckstead et al. 2004). Neurotensin dose-dependently activated an inward current in DA neurons and inhibited the D2R IPSC (Fig. 1A-C). Neurotensin also caused an increase in noise at all doses tested (Fig. 1C). The calculated maximal current and maximal inhibition of the D2R IPSC were 774.9 pA and 89.45 % respectively, and the EC50 values of the effects of neurotensin on VTA DA neurons were 208.7 nM for the inward current and 4.38 nM for the inhibition of the D2R IPSC (Fig. 1A-B). The inhibition of the D2R IPSC positively correlated with the magnitude of the inward current (r=0.672, p=0.0006, Fig. 1D), suggesting that neurotensin may activate the inward current and inhibit the D2R IPSC in VTA DA neurons through a common mechanism.

The timing of the activation of the inward current and the inhibition of the D2R IPSC differed however. The inward current caused by 100 nM neurotensin reached its peak quickly, whereas the onset and peak of the inhibition of the D2R IPSC was delayed compared to the inward current (Fig. 2A-C). The washout and recovery of the effects of neurotensin also differed as the neurotensin current and the increase in noise slowly reversed (in ~10-15 minutes), while the inhibition of the D2R IPSC never recovered during the drug washout period (Fig. 2A,C). Furthermore, the D2R IPSC remained inhibited 10 minutes after neurotensin application when the neurotensin current had almost completely recovered suggesting that the inward current is not simply occluding the D2R IPSC (Fig. 2A-C). Thus, although the magnitude of the inward current correlated with the amount of inhibition of the D2R IPSC, there were differences in the timing of the two effects of neurotensin suggesting that they may actually be mediated through different mechanisms.
We next sought to confirm whether the neurotensin caused inward current and inhibition of the D2R IPSC were independent by inhibiting the inward current and measuring the effect of neurotensin on the D2R ISPC. Thus, we attempted to confirm previous experiments identifying the ion channels mediating the neurotensin-induced inward current in DA neurons. The neurotensin-induced current obtained from slow voltage ramps showed a unique IV relationship with outward rectification and a extended zero slope region around the reversal potential, which was calculated to be \(-36 \pm 6.6\) mV (Fig. 3). This unique IV curve, combined with previous reports demonstrating that neurotensin activates a slow nonselective cation conductance permeable to Na\(^+\), K\(^+\), and Cs\(^+\) (Chien et al. 1996; Farkas et al. 1996; Jiang et al. 1994), suggests that neurotensin may be activating a member of the transient receptor potential C channel (TrpC) family (Kim et al. 2012; Zhang and Trebak 2014). Thus, we tested whether SKF 96365, a TrpC channel blocker that was previously shown to block the neurotensin caused increase in firing frequency in DA neurons (St-Gelais et al. 2004), could inhibit the neurotensin induced inward current in VTA DA neurons. SKF 96365 (100 μM) partially inhibited the neurotensin-induced inward current (Fig. 4C-D). Although the peak inward current caused by neurotensin (100 nM) was only slightly decreased by SKF 96365, the sustained neurotensin-induced current rapidly decreased, and the duration of the neurotensin-induced inward current was significantly shortened (Fig. 4C-D) (significant main effects of treatment (F(1, 9)=6.079, p<0.05), and time (F(20, 175)=13.888, p<0.001), and a significant treatment×time interaction (F(20,175)=2.583, p<0.001). In addition, the increase in noise caused by neurotensin also recovered more quickly during the washout and recovery of the neurotensin-induced current. We also tested whether SKF 96365 affected the ability of neurotensin to inhibit the D2R IPSC. SKF 96365 had no effect on the ability of neurotensin to inhibit the D2R IPSC however, as the
peak inhibition of the D2R IPSC caused by neurotensin was unaffected (Fig. 4E-F). The D2R IPSC also remained inhibited throughout the experiment even when the neurotensin current recovered (Fig. 4A-B) suggesting that the inward current and the inhibition of the D2R IPSC are likely independent and that the inward current caused by neurotensin does not simply occlude the D2R IPSC.

We next sought to confirm that the effects of neurotensin on the D2R IPSC were mediated post-synaptically and not through alterations in DA release, by testing whether neurotensin also inhibited the D2R-mediated current generated by the iontophoretic application of DA. As predicted, neurotensin inhibited the iontophoresis evoked D2R current (Fig. 5A) to the same magnitude as the electrically evoked D2R IPSC (Fig. 5B-C). Thus, neurotensin appears to act at the post-synaptic membrane to inhibit the D2R IPSC and not through a pre-synaptic change in DA release.

We next attempted to identify the mechanism by which neurotensin inhibits the D2R IPSC. Previous studies suggested that neurotensin inhibits D2R signaling through a Ca\(^{2+}\) and PKC dependent mechanism (Jomphe et al. 2006; Nimitvilai et al. 2013; Thibault et al. 2011). Therefore we tested the role of the release of Ca\(^{2+}\) from intracellular stores in the neurotensin caused inhibition of the D2R IPSC through the use of the reversible sarcoplasmic reticulum Ca\(^{2+}\) ATPase inhibitor, cyclopiazonic acid (CPA). Pretreatment of brain slices with CPA (10 \(\mu\)M) for at least 15 minutes prior to neurotensin application had no effect on the neurotensin (100 nM) inhibition of the D2R IPSC (Fig. 6A-B). In addition CPA had no effect on the peak inward current induced by neurotensin (Fig. 6C-D). We also tested whether neurotensin inhibits the D2R IPSC through a PKC-dependent process through the use of the non-specific PKC inhibitor, chelerythrine. Chelerythrine (10 \(\mu\)M) also had no effect on the neurotensin-induced inhibition of
the D2R IPSC (Fig. 6A-B), and there was no significant difference between the peak inward current induced by neurotensin after chelerythrine treatment compared with neurotensin alone (Fig. 6C-D). Thus, in contrast to previous reports (Jomphe et al. 2006; Nimitvilai et al. 2013; Thibault et al. 2011) the inhibition of the D2R IPSC in VTA DA neurons by neurotensin does not appear to depend on PKC activation, nor does it depend on release of Ca\(^{2+}\) from intracellular stores.

We next tested whether the inhibition of the D2R IPSC was specific to neurotensin or could be achieved by activation of other Gq-coupled receptors by examining whether activation of metabotropic glutamate receptors or muscarinic acetylcholine receptors also inhibited the D2R IPSC. To activate muscarinic receptors, acetylcholine (ACh) was added in the presence of mecamylamine (30 \(\mu\)M), a nicotinic acetylcholine receptor antagonist. For this experiment, we chose doses of the metabotropic glutamate receptor agonist, DHPG (10 \(\mu\)M), ACh (1 mM), and neurotensin (10 nM) that caused approximately the same peak inward current to allow for direct comparison of their effects on the D2R IPSC (Fig. 7A-D). There were no significant differences between the peak current activated by DHPG, ACh, and neurotensin (F(2,16)=1.217, p=0.322) (Fig. 7D), but there were significant differences in the inhibition of the D2R IPSC, as neurotensin inhibited the D2R IPSC to a significantly greater extent than DHPG or ACh (F(2,16)=5.181, p<0.05) (Fig. 7E). In addition, the effects of DHPG and ACh on the D2R IPSC started to reverse upon removal of the agonist, whereas the effects of neurotensin on the D2R IPSC showed no reversal (Fig. 7A-C). Thus, it appears that activation of Gq-coupled receptors can inhibit the D2R IPSC, but activation of NTS1 appears to engage an additional mechanism that causes significantly greater inhibition of the D2R IPSC.
We next sought to test whether the ability of neurotensin to inhibit the D2R IPSC was specific to D2Rs or if it would also affect other inhibitory responses in VTA DA neurons. GABA_B receptors and D2Rs both inhibit VTA DA neurons through the activation of G-protein coupled inward rectifying potassium channels (GIRK channels). Thus, we next tested whether neurotensin also affected the IPSC generated by activation of GABA_B receptors (GABA_B IPSC) (Fig. 8A-B). Interestingly, neurotensin inhibited the GABA_B IPSC to a similar degree as the D2R IPSC (Fig. 8C). This was true for both the maximal dose (100 nM) of neurotensin that fully inhibited the D2R IPSC, and an intermediate dose (10 nM) closer to the EC50 value for neurotensin inhibition of the D2R IPSC (Fig. 8C). Thus, neurotensin does not appear to specifically inhibit the D2R IPSC in VTA DA neurons but can also inhibit the GABA_B IPSC, possibly through a common mechanism.

We next sought to examine the similarity of the inhibition of the D2R IPSC and the GABA_B IPSC in more detail. The D2R and GABA_B IPSCs differ in their sensitivity to intracellular Ca^{2+} levels as the D2R IPSC shows increased desensitization and long-term depression when intracellular free Ca^{2+} levels are weakly buffered, where as the GABA_B IPSC is insensitive to changes in intracellular Ca^{2+} (Beckstead and Williams 2007). Thus, we next tested whether the effects of neurotensin on the D2R and GABA_B IPSCs were affected by basal intracellular Ca^{2+} levels by switching from a high Ca^{2+} buffering internal solution (containing 10 mM BAPTA = low levels of basal free intracellular Ca^{2+}) to a low Ca^{2+} buffering internal solution (containing 0.1 mM EGTA = higher levels of basal free intracellular Ca^{2+}). A dose of neurotensin near the EC50 value for inhibition of the D2R IPSC was used in these experiments to allow for the identification of potential increases or decreases in the effect of neurotensin on the D2R and GABA_B IPSCs with the reduced calcium buffering internal solution. Neurotensin
(10 nM) still caused an inward current and slightly reduced both the D2R and GABA_B IPSCs when measured with a 0.1 mM EGTA internal solution (Fig. 9). Interestingly, the inward current induced by neurotensin was significantly reduced compared to the inward current with the internal solution containing 10 mM BAPTA (Fig. 9A-B) (significant main effects of treatment (F(1, 15)=23.357, p<0.001) and time (F(19,250)=33.674, p<0.001), and significant treatment×time interaction (F(19,250)=14.087, p<0.001). The neurotensin-caused inhibition of both the D2R and GABA_B IPSCs were also significantly reduced with the 0.1 mM EGTA internal solution compared to the 10 mM BAPTA internal solution with no differences in the magnitude of inhibition of the D2R IPSC versus the GABA_B IPSC (Fig. 9C-F) (D2R IPSC: significant main effects of treatment (F(1, 17)=32.102, p<0.001) and time (F(19, 288)=33.157, p<0.001), and a significant treatment×time interaction (F(19,288)=11.708, p<0.001); GABA_B IPSC: significant main effects of treatment (F(1,12)=17.439, p=0.001) and time (F(20, 240)=18.321, p<0.001), and a significant treatment×time interaction (F(20,240)=8.165, p<0.001).

Thus, the effects of neurotensin on DA neurons were greater when intracellular Ca^{2+} was strongly buffered and resting levels of free intracellular Ca^{2+} were low, and were attenuated when intracellular Ca^{2+} was weakly buffered and resting levels of free intracellular Ca^{2+} were high.

**Discussion**

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

The effects of neurotensin on the inward current and the D2R and GABA\textsubscript{B} IPSCs appear to be independent and mediated by different mechanisms. The timing of neurotensin inhibition of the D2R IPSC was significantly delayed compared to the inward current caused by neurotensin, and the neurotensin-induced current recovered during the wash out period after neurotensin application, whereas the inhibition of the D2R IPSC did not. The EC\textsubscript{50} values for the two effects of neurotensin were also different further supporting the argument that the two effects of neurotensin occur through separate mechanisms. The EC\textsubscript{50} value for inhibition of the D2R IPSC by neurotensin was \textasciitilde 4 nM, much lower than the EC\textsubscript{50} value for the neurotensin induced current (\textasciitilde 200 nM), and lower doses of neurotensin (1-10 nM) have often been used previously to examine the effect of neurotensin on D2R signaling (Jomphe et al. 2006; Nimitvilai et al. 2012; Shi and Bunney 1990; Shi and Bunney 1991; 1992; Werkman et al. 2000) while higher doses of neurotensin (1 nM to 5 \( \mu \)M) have been used to examine the neurotensin induced inward current in DA neurons (Farkas et al. 1996; Jiang et al. 1994; Jomphe et al. 2006; Shi and Bunney 1991; St-Gelais et al. 2004; Werkman et al. 2000). In addition, partially inhibiting the neurotensin current with SKF 96365 did not have any effect on the neurotensin caused inhibition of the D2R IPSC. Finally, ACh, DHPG, and neurotensin caused inward currents that were similar in magnitude, but neurotensin had a much larger effect on the D2R IPSC. If the inward current caused the inhibition of the D2R IPSC then it would be expected that neurotensin, ACh, and DHPG would inhibit the D2R IPSC to the same magnitude when activating inward currents of the same size. In agreement with our findings, it was previously reported that neurotensin reduces quinpirole (D2R agonist) induced inhibition of DA activity even when the excitatory
of occlusion or net excitation, and that these are independent effects downstream of NTS1 activation.

The neurotensin-induced inward current has been characterized as a slow non-selective cation current that is equally permeable to both Na\(^+\) and K\(^+\), a characteristic of Trp channels (Farkas et al. 1996). The neurotensin-induced current in DA neurons was similar to that generated by activation of specific TrpC channels expressed in HEK-293 cells (Kim et al. 2012; Zhang and Trebak 2014) suggesting that neurotensin may be activating a member of the TrpC family, potentially through the activation of phospholipase C and release of DAG (Harteneck and Gollasch 2011; Pena and Ordaz 2008). We found that the TrpC channel blocker SKF 96365 significantly shortened the duration of the neurotensin-induced current, which is in agreement with a previous report showing that SKF 96365 blocks the neurotensin-caused increase in DA neuron firing frequency (St-Gelais et al. 2004). Thus, it appears that the neurotensin activated inward current is at least partially mediated by activation of TrpC channels in VTA DA neurons.

The majority of evidence suggests that the effects of neurotensin on DA neurons are mediated by signals that are downstream of PLC activation. Previously it was reported that the neurotensin-induced inward current and increase in firing frequency is dependent on Ca\(^{2+}\) and the IP3 receptor (Jomphe et al. 2006; St-Gelais et al. 2004; Wu et al. 1995). Another study found that the neurotensin-induced inward current was not dependent on Ca\(^{2+}\) however, as the neurotensin current was not affected by buffering intracellular Ca\(^{2+}\) with 20 mM BAPTA (Farkas et al. 1996). In addition it has also been reported that neurotensin inhibits D2R signaling through a PKC and Ca\(^{2+}\) dependent mechanism (Jomphe et al. 2006; Nimitvilai et al. 2013; Thibault et al.
In contrast to these previous reports, we found that the neurotensin inhibition of the D2R IPSC and the neurotensin-induced current were not dependent on PKC or Ca$^{2+}$, and the effects of neurotensin were actually potentiated when intracellular Ca$^{2+}$ was buffered with 10 mM BAPTA. In addition the neurotensin inhibition of the D2R IPSC and the inward current were not dependent on Ca$^{2+}$ release from intracellular stores. Interestingly, it has also been reported that neurotensin reduces the DA-caused inhibition of DA neuronal firing through the cAMP pathway and not through a PKC dependent mechanism (Shi and Bunney 1992). Additional experiments are needed to resolve these differences however and to determine if the effects of neurotensin occur through dual pathways.

Although neurotensin likely inhibits the D2R IPSC via activation of Gq coupled signaling through a mechanism similar to metabotropic glutamate receptors and muscarinic acetylcholine receptors, here we have shown that there appears to be an additional mechanism activated by neurotensin to inhibit the D2R IPSC to a larger extent than other Gq coupled receptors. Interestingly, we also found that the effects of neurotensin were not specific to the D2R IPSC as neurotensin also inhibited the GABA$_B$ IPSC to the same magnitude. This suggests that the effects of neurotensin are likely not due to modulation of D2R activity by direct heterodimer interactions with NTS1 as has been observed in HEK-293 cells (Borroto-Escuela et al. 2013; Koschatzky et al. 2011). Previously it was reported that neurotensin does not block GABA caused inhibition of DA neuronal firing (Shi and Bunney 1991). GABA inhibits DA neurons through the activation of both GABA$_A$ and GABA$_B$ receptors however, so neurotensin may only block the inhibition produced by GABA$_B$ receptors and not GABA$_A$ receptors, which could explain the differences in these results. Thus the results presented here suggest that neurotensin inhibits both GABA$_B$ and D2R signaling in VTA DA neurons.
In these studies, we have shown a novel mechanism for modulation of VTA DA neuron activity by neurotensin: inhibition of GABA$_B$ IPSCs. GABA$_B$ receptors and D2Rs both activate GIRK channels to inhibit DA neurons, so it is possible that neurotensin modulates GIRK channel activity downstream of both D2R and GABA$_B$ activation to reduce D2R and GABA$_B$ caused inhibition of DA neurons (Fig. 10). In support of this notion, it was previously shown that neurotensin blocks D2R signaling downstream of the D2R (Farkas et al. 1997). In addition, it was recently reported that inducing high frequency bursting or depolarization in VTA DA neurons causes potentiation of GIRK currents and this potentiation was due to modulation to the GIRK channels themselves rather than regulation of the GABA$_B$ or D2 receptors (Lalive et al. 2014). Thus, neurotensin may reduce both D2R and GABA$_B$ mediated GIRK currents by causing direct modulation of GIRK channels, although further experiments will be required to test this hypothesis.

D2R IPSC desensitization and long-term depression have been reported to increase when intracellular Ca$^{2+}$ is weakly buffered and free intracellular Ca$^{2+}$ levels are high, whereas GABA$_B$ IPSCs do not show the same sensitivity to intracellular Ca$^{2+}$ levels (Beckstead and Williams 2007). Combined with the ability of neurotensin to increase Ca$^{2+}$ in DA neurons (Jomphe et al. 2006; St-Gelais et al. 2004), these results suggest that reducing the Ca$^{2+}$ buffering capacity of the internal solution would result in a larger effect of neurotensin on the D2R IPSC but not the GABA$_B$ IPSC. Surprisingly, the neurotensin-caused inhibition of both the GABA$_B$ and D2R IPSCs were significantly attenuated with low intracellular Ca$^{2+}$ buffering (i.e. 0.1 mM EGTA solution). Thus, neurotensin appears to inhibit GIRK current activation downstream of D2R and GABA$_B$ receptors through a Ca$^{2+}$ sensitive mechanism, whereby low levels of free intracellular calcium are required for the full inhibition of GIRK channel activation (Fig. 10). Alternatively,
it is also possible that the NTS1 receptor is Ca\textsuperscript{2+} sensitive. We found that the neurotensin-
induced inward current was also significantly reduced with low intracellular Ca\textsuperscript{2+} buffering.
Thus, all effects of neurotensin in DA neurons were reduced with low intracellular Ca\textsuperscript{2+} buffering
and high intracellular levels of free Ca\textsuperscript{2+}. Therefore, it is possible that the NTS1 receptor may be
sensitive to free intracellular Ca\textsuperscript{2+} levels and may desensitize or internalize with high levels of
free intracellular Ca\textsuperscript{2+} resulting in a reduced effect of neurotensin, although future studies will be
required to test this hypothesis.

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

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**Figure captions:**

Fig. 1: Neurotensin dose-dependently activates an inward current and inhibits the D2R IPSC in VTA DA neurons.  A. Dose-response curve of the inward current induced by neurotensin.  B. Dose-response curve of the inhibitory effect of neurotensin on the D2R IPSC.  C. Sample traces of the neurotensin (NT) induced current (left) and inhibition of the D2R IPSC (right).  D. The
size of the inward current is positively correlated to the amount of inhibition of the D2R IPSC.

Bars in C indicate time of neurotensin application. n=5-7 cells from 4-7 mice for each dose.

Scale Bars: 50 pA/2 min (1nM NT current); 100 pA/2 min (10 nM NT current); 200 pA/2 min (100 nM NT current); 20 pA/500 ms (NT inhibition of D2R IPSC).

Fig. 2: The neurotensin-induced inward current preceeds the inhibition of the D2R IPSC. A. Sample cell of the effects of neurotensin (100 nM) on the inward current and the D2R IPSC. B. Sample traces of the D2R IPSCs in A before neurotensin (a; black trace), during the peak of the neurotensin inward current (b; grey trace), and at 8-10 minutes after neurotensin washout (c; grey trace). C. Mean responses of the effects of neurotensin (100 nM) on the inward current and the D2R IPSC. Bars in A and C indicate time of neurotensin application. n=6 cells from 6 mice.

Fig. 3: Current -Voltage relationship of the neurotensin-induced current in VTA DA neurons. A. Sample current traces resulting from slow voltage ramps (-120 mV to +20 mV at 140 mV s⁻¹) before (black trace) and after neurotensin (100 nM; grey trace). B. Sample trace of the net neurotensin (100 nM) induced current. C. Mean current-voltage relationship of the neurotensin (100 nM) induced current. n=7 neurons from 4 mice. Scale Bars: 1 nA/200 ms

Fig. 4: The TrpC channel inhibitor, SKF 96365, partially blocks the neurotensin-induced inward current but does not affect the inhibition of the D2R IPSC. A. Sample cell of the effects of neurotensin (100 nM) on the inward current and the D2R IPSC in the presence of SKF 96365 (100 μM). Inset: sample trace of the D2R IPSC before neurotensin (a; black trace) and 8-10
minutes after neurotensin washout (b; grey trace) in the presence of SKF 96365. B. Mean responses of the effects of neurotensin (100 nM) on the inward current and the D2R IPSC in the presence of SKF 96365 (100 μM). C. Mean inward current generated by neurotensin (100 nM) in the absence and presence of SKF 96365 (100 μM). D. Mean neurotensin-induced current at the peak and 5 minutes after neurotensin washout in the presence and absence of SKF 96365. E-F. Mean effect of neurotensin (100 nM) on the D2R IPSC in the absence and presence of SKF 96365 (100 μM). Bars in A-C and E indicate time of neurotensin and SKF 96365 application. n=5-6 neurons from 4-6 mice for each group. Scale bar: 20 pA/500 ms. *p<0.05 vs neurotensin alone

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

Fig. 6: Neurotensin inhibition of the D2R IPSC and the neurotensin-induced current are not dependent on PKC activity or release of Ca^{2+} from intracellular stores. A-B. Mean inhibition of the D2R IPSC caused by neurotensin (100 nM) in the presence and absence of CPA (10 μM) or chelerythrine (CHE) (10 μM). C-D. Mean neurotensin (100 nM) induced current in the presence and absence of CPA (10 μM) or CHE (10 μM). Bars in A and C indicate time of neurotensin application. n=5-6 cells, from 4-6 mice for each group.
Fig. 7: Neurotensin inhibits the D2R IPSC significantly more than DHPG or acetylcholine. A. Mean inward current and inhibition of the D2R IPSC caused by DHPG (10 μM). Inset: Sample trace of the D2R IPSC before DHPG (a; black trace) and at 7-11 minutes after applying DHPG (b; grey trace). B. Mean inward current and inhibition of the D2R IPSC caused by ACh (1 mM) in the presence of mecamylamine (30 μM). Inset: Sample trace of the D2R IPSC before ACh (a; black trace) and at 7-11 minutes after applying ACh (b; grey trace). C. Mean inward current and inhibition of the D2R IPSC caused by neurotensin (10 nM). Inset: Sample trace of the D2R IPSC before neurotensin (a; black trace) and at 7-11 minutes after applying neurotensin (b; grey trace). D. The peak inward currents caused by DHPG (10 μM), ACh (1 mM), and neurotensin (10 nM) were not significantly different. E. Neurotensin (10 nM) inhibited the D2R IPSC significantly more than DHPG and ACh. Bars in A-C indicate time of drug application. DHPG: n=5 cells from 4 mice; ACh: n=8 cells from 7 mice; neurotensin: n=6 cells from 5 mice. Scale bars: 30 pA/500 ms. *p<0.05 vs neurotensin

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

Fig. 9: Reduced buffering of intracellular calcium attenuates the effects of neurotensin. A-B. Mean neurotensin (10 nM) induced inward current with an internal solution containing 10 mM
BAPTA or 0.1 mM EGTA. C-F. Sample traces (C,E) and mean effect (D,F) of neurotensin (10^6 nM; grey trace) on the D2R IPSC (C-D) and GABA_B IPSC (E-F) using internal solutions containing 10 mM BAPTA or 0.1 mM EGTA. Bars in A, D, & F indicate time of neurotensin application. n=5-12 cells from 4-11 mice for each group. Scale Bars: 20 pA/400 ms (C); 20 pA/200 ms (D). #p<0.05, *p≤0.001

Fig. 10: Diagram of the proposed model of neurotensin inhibition of GIRK currents activated by D2 and GABA_B receptors. Activation of NTS1 with neurotensin in VTA DA neurons causes robust inhibition of D2R and GABA_B GIRK currents when the relative levels of free intracellular Ca^{2+} are low due to strong Ca^{2+} buffering, while neurotensin induced inhibition of D2R and GABA_B GIRK currents is significantly attenuated when relative levels of free intracellular Ca^{2+} are higher due to weak Ca buffering.
Fig. 1

A

Inward current (pA)

LOG [Neurotensin]

B

D2R IPSC (% inhibition)

LOG [Neurotensin]

C

1 nM NT

10 nM NT

100 nM NT

Control  NT

D

Inward current (pA)

D2R IPSC (% inhibition)

r=0.672, p=0.0006
Fig. 3

A 

B 

C 

Control 

NT (100 nM) 

Voltage (mV) 

Current (pA)
Fig. 5

Panel A: Comparison of D2R Current between Control and NT (100 nM).

Panel B: Graph showing the effect of NT (100 nM) on D2R Current and D2R IPSC over time. Symbols indicate significant differences.

Panel C: Bar graph comparing DA Ionto Current and D2R IPSC with n.s. indicating no significant difference.