Neurotensin Induces Presynaptic Depression of D₂ Dopamine Autoreceptor-Mediated Neurotransmission in Midbrain Dopaminergic Neurons

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Increased dopaminergic signaling is a hallmark of severe mesencephalic pathologies such as schizophrenia and psychostimulant abuse. Activity of midbrain dopaminergic neurons is under strict control of inhibitory D₂ autoreceptors. Application of the modulatory peptide neurotensin (NT) to midbrain dopaminergic neurons transiently increases activity by decreasing D₂ dopamine autoreceptor function, yet little is known about the mechanisms that underlie long-lasting effects. Here, we performed patch-clamp electrophysiology and fast-scan cyclic voltammetry in mouse brain slices to determine the effects of NT on dopamine autoreceptor-mediated neurotransmission. Application of the active peptide fragment NT_{8–13} produced synaptic depression that exhibited short- and long-term components. Sustained depression of D₂ autoreceptor signaling required activation of the type 2 NT receptor and the protein phosphatase calcineurin. NT application increased paired-pulse ratios and decreased extracellular levels of somatodendritic dopamine, consistent with a decrease in presynaptic dopamine release. Surprisingly, we observed that electrically induced long-term depression of dopaminergic neurotransmission that we reported previously was also dependent on type 2 NT receptors and calcineurin. Because electrically induced depression, but not NT-induced depression, was blocked by postsynaptic calcium chelation, our findings suggest that endogenous NT may act through a local circuit to decrease presynaptic dopamine release. The current research provides a mechanism through which augmented NT release can produce a long-lasting increase in membrane excitability of midbrain dopamine neurons.

Key words: calcineurin; Dopamine; IPSC; neurotensin; plasticity; presynaptic

Significance Statement

Whereas plasticity of glutamate synapses in the brain has been studied extensively, demonstrations of plasticity at dopaminergic synapses have been more elusive. By quantifying inhibitory neurotransmission between midbrain dopaminergic neurons in brain slices from mice we have discovered that the modulatory peptide neurotensin can induce a persistent synaptic depression by decreasing dopamine release. This depression of inhibitory synaptic input would be expected to increase excitability of dopaminergic neurons. Induction of the plasticity can be pharmacologically blocked by antagonists of either the protein phosphatase calcineurin or neurotensin receptors, and persists surprisingly long after a brief exposure to the peptide. Since neurotensin–dopamine interactions have been implicated in hyperdopaminergic pathologies, these findings describe a synaptic mechanism that could contribute to addiction and/or schizophrenia.

Introduction

Midbrain dopaminergic neurons are key to voluntary movement and reward-related behaviors, and disruption of their function is associated with Parkinson's disease, drug addiction, and schizophrenia (Wise, 2004; Chinta and Andersen, 2005; Pierce and Ku-

maresan, 2006; Berridge, 2007; Howes and Kapur, 2009; Schultz, 2010). Dopaminergic neurons release dopamine not only from axon terminals, but also in somatodendritic areas in the midbrain (Geffen et al., 1976; Kalivas et al., 1989). Locally released dopamine can bind to D₂ type autoreceptors to inhibit dopamine

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neuron impulse activity through activation of G-protein-coupled potassium (GIRK) channels (Lacey et al., 1987; Pucak and Grace, 1994; Beckstead et al., 2004). This unique form of neurotransmission can be studied in single neurons by electrically evoking dopamine-mediated IPSCs (D₂-IPSCs; Beckstead et al., 2004). Our previous work suggests that this form of synaptic transmission is subject to multiple forms of plasticity, including a stimulation-induced long-term depression of dopaminergic neurotransmission (LTD_{DA}) that can increase dopaminergic neuron excitability (Beckstead and Williams, 2007).

Neurotensin (NT) is a tridecapeptide that is expressed in diverse regions throughout the brain. NT modulates dopaminergic function, and dysregulation of NT signaling is associated with dopaminergic pathologies (Boules et al., 2013). Elevated levels of NT in the midbrain promote motivated behavior, and NT receptor activation in the ventral tegmental area (VTA) is required to induce behavioral sensitization to amphetamine (Panayi et al., 2005; Kempadoo et al., 2013). Although NT can produce long-lasting effects on behavior (Rompré, 1997) the cellular mechanisms responsible for these effects are not well understood.

Midbrain dopaminergic neurons express G-protein-coupled neurotensin type 1 and 2 receptors (NTS1/2; Vincent et al., 1999) as well as the non-G-protein-coupled NTS3 receptor (Sarret et al., 2003; Xia et al., 2013). Although dopamine neurons receive strong input from NT-positive fibers (Geisler and Zahm, 2006), NT-like immunoreactivity is also observed within dopaminergic neurons themselves, suggesting that NT may also produce local, somatodendritic effects (Hökfelt et al., 1984). Consistent with behavioral stimulation, application of NT to the midbrain increases dopaminergic neuron firing and enhances terminal dopamine release (Okuma et al., 1983; Hétier et al., 1988; Fawaz et al., 2009). One likely mechanism for the excitatory action of NT on these neurons is through a decrease in function of D₂ dopamine autoreceptors. Exogenous NT application induces transient, protein kinase C (PKC)-dependent internalization of D₂ receptors when expressed in HEK cells (Thibault et al., 2011), and NT-induced enhancement of dopamine overflow in the nucleus accumbens is prevented by D₂ receptor blockade (Fawaz et al., 2009). However, the effects of NT on the presynaptic and postsynaptic determinants of dendrodendritic dopamine neurotransmission have not been studied.

Here we describe the effects of NT on dopamine autoreceptor-mediated IPSCs in midbrain dopaminergic neurons. Exogenous application of the active peptide fragment NT_{8–13} depressed D₂-IPSCs by reducing dopamine release, providing a mechanism that could explain long-lasting behavioral effects of this modulatory neuropeptide. Additionally, both NT-induced and electrically induced depression required calcineurin and NTS2 receptor activity. These results suggest that locally released NT can produce persistent adaptations in dopaminergic synaptic transmission.

Materials and Methods

Animals. Male DBA/2J mice were either purchased from The Jackson Laboratory or were the first generation offspring of previously purchased mice. Animals were group-housed under a reversed light/dark cycle (lights off from 9:00 A.M. to 7:00 P.M.). Humidity and temperature were controlled in the housing facility, and food and water were available *ad libitum*. All experiments were reviewed and approved by the Animal Care and Use Committees at the respective institutions.

Brain slice electrophysiology. On the day of the experiment, mice (postnatal days 37–70) were anesthetized with isoflurane and immediately decapitated. The brains were quickly extracted and placed in ice-cold carboxygenated (95% O₂ and 5% CO₂) artificial CSF (ACSF) containing

the following (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 2.4 CaCl₂, 1.4 NaH₂PO₄, 25 NaHCO₃, and 11 D-glucose. Kynurenic acid (1 mM) was added to the buffer that was used for the slicing procedure. Horizontal midbrain slices (200 μm) containing the substantia nigra pars compacta (SNc) and VTA were obtained using a vibrating microtome (Leica). Slices were incubated for at least 25 min at 34–36°C with carboxygenated ACSF that also contained the NMDA receptor antagonist MK-801 (10 μM).

Slices were placed in a recording chamber attached to an upright microscope (Nikon Instruments) and maintained at 34–36°C with ACSF perfused at a rate of 1.5 ml/min. Dopaminergic neurons were visually identified based on their location in relation to the midline, third cranial nerve, and the medial terminal nucleus of the accessory optic tract. Neurons were further identified physiologically by the presence of spontaneous pacemaker firing (1–5 Hz) with wide extracellular waveforms (>1.1 ms) and a hyperpolarization-activated current (*I_H*) of >100 pA. Recording pipettes (1.7–2.7 MΩ resistance) were constructed from thin wall capillaries (World Precision Instruments) with a PC-10 puller (Narishige International). Ionophoretic pipettes (100–150 MΩ) were made with a P-97 puller (Sutter Instruments). Whole-cell recordings were obtained using an internal solution containing the following (in mM): 115 K-methylsulfate, 20 NaCl, 1.5 MgCl₂, 10 HEPES, 2 ATP, 0.4 GTP, and either 10 BAPTA or 0.025 EGTA, pH 7.35–7.40, 269–272 mOsm. In our initial experiments, we found no effect of calcium chelation on the effects of NT_{8–13} either in the control condition or when slices were incubated with NT receptor antagonists, and thus these data were pooled. Afterward, all experiments were performed using an intracellular solution containing 0.025 mM EGTA.

D₂-IPSCs were evoked during voltage-clamp recordings (holding voltage, −55 mV) using electrical stimulation in the presence of the following receptor antagonists: picrotoxin (100 μM, GABA_A), CGP55845 (100 nM, GABA_B), DNQX (10 μM, AMPA), and hexamethonium (100 μM, nAChR). A bipolar stimulating electrode was inserted into the slice 50–200 μm caudal to the cell, and D₂-IPSCs were evoked with a train of three to five stimulations (0.5 ms) applied at 80–100 Hz once every 50 s. In some experiments, long-term depression of D₂-IPSCs was induced by low-frequency stimulation (LFS; 0.5 ms, 2 Hz, 300 s; Beckstead and Williams, 2007). Outward GIRK channel-mediated currents were also evoked by iontophoresis of dopamine (1 M in the pipette). Leakage from the iontophoretic pipette was prevented by application of a negative backing current, and dopamine was ejected as a cation with application of +150–220 nA for 30–200 ms with an ION-100 single-channel iontophoresis generator (Dagan Corporation). A five-trace stable baseline was obtained before bath perfusion of NT (10, 30, 100, or 300 nM for the concentration-response experiment, 100 nM for all following experiments) for 5 min, followed by at least 10 min of washout. Brain slices were preincubated in ACSF containing cyclosporin A (1 μM), SR48692 (1 μM), or SR142948 (1 μM) for the calcineurin-inhibition and NT receptor antagonist experiments, and in two experiments SR142948 was also bath perfused. For the PKC inhibition experiment, staurosporine (1 μM) was bath perfused for 10 min before coapplication of NT (100 nM) and staurosporine (1 μM).

Fast-scan cyclic voltammetry. Dopamine release was measured by fast-scan cyclic voltammetry (FSCV; Ford et al., 2010). Data were acquired using TarHeel software paired with custom hardware and analyzed with Axograph X (Axograph Scientific). Glass-encased carbon fiber electrodes (34–700, Goodfellow) with an exposed diameter of 7 μm were cut to an exposed length of 60–80 μm. Before use, the exposed carbon fiber tip was soaked in isopropanol purified with activated carbon for at least 20 min. The tip of the fiber was placed directly into the VTA ~50 μm below the surface of the slice. While holding the fiber at −0.4 V, a triangular waveform (−0.4 to 1.3 V, vs Ag/AgCl at 400 V/s) was applied at 10 Hz. Dopamine release was evoked by a monopolar glass stimulating electrode placed ~100 μm away from the fiber. The time course of dopamine was determined by plotting the peak oxidation potential versus time. When stimulating, recordings were made 5 min apart to prevent rundown of the signal. After experimentation, each electrode was calibrated against known concentrations of dopamine. FSCV data were collected in the presence of picrotoxin (100 μM), CGP55845 (200 nM), and DNQX (10

Figure 1. NT₈₋₁₃ induces a depression of dopamine synaptic currents that is apparently NTS2 dependent. **A**, D₂-IPSCs were recorded in dopaminergic neurons in the ventral midbrain, and bath perfusion of NT produced a sustained decrease in current amplitude. Sample traces. Left, D₂-IPSC before (black) and after (orange) perfusion of D₂ type receptor antagonist sulpiride (150 nM). Right, D₂-IPSC before (black) and after (dark blue) bath perfusion of NT₈₋₁₃ (100 nM). **B**, Bath perfusion of the active fragment NT₈₋₁₃ (100 nM, 5 min) induced a sustained depression of dopamine-mediated synaptic currents that persisted for the remainder of the patch-clamp recording. Horizontal bars indicate time points analyzed in **C** and **G**. **C**, The relationship between response and the log [dose] of NT₈₋₁₃ 12–15 min after washout (horizontal bar in **B**) was fitted with a sigmoidal curve to indicate an EC₅₀ value of 33.0 nM. **D**, NT-induced depression in cells from the SNc is prevented by preincubation of the brain slice with the nonselective NTS1/2 receptor antagonist SR142948 (1 μ M, red), but not by the NTS1-selective antagonist SR48692 (1 μ M, green). **E**, NT₈₋₁₃ (100 nM) induced depression in both the SNc and the VTA, and in both regions is blocked by preincubation with the NTS1/2 antagonist SR142948. Horizontal bar in **D** indicates the points that were sampled for summary data. **F**, Application of 10–300 nM NT₈₋₁₃ induced a depression of dopamine iontophoresis-mediated outward currents that did not persist over time. The bar indicates points that were analyzed for summary data in **G**. Inset, Sample trace showing a D₂-mediated outward current evoked by dopamine iontophoresis before (average of minutes 1–4, black) and after (average of minutes 11–14, blue) NT₈₋₁₃ (100 nM) bath perfusion. **G**, Concentration response (10–300 nM) of immediate NT₈₋₁₃-induced depression (horizontal bars in **B**, **F**) of D₂-mediated currents evoked by either electrical stimulation (IPSCs) or iontophoresis of dopamine. **H**, Bath perfusion of the NTS1/2 receptor antagonist SR142948 (1 μ M) after washout of NT₈₋₁₃ (100 nM) caused IPSC amplitudes to return to baseline levels. **p* < 0.05.

Figure 2. NT₈₋₁₃ application decreases somatodendritic dopamine release. **A**, Sample trace of paired-pulse stimulation (2 trains of 5 stimulations applied at 100 Hz, 2 s intertrain interval; Beckstead et al., 2007) before and immediately after NT₈₋₁₃ (100 nM) bath perfusion. **B**, Paired-pulse ratios, calculated as amplitude of peak 2/amplitude of peak 1, were increased after bath perfusion of NT₈₋₁₃ (100 nM). **C**, Left, Sample FSCV trace at baseline (black) and 5 min after NT₈₋₁₃ (100 nM, blue) washout. Right, Sample FSCV trace at baseline (black) and 5 min after NT₈₋₁₃ (100 nM, red) washout made in the presence of the NTS1/2 antagonist SR142948 (1 μ M). Inset, A cyclic voltammogram indicating oxidation and reduction peaks consistent with a dopamine signal. **D**, Time course of NT₈₋₁₃ effects on peak [DA]₀. The gray box denotes the time of NT₈₋₁₃ application. Blue circles denote recordings performed in sulpiride (200 nM) only; red circles denote recordings performed in both sulpiride (200 nM) and SR142948 (1 μ M), timing indicated by red bar. **E**, Percentage change in peak [DA]₀ measured from 5 to 10 min after NT₈₋₁₃ washout. NT₈₋₁₃ reduced the peak amplitude of [DA]₀ by $16 \pm 3\%$ compared to $3 \pm 1\%$ when in the presence of antagonist SR142948; $p = 0.004$. * $p < 0.05$; ** $p < 0.01$.

μ M), and slices were incubated in MK-801 (10 μ M) for 1 h before recording. Dopamine release was evoked by a five-stimulation train (0.5 ms pulses; 40–60 μ A; 100 Hz) applied via a monopolar glass stimulating electrode placed ~ 100 μ m away from the fiber. Cyclic voltammogram currents were obtained by subtracting the average of 10 voltammograms obtained before stimulation from each voltammogram obtained after stimulation.

Drugs. Kynurenic acid, MK-801, DNQX, dopamine, sulpiride, hexamethonium, and picrotoxin were purchased from Sigma-Aldrich. Taurine, cyclosporin A, SR48692, SR142948, and CGP55845 were purchased from Tocris Bioscience. The active fragment NT₈₋₁₃ was purchased from American Peptide Company.

Statistical analyses. Data were collected on a Macintosh computer (Apple) using Axograph version 1.3.5 (Axograph Scientific) and LabChart

(AD Instruments). One-way or two-way ANOVAs or Student's *t* tests were used for between- and within-group comparisons. Tukey's *post hoc* tests were performed subsequent to significant ANOVAs. Data are presented as mean $\pm$ SEM. In all cases, α was set a priori at 0.05.

Results

NT₈₋₁₃ induces sustained depression of D₂-IPSCs

We performed patch-clamp recordings of SNc and VTA dopamine neurons to determine the effect of NT receptor activation on dopamine autoreceptor-mediated neurotransmission (Beckstead et al., 2004). Consistent with previous reports, exogenous application of the active peptide fragment NT₈₋₁₃ (100 nM) induced a modest inward current in dopaminergic neurons of the SNc during bath perfusion (Wu et al., 1995; Jomphe et al., 2006; data not shown). Additionally, a 5 min application of NT₈₋₁₃ induced depression of D₂-IPSCs that persisted for as long as we could maintain the patch-clamp recording (Fig. 1A,B). Unlike previously reported electrically evoked LTD_{DA} (Beckstead and Williams, 2007), the NT₈₋₁₃-induced depression was not blocked by chelating calcium in the neuron that was being recorded (average of minutes 15–25; chelator in intracellular solution, 0.025 mM EGTA, $36.0 \pm 8.45\%$ reduction; 10 mM BAPTA, $51.9 \pm 3.86\%$ reduction; $t_{(15)} = 1.78$; $p = 0.094$); thus, data from these experiments were pooled. Dose-response analysis using a sigmoidal curve fit indicated an EC₅₀ value of 33.0 nM for NT₈₋₁₃ (Fig. 1C). Since dopaminergic neurons express both NTS1 and NTS2 receptors, we sought to determine whether one of these subtypes was responsible for NT₈₋₁₃-induced plasticity. The persistence of depression was not eliminated by preincubation of brain slices with the NTS1 antagonist SR48692 (1 μ M, $31.4 \pm 5.03\%$ reduction), but was blocked by preincubation with the mixed NTS1/2 antagonist SR142948 (1 μ M; $-1.93 \pm 3.88\%$ reduction) consistent with an NTS2-dependent effect (Fig. 1D). A one-way ANOVA on normalized IPSC amplitudes, measured as the average of minute 22–25 within single cells from the SNc, revealed a significant effect of

treatment on the persistence of the depression ($F_{(2,17)} = 8.39$; $p = 0.0029$), and Tukey's *post hoc* analysis indicated a role for NTS2 receptors in the plasticity (SR142948 compared to controls, $p < 0.001$; compared to SR48692, $p < 0.05$). Additionally, NT₈₋₁₃ produced a short-lived reduction in IPSC amplitude even in the presence of the antagonists, suggesting an effect independent of NTS1/2 receptors. To determine whether NT-induced plasticity was specific to the SNc, we also obtained recordings of D₂-IPSCs in the VTA and attempted to block NT effects with the NTS1/2 antagonist SR142948 (1 μ M). Recordings from the VTA yielded results very similar to those obtained in the SNc (Fig. 1E; average of minutes 22–25;

$t_{(4)} = 6.19$; $p = 0.0035$). The results suggest that NT₈₋₁₃ can induce an NTS2-dependent depression of D₂-IPSCs in both the SNc and the VTA.

To determine whether NT₈₋₁₃-induced depression of D₂-IPSCs was due to changes in postsynaptic D₂ autoreceptor signaling, we next elicited D₂ autoreceptor-mediated outward currents in the SNc with episodic (once every 50 s) iontophoresis of exogenous dopamine. Bath perfusion of NT₈₋₁₃ (10–300 nM) induced depression of these currents immediately following perfusion; however, in this case, the effect recovered toward baseline after perfusion of the drug ceased (Fig. 1F). The reduction in normalized current amplitudes exhibited a very similar concentration response at time points early in the washout when compared to the reduction in IPSC amplitude (Fig. 1G). Bath perfusion of the nonselective NTS1/2 antagonist SR142948 alone (1 μ M for 8–10 min) in the absence of NT₈₋₁₃ did not substantively affect normalized D₂-IPSC amplitudes ($114 \pm 13.4\%$ of baseline amplitude; $t_{(7)} = 1.33$; $p = 0.23$; data not shown). Surprisingly however, bath perfusion of the NTS1/2 receptor antagonist SR142928 (1 μ M) immediately following NT₈₋₁₃ (100 nM) caused recovery of the depression (Fig. 1H), suggesting a role for sustained NT receptor activation by a brief exposure to NT₈₋₁₃. Overall, these data indicate that NT₈₋₁₃ produces a reduction of D₂-IPSC amplitudes that has two phases: an early phase whose locus is postsynaptic and a late phase whose persistence depends on NTS2 receptor-activation and could be expressed presynaptically.

NT₈₋₁₃ decreases somatodendritic release of dopamine

Our early experiments indicated that NT₈₋₁₃ persistently depressed dopamine currents only when they were elicited by release of endogenous dopamine and not by dopamine iontophoresis. We next sought to more directly determine whether NT₈₋₁₃ decreases dopamine release. We first tested this by measuring paired-pulse ratios, which generally exhibit a negative correlation with neurotransmitter release probability. Paired-pulse ratios of D₂-IPSCs were calculated as the amplitude of peak 2 divided by the amplitude of peak 1 (two trains of five stimulations applied at 100 Hz, 2 s intertrain interval; Beckstead et al., 2007). A paired t test revealed a significant increase in paired-pulse ratio immediately after perfusion of NT₈₋₁₃ (average of first 4 min after perfusion; $t_{(6)} = 2.59$; $p = 0.041$; Fig. 2A,B) and 10–15 min after wash ($t_{(6)} = 2.64$; $p = 0.046$). This finding is consistent with a presynaptic effect of NT and possibly a reduction in dopamine release.

The effect of NT₈₋₁₃ on dopamine release in the midbrain was more directly assessed using FSCV. Dopamine release was evoked by trains of electrical stimulation (five stimulations at 100 Hz), and the peak concentration of the resulting extracellular dopamine transient ([DA]_O) was measured with a carbon fiber electrode placed into the VTA. For these experiments, sulpiride (200 nM) was included in the bath solution to remove potentially confounding effects of presynaptic D₂ receptors. After achieving a

Figure 3. NT₈₋₁₃-induced depression requires activation of the protein phosphatase calcineurin. **A**, NT₈₋₁₃-induced depression (blue) was blocked by preincubation of brain slices with the calcineurin inhibitor cyclosporin A (1 μ M, orange). **B**, Summary averages were determined for the time points indicated by the horizontal bar in **A**. NT₈₋₁₃-induced plasticity was abolished in slices that had been incubated with cyclosporin A ($p = 0.0078$). **C**, Incubation of brain slices with cyclosporin A (1 μ M) did not block NT₈₋₁₃-induced (100 nM) short-term depression of D₂ receptor-mediated outward currents activated by iontophoresis of dopamine. **D**, Summary averages were determined for the time points indicated by the horizontal bar in **C**. Preincubation of slices with cyclosporin A actually produced a slight increase in depression during early washout ($p = 0.04$). * $p < 0.05$; ** $p < 0.01$.

stable baseline, NT₈₋₁₃ (100 nM) was applied for 5 min and then washed out. Paired t tests revealed a significant reduction of peak [DA]_O between baseline (47 ± 3 nM) and after NT₈₋₁₃ application (measured at 5 and 10 min after washout; 39 ± 3 nM; $t_{(6)} = 4.93$; $p = 0.0026$). This reduction in peak [DA]_O was observable for at least 15 min after NT was washed out. When slices were pretreated with the nonselective NTS1/2 antagonist SR142948 (1 μ M), no significant effect on the peak [DA]_O by NT₈₋₁₃ (100 nM) was observed (baseline, 42 ± 6 nM; NT + SR142948, 41 ± 6 nM; $t_{(6)} = 1.55$; $p = 0.17$; Fig. 2C–E). Thus, treatment with NT₈₋₁₃ significantly reduced the peak [DA]_O measurable by FSCV by $16 \pm 3\%$ (versus $3 \pm 2\%$ in SR142948; $p = 0.002$). In a separate experiment with no sulpiride present, we again observed a small but significant decrease in peak [DA]_O in response to 100 nM NT₈₋₁₃ ($13 \pm 3\%$ reduction from baseline; $t_{(11)} = 3.043$; $p = 0.0075$; data not shown). The application of NT₈₋₁₃ thus significantly lowered extracellular dopamine levels in a small but reproducible manner, suggesting that NT₈₋₁₃ decreases somatodendritic dopamine release in the midbrain.

NT₈₋₁₃-induced depression requires activation of the protein phosphatase calcineurin

Application of NT to midbrain dopamine neurons increases intracellular levels of calcium and enhances calcium/calmodulin signaling (Kasckow et al., 1991; St-Gelais et al., 2004). Concurrent binding of calcium and calcium/calmodulin fully activates the protein phosphatase calcineurin, which at glutamatergic synapses decreases presynaptic release and induces LTD (Perrino et al., 1995; Groth et al., 2003). We thus tested whether calcineurin was also responsible for NT₈₋₁₃-induced dopaminergic synaptic plasticity. Continuous incubation of slices with the calcineurin inhibitor cyclosporin A (1 μ M) prevented the NT₈₋₁₃-induced

Figure 4. Low-frequency stimulation-induced LTD_{DA} requires the activation of NTS2 and the protein phosphatase calcineurin. **A**, As reported previously (Beckstead and Williams, 2007), LFS (0.5 ms, 2 Hz for 300 s) induced a persistent depression of D₂-IPSCs (black). Preincubation of brain slices with the nonselective NTS1/2 receptor antagonist SR142948 (1 μ M, red) prevented induction of LTD_{DA}, whereas no effect of the NTS1-selective antagonist SR48692 (1 μ M, green) was observed. **B**, Summary averages were determined for the time points indicated by the horizontal bar in **A**. The average reduction in IPSC amplitude was significantly different for cells that had been incubated with SR142948 (1 μ M; red) versus SR48692 (1 μ M; green; $p < 0.01$) and controls (black; $p < 0.001$). **C**, LFS-induced LTD_{DA} (black) was blocked by preincubation of brain slices in the calcineurin inhibitor cyclosporin A (1 μ M; orange). **D**, Summary averages were determined for the time points indicated by the horizontal bar in **C**. The average reduction of IPSC amplitude was significantly lower in slices that were incubated in cyclosporin A (orange; $p = 0.007$). **E**, The amplitude of D₂-IPSCs returned to baseline levels when the NTS1/2 antagonist SR142948 was applied after induction of LTD. **F**, Summary averages were determined for the time points indicated by the horizontal bar in **E**. The average reduction was significantly lower in slices that had been exposed to SR142948 (red; $p = 0.012$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

reduction of D₂-IPSCs (minutes 22–25, control, $32.9 \pm 8.44\%$; cyclosporin A, $-19.0 \pm 13.9\%$; $t_{(9)} = 3.4$; $p = 0.0078$; Fig. 3A, horizontal bar, B). We next used iontophoresis to determine whether cyclosporin A also affects the transient NT_{8–13}-mediated depression of autoreceptor currents induced by dopamine iontophoresis. Continuous incubation of brain slices with cyclosporin A did not inhibit the early reduction of normalized D₂ currents; in fact, an unpaired t test indicated a slight increase in amplitude of the depression at early time points immediately after washout (control, $53.3 \pm 7.18\%$; cyclosporin A, $76.7 \pm 4.01\%$; $t_{(8)} = 2.45$; $p = 0.04$; Fig. 3C, horizontal bar, D). This suggests that calcineurin is unlikely

to play a postsynaptic role in neurotensin-induced depression, but is more likely involved in the persistent presynaptic effects of NT_{8–13}.

LTD_{DA} induced by low-frequency electrical stimulation also requires calcineurin and prolonged activation of NT receptors

We reported previously that LFS-induced LTD_{DA} can be blocked by postsynaptic calcium chelation (Beckstead and Williams, 2007). However, our present observations indicate that prolonged NT_{8–13}-induced depression is predominantly expressed presynaptically and is not blocked by chelating calcium in the postsynaptic neuron. To determine whether the two forms of depression share overlapping mechanisms, we next used LFS (2 Hz stimulation, 300 s) to induce LTD_{DA} in slices that were incubated with NTS1 or NTS1/2 antagonists. We found that LFS-induced LTD_{DA} was abolished in slices that were preincubated with the NTS1/2 receptor antagonist SR142948, but not the NTS1-selective antagonist SR48692 (Fig. 4A). A one-way ANOVA revealed a significant effect of treatment on LTD_{DA} ($F_{(2,14)} = 0.48$; $p = 0.0005$; Fig. 4B). Tukey's *post hoc* analysis showed a significant difference in depression of D₂-IPSCs in cells from slices that were incubated with SR142948 ($-3.85 \pm 11.2\%$ reduction), compared to controls ($53.5 \pm 4.87\%$ reduction; $p < 0.001$) and compared to cells from slices that were incubated with SR48692 ($51.1 \pm 8.94\%$ reduction; $p < 0.01$). This suggests that LFS-induced LTD_{DA} requires NTS2 activation, and endogenous NT mediates this form of plasticity.

We next tested whether LFS-induced LTD_{DA} also requires activation of calcineurin, similar to NT_{8–13}-induced depression, by preincubating brain slices in the calcineurin inhibitor cyclosporin A (1 μ M). Indeed, an unpaired t test revealed a significant effect of treatment on normalized D₂-IPSC amplitude (minutes 22–25, control, $53.5 \pm 4.87\%$ reduction; cyclosporin A, $9.43 \pm 11.0\%$ reduction; $t_{(6)} = 4.01$; $p = 0.0071$; Fig. 4C, horizontal bar, D). Finally, we tested whether the nonselective NT receptor antagonist SR142948 applied after the LFS protocol could reverse electrically evoked LTD_{DA}. An unpaired t test revealed a significant antagonist-induced decrease in reduction (minutes 22–25, control, $37.9 \pm 4.74\%$ reduction; SR142948, $-8.34 \pm 13.9\%$ reduction; $t_{(16)} = 2.85$; $p = 0.0116$; Fig. 4E, horizontal bar, F), suggesting persistent activation of NT receptors in the absence of high concentrations of the peptide. The similarities between the two forms of plasticity combined with the calcium dependence of electrically induced LT-

D_{DA} suggest that bath-perfused NT may bypass an early calcium-dependent step in the induction of LTD_{DA}.

NT₈₋₁₃-induced short-term depression of outward currents is PKC dependent

NT transiently desensitizes D₂ receptors through PKC-mediated phosphorylation when tested in a HEK cell expression system (Thibault et al., 2011). We finally sought to determine whether PKC is also responsible for the short-term depression of autoreceptor-mediated currents produced by NT₈₋₁₃. An unpaired *t* test revealed that incubation of slices with the PKC-inhibitor staurosporine significantly decreased NT₈₋₁₃-induced short-term depression (measured 1–4 min into washout) of outward currents evoked with dopamine iontophoresis (control, 55.3 ± 8.22% reduction; staurosporine, 26.2 ± 9.19% reduction; *t*₍₈₎ = 2.36; *p* = 0.046; Fig. 5A, horizontal bar, B). Consistent with previously published work, this indicates that the short-term, postsynaptic depression of autoreceptors is mediated at least in part by PKC.

Discussion

NT receptor-dependent depression of D₂-IPSCs

Previous work has established multiple roles for NT in the ventral midbrain, which is densely innervated by NT-positive fibers (Jennes et al., 1982; Geisler and Zahm, 2006). In the VTA, NT is behaviorally reinforcing, as it induces hyperactivity, supports self-administration, produces conditioned place preference, and affects intracranial self-stimulation responding (Kalivas et al., 1983; Glimcher et al., 1984, 1987; Rompré, 1995). Consistent with this, dopaminergic neurons in both the SN and the VTA express receptors for, and are excited by, NT (Szigethy and Beaudet, 1989; Stowe and Nemeroff, 1991). High concentrations of NT directly depolarize dopaminergic neurons through a nonselective cation conductance that is IP₃ receptor dependent (Wu et al., 1995; St-Gelais et al., 2004). Low concentrations of NT blunt the ability of D₂ dopamine autoreceptor agonists to inhibit neuron firing (Shi and Bunney, 1991; Nalivaiko et al., 1998), but this effect is not IP₃ receptor dependent (Jomphe et al., 2006). Work in isolated and cultured dopaminergic neurons suggests that effects on autoreceptors are blocked by chelating intracellular calcium and are PKC dependent (Wu and Wang, 1995; Jomphe et al., 2006). PKC and NT modulate the reversal of dopamine-induced inhibition of firing of VTA dopamine neurons (Nimitvilai et al., 2012, 2013), and NTS1 receptor activation can cause internalization of D₂ receptors through PKC and β -arrestin 1 (Thibault et al., 2011).

We extended this work by determining the effects of NT on D₂-IPSCs, which provide a real-time measure of the physiological consequences of endogenous dopamine release. We found that NT induces depression of D₂-IPSCs in dopamine cells of the SNc and VTA, and requires activation of NTS2 receptors. In the SNc, NTS2 is mostly expressed on neuronal processes rather than at the cell bodies (Sarret et al., 2003), placing them in an ideal position to modulate dendrodendritic neurotransmission. Increased firing of dopaminergic neurons has been attributed previously to the type 1 receptor (St-Gelais et al., 2004), suggesting that NTS1 and NTS2 receptors maintain distinct physiological roles in do-

Figure 5. Transient NT₈₋₁₃-induced depression of autoreceptor-mediated currents is mediated by protein kinase C. **A**, Preapplication of the PKC inhibitor staurosporine (1 μ M, white) significantly decreased NT₈₋₁₃-induced depression of currents evoked by iontophoresis. **B**, Reduction in normalized amplitude (percentage) was determined for the time points indicated by the horizontal bar in **A**. Staurosporine significantly decreased the transient NT₈₋₁₃-induced reduction in current amplitude (control, blue; staurosporine, white; *p* = 0.046). **p* < 0.05.

pamine neurons. We should note that NT₈₋₁₃ induced an immediate depression of D₂-IPSCs in SNc slices that had been incubated in the NTS1/2 receptor antagonist. This could be mediated by the non-G-protein-coupled NTS3 receptor. Substantial immunoreactivity for NTS3 has been reported in cell bodies and dendrites of tyrosine hydroxylase-positive neurons in the SNc, and to a lesser degree in the SN pars reticulata and VTA (Sarret et al., 2003; Xia et al., 2013). Future studies with receptor knock-outs and/or more selective antagonists will be needed to further elucidate the roles of the distinct receptors.

Application of the NTS1/2 receptor antagonist SR142948 either during washout of NT₈₋₁₃ or after LFS surprisingly reversed depression of D₂-IPSCs (Fig. 1H,E). Whereas some studies suggest that exogenously applied NT has transient effects on dopaminergic neurons that reverse upon washout (Wu et al., 1995; Nalivaiko et al., 1998; Jomphe et al., 2006; Fawaz et al., 2009), prolonged effects on glutamatergic neurotransmission have also been reported (Kortleven et al., 2012; Kempadoo et al., 2013). The persistent effects of both endogenous NT and exogenous NT₈₋₁₃ could indicate that brief agonist exposure produces persistent receptor activation through an undetermined mechanism and that SR142948 exhibits inverse agonist properties. It could also indicate slow unbinding of the agonist, which would be unexpected given the slow but consistent washout of NT₈₋₁₃ effects on iontophoresis (Fig. 1F) and the relatively low affinity of NTS2 receptors (Mazella et al., 1996). Regardless of the receptor mechanisms at work, the data are consistent with a physiological role for persistent plasticity of dendritic dopamine synapses in response to brief exposure to a high concentration of NT.

Presynaptic effects of neurotensin on dopamine neurotransmission

We observed that NT₈₋₁₃ persistently reduced D₂ receptor-mediated currents when they were evoked by endogenous, but not exogenous, dopamine, suggesting that NT₈₋₁₃ can act presynaptically to reduce dopamine release. We also observed an increase in paired-pulse ratios immediately after NT₈₋₁₃ perfusion as well as after washout, consistent with a decrease in release probability. IPSC kinetics necessitated the use of a 2 s interval between pulse trains during these experiments. Although this is longer than typically used to study release of fast transmitters, we showed previously that this protocol induces a paired-pulse depression whose locus is presynaptic (Beckstead et al., 2007). Furthermore, the fact that NT₈₋₁₃ consistently increased this ratio is strong evidence of a presynaptic effect on neurotransmitter release.

We also confirmed using fast-scan cyclic voltammetry that NT_{8–13} decreases dopamine release. In the SNc, both serotonin and dopamine contribute to the electrochemical signal following electrical stimulation (John et al., 2006); thus, our electrochemical experiments were necessarily conducted in the VTA to obtain a pure dopamine signal. The NT_{8–13}-induced decrease in extracellular dopamine was smaller in amplitude than the decrease in amplitude of D₂-IPSCs. This could be due to the relatively slow temporal resolution of voltammetry (10 Hz) or other differences between the two techniques. For instance, when using FSCV, adsorption of dopamine occurs on the carbon fiber between stimulations, whereas no matching phenomenon occurs with postsynaptic receptors. Experimentally, the IPSC is stable when measured every 50 s, whereas stimulating at that frequency causes the FSCV signal to run down dramatically. Furthermore, the receptors underlying the D₂-IPSC are near dopamine release sites (<1 μ m) and encode dopamine transients that peak around 100 μ M and last tens of milliseconds. In contrast, carbon fiber FSCV requires synaptic overflow and extended diffusion resulting in a seconds-long signal that peaks between 100 and 200 nM dopamine (Ford et al., 2010; Courtney and Ford, 2014). Because FSCV and D₂-IPSCs measure dopamine at different points during its spatial-temporal diffusion, it is hard to correlate changes to the bulk dopamine transient measured by FSCV with changes in the synaptic transient of dopamine interacting with D₂ receptors during the IPSC (Ford et al., 2010; Courtney and Ford, 2014). Despite these limitations, the decreased extracellular dopamine measured in the presence and absence of sulpiride, but not in the presence of SR142948, indicates that NT_{8–13} causes a reduction in dopamine release.

Although speculation, the NT_{8–13}-induced decrease in dopamine release could be mediated through synapsin. NT increases levels of intracellular calcium and has been shown in the striatum to stimulate calcium/calmodulin, which activates calcineurin (Kasckow et al., 1991). At glutamatergic synapses, this protein phosphatase can dephosphorylate synapsin, which in its phosphorylated state allows trafficking of synaptic vesicles from the reserve to the readily releasable pool. Dephosphorylation of synapsin could thus prevent trafficking and decrease neurotransmitter release, resulting in synaptic depression (Hilfiker et al., 1999; Groth et al., 2003).

LTD_{DA} also requires activation of NTS2 and calcineurin

In the initial report of LTD_{DA} (Beckstead and Williams, 2007), we used low-frequency electrical stimulation to induce a persistent depression of D₂-IPSCs. At the time, we were unable to attribute the LTD to a specific cellular mechanism, and since application of a high concentration of dopamine mimicked the LTD, we postulated a role for postsynaptic receptor desensitization. Surprisingly, we find here that both NT- and LFS-induced depression can be blocked either with a calcineurin inhibitor or a NT receptor antagonist. The only difference experimentally between the two forms of depression is that calcium chelation blocks induction of plasticity by LFS, but not NT_{8–13}. We cannot definitively say what causes this; however, if we are indeed studying a unitary form of plasticity, then bath perfusion of NT_{8–13} can bypass an early step in LTD induction that requires postsynaptic calcium. Since postsynaptic calcium is typically required for retrograde neurotransmitter signaling, one intriguing possibility is that NT could act as a retrograde messenger that decreases presynaptic dopamine release. Although the mesencephalon receives strong NT input from diverse brain regions (Geisler and Zahm, 2006), NT-like immunoreactivity has been observed in dopamine neu-

rons themselves (Hökfelt et al., 1984). Stimulus-evoked NT release in rat brain tissue is calcium dependent (Iversen et al., 1978), and a previous study indicated that NT can act through a local circuit to affect GABA release in the bed nucleus of the stria terminalis (Krawczyk et al., 2013). Future work using cell type-specific approaches will determine the source of NT that can cause plasticity of D₂-IPSCs.

It is also possible that more classical retrograde messengers such as endocannabinoids could play a role in LTD_{DA}. Indeed, Δ^9 -tetrahydrocannabinol increases firing rate and burst firing of VTA and SNc dopamine neurons *in vivo* (French et al., 1997) by acting on the CB₁ receptor (Gessa et al., 1998). Furthermore, NT induces LTD of glutamatergic EPSCs in the striatum and in dopamine neurons in the VTA that can be blocked by the CB₁ antagonist AM 251 (Yin et al., 2008; Kortleven et al., 2012). We do not believe, however, that cannabinoid signaling is responsible for depression of D₂-IPSCs. Midbrain dopaminergic neurons do not express CB₁ receptors (Herkenham et al., 1990), and preincubation of brain slices with the CB₁ antagonist AM 251 (1 μ M) did not prevent NT-induced depression of D₂-IPSCs (immediate reduction, 43.7 $\pm$ 6.05%, n = 9; prolonged reduction, 43.6 $\pm$ 3.11%, n = 2; data not shown).

In summary, our results suggest that the transmitter peptide NT can induce a presynaptically expressed depression of D₂-IPSCs. The effects of NT on dopamine autoreceptor signaling provide a mechanism for a prolonged increase in dopaminergic output and could be important for pathologies of the mesencephalic dopamine system such as schizophrenia and drug abuse.

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