



Neurotensin inhibits glutamate-mediated synaptic inputs onto ventral tegmental area dopamine neurons through the release of the endocannabinoid 2-AG

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ABSTRACT

Neurotensin (NT), a neuropeptide abundant in the ventral midbrain, is known to act as a key regulator of the mesolimbic dopamine (DA) system, originating in the ventral tegmental area (VTA). NT activates metabotropic receptors coupled to Gq heterotrimeric G proteins, a signaling pathway often triggering endocannabinoid (EC) production in the brain. Because ECs act as negative regulators of many glutamate synapses and have also been shown recently to gate LTP induction in the VTA, we examined the hypothesis that NT regulates glutamate-mediated synaptic inputs to VTA DA neurons. We performed whole cell patch-clamp recordings in VTA DA neurons in TH-EGFP transgenic mouse brain slices and found that NT induces a long-lasting decrease of the EPSC amplitude that was mediated by the type 1 NT receptor. An antagonist of the CB1 EC receptor blocked this decrease. This effect of NT was not dependent on intracellular calcium, but required G-protein activation and phospholipase C. Blockade of the CB1 receptor after the induction of EPSC depression reversed synaptic depression, an effect not mimicked by blocking NT receptors, thus suggesting the occurrence of prolonged EC production and release. The EC responsible for synaptic depression was identified as 2-arachidonoylglycerol, the same EC known to gate LTP induction in VTA DA neurons. However, blocking NT receptors during LTP induction did not facilitate LTP induction, suggesting that endogenously released NT is not a major source of EC production during LTP inducing stimulations.

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1. Introduction

The 13 amino-acid peptide neurotensin (NT) is abundant in the mammalian nervous system. It was first discovered and isolated from the bovine hypothalamus in 1973 (Carraway and Leeman, 1973). A large body of work has highlighted NT as a key regulator of the midbrain DA system, and notably of ventral tegmental area (VTA) DA neurons (Nemeroff et al., 1982; Quirion, 1983; Hökfelt et al., 1984; Dana et al., 1989; Rostene et al., 1997; Binder et al., 2001). NT is recognized by at least three receptors, the most abundant being NTS1 and NTS2. NTS1, which has the highest affinity for NT, is a Gq-coupled receptor that is highly expressed by VTA DA neurons both in the somatodendritic and axonal compartments (a.o. Young and Kuhar, 1981), where it can act to alter DA neuron excitability and DA release. The possible

implication of NT in DA-associated illnesses such as schizophrenia and drug addiction has received significant attention (For review, see: Binder et al., 2001; St-Gelais et al., 2006).

NT induces an increase in intracellular calcium in cultured midbrain DA neurons acting through the NTS1 receptor and a downstream pathway implicating phospholipase C and cationic channel activation, which leads to an increased firing rate in these neurons (St-Gelais et al., 2004). Considering that activation of G-protein coupled receptors such as the orexin (Haj-Dahmane and Shen, 2005), mGluR1 (Maejima et al., 2001) and cholecystokinin receptors (Foldy et al., 2007) can lead to endocannabinoid (EC) production and release (for review, see (Gyombolai et al., 2011)), it may be reasonably hypothesized that NT-mediated signaling cascades also lead to the synthesis and release of ECs by VTA DA neurons. This hypothesis is reinforced by the recent demonstration that NT causes a decrease in evoked glutamate-mediated excitatory post-synaptic currents (EPSCs) in the striatum through EC release and retrograde activation of CB1 receptors (Yin et al., 2008).

Recently, we reported that ECs, in particular 2-arachidonoylglycerol, can gate the induction of long term

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potentiation (LTP) in DA neurons of the VTA (Kortleven et al., 2011). LTP in this area of the brain is otherwise known to occur after injection of cocaine (Ungless et al., 2001) and other drugs of abuse (Saal et al., 2003), and when inhibited in KO mice, causes alterations of drug-seeking behaviors (Engblom et al., 2008; Zweifel et al., 2008). Recent work has also revealed that LTP in non-dopaminergic neurons of the VTA is even more directly linked to such drug-seeking behaviors (Luo et al., 2010). Therefore, understanding the mechanisms of LTP in the VTA may prove to be critical to our understanding of drug addiction. Induction of LTP at glutamatergic synapses in VTA DA neurons *in vitro* has proven to be rather difficult. However, GABA_A and EC receptors have been identified recently as able to gate LTP of AMPA-mediated glutamatergic transmission in this structure (Liu et al., 2005; Luu and Malenka, 2008; Kortleven et al., 2011). Identification of the endogenous signals that lead to EC release by DA neurons and that may regulate LTP induction is required to fully understand the mechanisms of LTP induction at midbrain glutamatergic synapses, whether it is induced by physiological signals or by drugs of abuse. Here we examined the hypothesis that NT regulates EC release and that endogenously released NT acts as a negative regulator of LTP induced by a spike time dependent pairing protocol.

2. Material and methods

2.1. Animals

All experiments were approved by the animal ethics committee (CDEA) of the Université de Montréal. Animal discomfort and suffering was kept to an absolute minimum. Mice (P14–P21) of the TH-eGFP/21-31 line that express the enhanced green fluorescent protein (eGFP) gene under the control of the tyrosine hydroxylase (TH) promoter (Sawamoto et al., 2001) were used in electrophysiological experiments to select VTA DA neurons for recording (Jomphe et al., 2005).

2.2. Drugs

SR95531 (2 μ M) and AM251 (4 and 10 μ M) were obtained from Ascent Scientific (Bristol, UK). Tetrahydrolipstatin (2 μ M; intra-pipette) and U-73122 (10 μ M; intra-pipette) were from Calbiochem (San Diego, CA, USA). Neurotensin 8–13 (50 nM), BAPTA (20 mM; intra-pipette) and GDP- β S (1 mM; intra-pipette) were obtained from Sigma-Aldrich (St-Louis, MO, USA). Finally, SR48692 (500 nM) and SR142948A (500 nM) were supplied by Sanofi-Synthelabo (Paris, France).

2.3. Slice preparation

Mice were anaesthetized with halothane and immediately killed by decapitation. The brain was rapidly removed and placed in ice-cold carboxygenated (95% O₂ and 5% CO₂) cutting-solution (glycerol-containing artificial cerebrospinal fluid (G-ACSF)) containing (in mM): Glycerol (250), KCl (3.5), MgCl₂ (1.3), CaCl₂ (2.0), NaHPO₄ (1.2), glucose (11), NaHCO₃ (26), pH 7.35. Solutions in which NaCl is replaced with glycerol have been proposed to increase slice quality when compared to sucrose-containing cutting solutions (Ye et al., 2006). Horizontal slices (200–220 μ m) were cut in the same G-ACSF solution with a Leica VT1000S vibrating microtome (Leica Microsystems, Wetzlar, Germany) and placed in a slice-saver containing carboxygenated artificial cerebrospinal fluid (ACSF) at room temperature (around 21–23 °C). ACSF contained (in mM): NaCl (126), KCl (3.5), MgCl₂ (1.3), CaCl₂ (2), NaHPO₄ (1.2), glucose (11), NaHCO₃ (26), pH 7.35, 305 mOsm. Slices remained untouched in the slice-saver for at least 1 h before being transferred to the recording chamber which was continuously perfused with carboxygenated ACSF (2 ml/min) heated to 30 \pm 1 °C with a TC-324B in-line heating unit (Warner Instruments, Hamden, CT, USA).

2.4. Electrophysiology

A concentric bipolar Pt/Ir stimulating electrode (FHC, Bodoïn, ME, USA) was placed in the rostral VTA and recordings were started after an equilibration period of 20 min. Recording electrodes (4–6 M Ω) were made from borosilicate capillaries (WPI, FL, USA) with a PP-830 micro-pipette puller (Narishige, Tokyo, Japan). Electrodes were filled with a solution containing (in mM): potassium methylsulfate (145), NaCl (10), EGTA (0.1), MgATP (2), GTP (Tris salt) (0.6), HEPES (10), phosphocreatine (10), pH 7.35, 300 mOsm.

Whole cell current- and voltage-clamp recordings were performed using a MultiClamp 700B amplifier and PClamp 10 acquisition software (Molecular Devices, Sunnyvale, CA, USA). Signals were filtered at 2 kHz, digitized at 20 kHz using

a Digidata 1440A digitizer and analyzed with Clampex software (Molecular Devices). Drugs were bath-applied. The GABA_A receptor antagonist SR95531 (2 μ M) was added to the ACSF solution during all recordings, with the notable exception of LTP-induction recordings, as it is known that inclusion of GABA_A receptor antagonists facilitates the induction of LTP in the VTA (Liu et al., 2005; Luu and Malenka, 2008; Kortleven et al., 2011).

During voltage-clamp recordings, extracellular stimulation was performed at 0.1 Hz for 25 min. Excitatory post-synaptic currents (EPSCs) were recorded from eGFP-expressing neurons that were voltage-clamped at –70 mV. NT was applied after a baseline period of 5 min and application was maintained for 10 min, followed by a 10 min washout period. When other drugs were used, they were present at least 5 min before the start of the recordings. The series resistance was compensated at 50% immediately before the start of the recordings.

In LTP experiments, neurons were current-clamped at roughly –70 mV using the multi-clamp commander 700B in current clamp mode. A 10 min baseline period of stimulation-evoked EPSPs (0.1 Hz) was obtained before LTP-induction took place. The LTP induction protocol was a spike-time dependent pairing protocol initially described in Liu et al. (2005), which was also used by others to demonstrate EC-gated LTP induction in VTA DA neurons (Kortleven et al., 2011). Briefly, extracellular stimulations (10 Hz) were followed 5 ms later by a 3 ms intracellular current injection of 2 nA to induce an action potential in the recorded neuron, that was therefore paired with the evoked EPSP. Such a single pairing was performed 5 times per train at 10 Hz, for twenty trains, with each train separated by 5 s. After LTP induction, EPSPs were once again evoked at 0.1 Hz for 20 min to assess the effect of LTP induction on EPSP-amplitude. After the pairing protocol, EPSPs were monitored for 20 min to evaluate if LTP was induced. Although this recording duration does not allow to reveal the full duration of LTP, we have shown previously that in the presence of CB1 receptor blockade, LTP is induced and detectable well within this period (Kortleven et al., 2011). The bridge balance (current-clamp) was monitored throughout all LTP recordings and neurons displaying a change of more than 30% in the bridge balance value were discarded.

2.5. Immunohistochemistry and imaging

Mice of either 14 or 21 days old were anaesthetized with halothane and killed by decapitation. Their brains were removed immediately and fixed for at least 24 h in 4% PFA. Sections (50 μ m) were then made using a vibrating microtome and placed into phosphate buffered saline (PBS). These slices were incubated overnight with a rabbit anti-NT antibody (1:5000; Immunostar, Hudson, WI, USA) and a guinea pig type 1 vesicular glutamate transporter (VGLUT1) (1:5000) or a mouse type 2 vesicular glutamate transporter (VGLUT2) antibody (1:1000; both Millipore, Billerica, MA, USA). The specificity of the NT antibody has been previously demonstrated (Geisler and Zahm, 2006). For each mouse, sections were selected in alternating fashion for VGLUT1 or VGLUT2 immunostaining, assuring a roughly equal number of sections for each VGLUT antibody. Slices were then incubated for 2 h with an Alexa Fluor 546 fluorescent anti-rabbit secondary antibody (1:500) to reveal the NT primary antibody, and an Alexa Fluor 647 anti-guinea pig or an Alexa Fluor 647 anti-mouse secondary antibody (1:500) to visualize VGLUT1 and VGLUT2 primary antibodies, respectively. All fluorescent secondary antibodies were obtained from Molecular Probes through Invitrogen (Carlsbad, CA, USA).

Confocal images of these slices were obtained with a laser scanning confocal microscope (Fluoview FV1000, Olympus) equipped with an Olympus 60 \times oil-immersion objective (N.A. 1.42). An argon laser (488 nm wavelength) was used for excitation of eGFP. Fluorescence was collected after passing through a 500–530 nm band-pass emission filter. The 543 nm wavelength of a helium-neon laser was used to reveal NT-staining, with fluorescence collection occurring through a 555–625 nm band-pass emission filter. The 633 nm wavelength of a helium-neon laser was used to reveal the VGLUT1 and -2 staining, with fluorescence collection through a 655–755 nm band-pass emission filter.

2.6. Colocalization analysis

Two image stacks (5 images per stack, 5 μ m step size) were obtained from the VTA for each hemisphere (1 rostral, 1 caudal), for a total of 4 stacks per section, whenever possible. A threshold was applied and 3 images per stack were analyzed. This analysis consisted of a particle analysis, where signal with a surface between 0.5 and 5 μ m² was considered a presumed axon terminal. This was then used to count the number and percentage of terminals that showed colocalization of NT and VGLUT signals.

2.7. Statistical analysis

The amplitude of recorded EPSCs and EPSPs was measured using Clampex software (Molecular Devices). In voltage-clamp experiments, the 5 min baseline period was normalized to the average of the first two and a half min of this same period. In LTP recordings, the 10 min of the baseline period was normalized to the first 5 min of this same baseline period. This was done to control for any drift occurring during the baseline period. During voltage-clamp experiments evaluating the effects of NT of EPSC amplitude, the effect of NT was quantified between min

10–17, the period showing the maximal decrease of EPSC amplitude in experiments in which NT was applied alone. This period was normalized and compared to the average of the baseline period. To quantify the induction of LTP in the LTP experiments, the last 10 min of the recordings were normalized and compared to the average of the baseline period. Two-tailed paired *t*-tests were performed, where appropriate, using Graphpad Prism software (Graphpad Software, La Jolla, CA, USA). Results were considered statistically significant when $p < 0.05$. Values are presented as average \pm SEM. All averages are expressed as percentages of baseline values.

3. Results

3.1. NT is present in the VTA and colocalizes with VGLUT1 and VGLUT2

Previous work has shown that NT in the VTA is present in axonal-like varicosities that are in close proximity to VTA DA neurons (Hökfelt et al., 1984) and those are likely to be activated by extracellular stimulation typically used to trigger LTP. We first wished to evaluate if NT was preferentially associated with VGLUT1-positive glutamate terminals mostly originating from cortical structures, or with VGLUT2-positive terminals originating mostly from subcortical structures. NT-immunopositive axonal-like varicosities were found to be abundant in the VTA and NT-immunoreactive signal colocalized with both VGLUT1 and VGLUT2 in a subset of axon terminals (Fig. 1).

Colocalization analysis showed that NT, while equally distributed among VGLUT1 and VGLUT2 terminals at 14 days ($2.09 \pm 0.22\%$ vs $2.40 \pm 0.21\%$ respectively; $p = 0.33$), is present significantly more frequently in VGLUT2-positive terminals than VGLUT1-positive terminals at 21 days ($7.61 \pm 0.90\%$ vs $2.67 \pm 0.32\%$ respectively; $p < 0.0001$). The appearance of this relative segregation was associated with a significant increase in NT expression in VGLUT2 immunoreactive terminals with age ($2.40 \pm 0.21\%$ vs $7.61 \pm 0.90\%$; $p < 0.0001$), suggesting a continued development of NT expression in VGLUT2, but not VGLUT1-positive terminals in juvenile mice.

3.2. NT reduces EPSC amplitude through the NTS1 receptor

Prior to patch-clamp recording, DA neurons were identified by their expression of eGFP. We have previously found that in VTA slices of these TH-eGFP transgenic mice, 87% of eGFP positive neurons can be confirmed to be TH positive after post-recording immunohistochemistry (Kortleven et al., 2011). Bath application of NT (50 nM) induced a slowly-developing, long-lasting reduction in EPSC amplitude ($73.64 \pm 2.90\%$ of pre-application values; $N = 17$; $p < 0.00001$; Fig. 2A), which did not show a significant recovery within the following 10 min washout period. This decrease in EPSC amplitude was not the result of pre- or post-synaptic rundown, as confirmed by control experiments performed without NT ($103.37 \pm 7.42\%$; $N = 9$; $p = 0.59$; Fig. 2B). In parallel with the decrease in EPSC amplitude, NT caused a significant increase in membrane current fluctuation, revealed as an increase in current variance over time (Fig. S1A), which was likely due to the opening of non-selective cationic channels in response to NT (Jiang et al., 1994). No such change in current variance was observed in control experiments of the same duration performed without NT (Fig. S1B).

The NT-induced decrease in EPSC amplitude was blocked by pre-application of the broad spectrum NT receptor antagonist SR142948A (500 nM) ($96.23 \pm 2.25\%$; $N = 7$; $p = 0.69$; Fig. 2C), which also blocked the increase in membrane variance (Fig. S1C). The NTS1-selective antagonist SR48692 (500 nM) also blocked NT-induced EPSC depression ($95.00 \pm 7.82\%$; $N = 7$; $p = 0.53$; Fig. 2D), suggesting that the NTS1 receptor is mainly responsible for this effect of NT. However, SR48692 did not block NT-induced

membrane variance elevation (Fig. S1D), suggesting that the increase in membrane variance is independent from the mechanism leading to synaptic depression, and probably mediated by NTS2 instead of NTS1.

3.3. NT-induced EPSC depression is mediated by 2-arachidonoylglycerol through a calcium-independent pathway

Because NT has been shown to induce EC release in the striatum (Yin et al., 2008), we sought to verify the possibility that this occurs in the VTA as well, leading to CB1 receptor activation on glutamate terminals. In the presence of the CB1 receptor antagonist AM251 (4 μ M), NT failed to induce a significant decrease of EPSC amplitude ($96.83 \pm 6.69\%$; $N = 9$; $p = 0.93$; Fig. 3A). This strongly suggests that ECs are involved in the synaptic depression induced by NT. The elevation in membrane current variance induced by NT was unaffected by AM251 (Fig. S1E).

Since NT has been previously shown to cause an increase of intracellular calcium in cultured VTA DA neurons (St-Gelais et al., 2004) and intracellular calcium has been shown to be required for G-protein receptor mediated 2-AG release in the cerebellum and for EC release in the hippocampus through phospholipase C (PLC) activation (Hashimoto et al., 2005; Maejima et al., 2005), we hypothesized that this increase in calcium in response to NT is required to trigger EC production and release. We tested this hypothesis by including BAPTA (20 mM) in the recording pipette. In the presence of BAPTA, NT still induced a significant decrease of EPSC amplitude ($80.53 \pm 7.00\%$; $N = 12$; $p = 0.012$; Fig. 3B), which was not significantly different compared to the effect of NT alone ($p = 0.32$). This result suggests that intracellular calcium elevation is not required for EC production and synaptic depression induced by NT. Surprisingly, in neurons loaded with BAPTA, NT-induced membrane current variance was much larger compared to NT alone ($p = 0.0002$; Fig. S1F), thus suggesting that NT-induced cationic channel activation is actually under negative control by intracellular calcium. This result also otherwise provides a positive control for the effectiveness of cell loading with BAPTA.

It has previously been shown that EC release induced by orexin A in the dorsal raphe nucleus is not blocked by loading neurons with BAPTA. Instead, it was found to be mediated through a presumably direct G-protein-PLC interaction (Haj-Dahmane and Shen, 2005). To evaluate whether a similar mechanism is implicated in the VTA, we added the non-hydrolyzable GDP analog GDP- β S to the pipette solution and found that it effectively blocked NT-induced EPSC depression ($95.66 \pm 6.50\%$; $N = 5$; $p = 0.77$; Fig. 3C). NT-induced membrane current variance elevation was also eliminated (Fig. S1G), compatible with the idea that another G-protein coupled receptor, presumably NTS2, is responsible for this second effect of NT. The PLC inhibitor U-73122 (10 μ M; intra-pipette) similarly blocked the effects of NT on EPSC amplitude ($99.76 \pm 8.46\%$; $N = 6$; $p = 0.97$; Fig. 3D) and membrane current variance (Fig. S1H). Finally, in an effort to identify the specific EC involved, we used tetrahydrolipstatin (THL, 2 μ M, intra-pipette), an inhibitor of the diacylglycerol-lipases (DAG-L), of which the form DAG-L alpha is mainly responsible for the production of 2-arachidonoylglycerol (2-AG) (Gao et al., 2010; Tanimura et al., 2010). THL blocked NT-mediated EPSC depression ($93.45 \pm 6.46\%$; $N = 5$; $p = 0.35$; Fig. 3E) but not NT-mediated increase in membrane current variance (Fig. S1I). This result suggests that the predominant EC involved in NT-mediated CB1 receptor activation and EPSC depression is 2-AG.

A summary of all pharmacological experiments is presented in Fig. 3F.

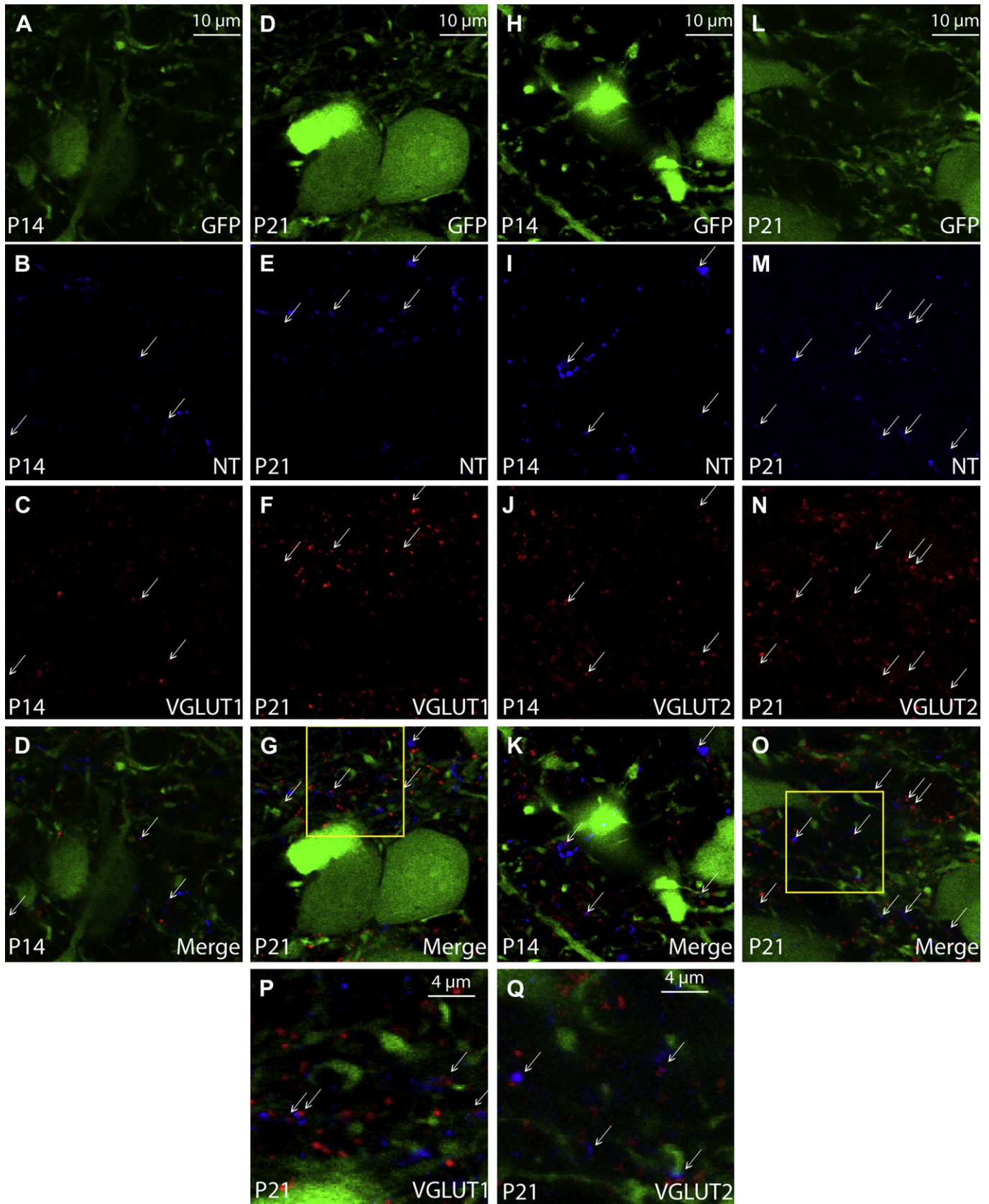


Fig. 1. A subset of glutamatergic terminals in the VTA expressing VGLUT1 and VGLUT2 contain neurotensin. **A.** Endogenous GFP signal in DAergic neurons in thin sections of the VTA of 14 days old TH-eGFP mice. **B.** NT-immunopositive terminals in the same section (blue). **C.** VGLUT1-immunopositive terminals in the same section (red). **D.** Merge of the three images, showing the presence of NT in VGLUT1-positive terminals of the VTA. **E–H.** Same as A–D, except that the sections are prepared from 21 days old TH-eGFP mice. **I–L.** Same as A–D, except that VGLUT2 immunoreactivity is shown in panels K and L instead of VGLUT1 immunoreactivity. **M–P.** Same as I–L, except that the sections are prepared from 21 days old TH-eGFP mice. **P and Q.** Magnified views of the areas identified by the yellow boxes of panels G and O respectively, to better show colocalization between the VGLUT and NT signals. White arrows indicate VGLUT1 or VGLUT2-positive terminals that are also NT-immunopositive. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

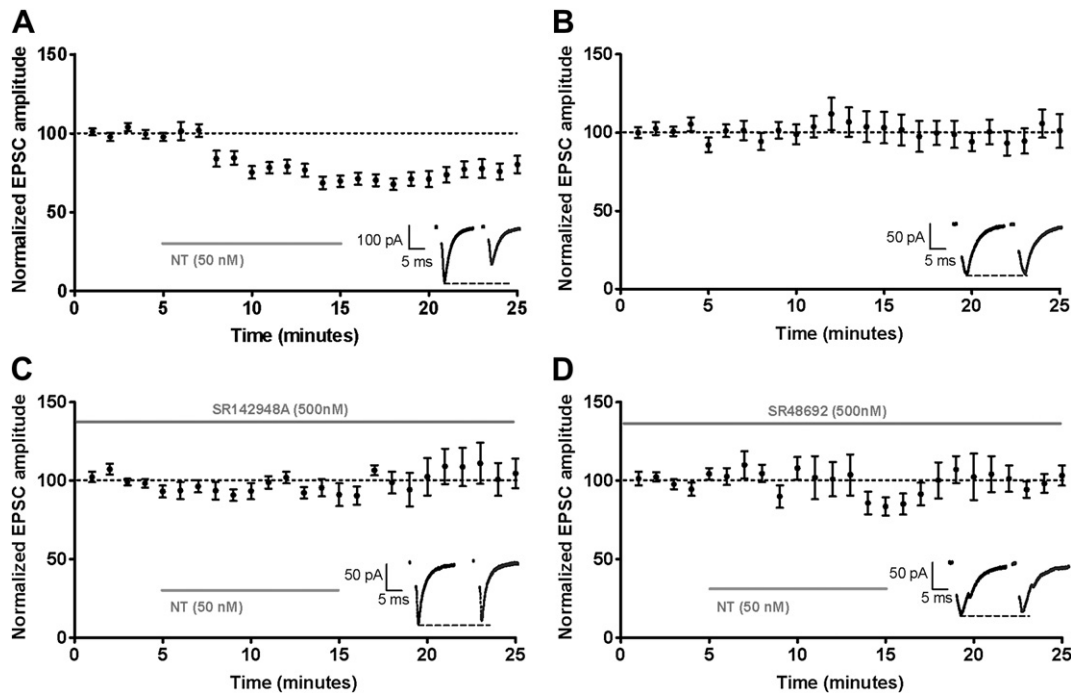


Fig. 2. Neurotensin induces a prolonged decrease in the amplitude of evoked excitatory post-synaptic currents in dopamine neurons of the VTA. **A.** Bath application of NT (50 nM) induced a long-lasting reduction of the amplitude of evoked EPSCs in DA neurons. This effect did not reverse during the 10 min NT washout period. **B.** The decrease in EPSC amplitude is unlikely to be due to rundown, as no significant decrease of this amplitude occurred when no NT was applied. **C.** In the presence of the broad spectrum NT receptor antagonist SR142948A (500 nM), NT failed to induce a significant decrease of EPSC amplitude. **D.** In the presence of the NTS1 receptor antagonist SR48692 (500 nM), NT failed to induce a significant decrease of EPSC amplitude. The insets show averaged example traces of evoked EPSCs before and following NT application.

3.4. Evidence for protracted EC release induced by NT

The synaptic depression caused by NT is long-lasting, with little recovery occurring during the 10 min washout period (Fig. 1A). This could be the result of a long-term depression-like phenomenon. Alternatively, it could result from protracted EC production and release in response to NT. To distinguish between these two hypotheses, we perfused the CB1 receptor antagonist AM251 (10 μ M) immediately after the end of the application of NT, once synaptic depression was at its peak ($74.34 \pm 3.77\%$; $N = 7$; $p = 0.0022$). Under such conditions, full recovery of EPSC amplitude was observed in the following 10 min period ($99.33 \pm 8.15\%$; $N = 7$; $p = 0.89$; compared to baseline; Fig. 4A). This was significantly different when compared to the values of the washout period during the experiments where NT was applied alone ($p = 0.019$).

Protracted EC production could in principle occur because of lingering NT due to poor washout. To test this possibility, we applied the broad spectrum NT receptor antagonist SR142948A (500 nM) immediately after the end of the 10 min NT application that once again caused a significant decrease in EPSC amplitude ($77.27 \pm 4.54\%$; $N = 4$; $p = 0.023$). Blockade of NT receptors during the NT washout period failed to promote recovery of EPSC amplitude ($69.5 \pm 5.6\%$; $N = 4$; $p = 0.022$ compared to baseline; Fig. 4B), compatible with the absence of residual NT.

3.5. Lack of evidence for NT-mediated EC release during an LTP-induction protocol

It has recently been reported that while spike time-dependent pairing fails to induce LTP in VTA DA neurons under baseline conditions, a significant LTP of glutamate inputs to DA neurons occurs after blockade of CB1 receptors or of 2-AG synthesis (Kortleven et al., 2011). Combined with the knowledge that NT is

present in some of the glutamatergic fibers that are stimulated during LTP induction, we hypothesized that part of the negative gating of LTP induction by ECs could result from NT release and the associated 2-AG production. To test this hypothesis, we evaluated if similarly to CB1 receptor blockade, a broad spectrum NT receptor antagonist would unmask LTP induction. We found that in the presence of SR142948A (500 nM), a treatment that is otherwise effective in blocking NT-induced EC release (Fig. 2C), no LTP could be induced by the spike time-dependent pairing protocol ($97.77 \pm 8.78\%$ of baseline values; $N = 5$; $p = 0.92$; Fig. 5A). However, when in the combined presence of both SR142948 and AM251 (4 μ M), a significant LTP was induced with this protocol ($130.74 \pm 7.17\%$ of baseline values; $N = 5$; $p = 0.016$; Fig. 5A). The normalized EPSP-amplitude recorded in the presence of the two antagonists was significantly different from the normalized EPSP-amplitude in the presence of SR142948A alone ($p = 0.02$; Fig. 5B).

4. Discussion

We have found that exogenous NT induced a significant reduction of evoked glutamate-mediated EPSC amplitude in VTA DA neurons, an effect which required ECs and specifically 2-AG. We conclude that NT acts through the NTS1 receptor to release 2-AG through a calcium-independent mechanism that requires G-protein mediated activation of PLC and subsequent DAG-lipase activity. These results suggest that NT release in the VTA has the potential to lead to EC release and local synaptic depression. However, our finding that an NT receptor antagonist alone does not unmask LTP induction in VTA DA neurons, unlike direct CB1 receptor blockade combined or not with an NT receptor antagonist, suggests that the spike time-dependent protocol often used to induce LTP in the VTA triggers EC release through a mechanism that is independent from NT.

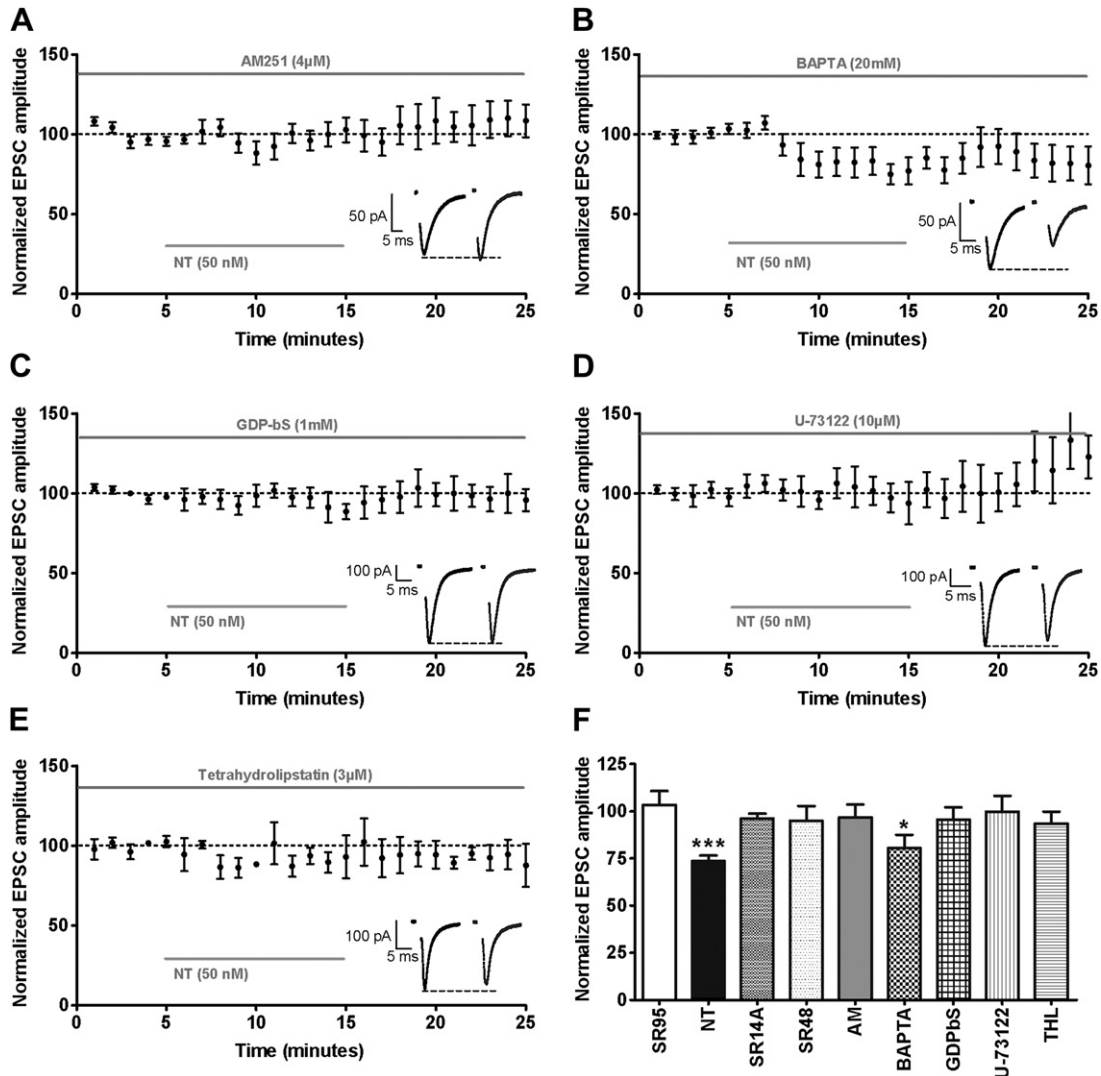


Fig. 3. Neurotensin decreases EPSC amplitude through 2-arachidonoylglycerol and the CB1 receptor, through a calcium-independent, but G-protein and phospholipase C-dependent mechanism. **A.** In the presence of the CB1 receptor antagonist AM251 (4 μ M), NT (50 nM) failed to induce a significant decrease in EPSC amplitude. **B.** The calcium chelator BAPTA (20 mM), added in the intra-pipette solution, failed to prevent the induction of synaptic depression by NT. **C.** Inhibiting G-proteins (GDP- β s; 1 mM in the pipette) blocked the synaptic depression normally induced by NT. **D.** The phospholipase C inhibitor U-73122 (10 μ M; intra-pipette) also blocked the NT-induced synaptic depression. **E.** Tetrahydrolipstatin (3 μ M; intra-pipette), a diacylglycerol-lipase inhibitor similarly prevented NT from inducing a decrease of EPSC amplitude. **F.** Summary graph showing the results of all pharmacological experiments. The insets show averaged example traces of evoked EPSCs before and following NT application. * $p < 0.05$; *** $p < 0.001$.

An abundant literature supports the existence of a tight regulatory interaction between the central NT and DA systems (for review, see: Binder et al., 2001). Apart from being present in DA neurons themselves, NT has been found to be present in punctate structures that are presumed to be afferent inputs to midbrain DA neurons (Uhl et al., 1979; Hökfelt et al., 1984). The bulk of NT-positive fibers in the VTA appear to come from the lateral pre-optic rostral lateral hypothalamic continuum and the medial pre-optic area (Geisler and Zahm, 2006). With a large proportion of VGLUT2-positive glutamatergic fibers that innervate the VTA also coming from similar regions (Geisler et al., 2007), it is therefore not unreasonable to hypothesize that a subset of such glutamatergic terminals co-release NT and glutamate. In the present study, we found that NT colocalized with VGLUT1 and -2 in the VTA in a small subset of terminals. In 14 day old mice, this relative expression of NT was similar in both VGLUT1- and VGLUT2-positive terminals. However, at 21 days, the proportion of VGLUT2-positive terminals co-expressing NT had increased significantly, whereas the proportion of VGLUT1-positive terminals co-expressing NT remained the

same. Interestingly, the increase in NT expression found here concurs with an increase of NTS1 expression during development, which reaches a plateau at p21 (Lepee-Logeoux et al., 1999). Together these findings suggest the possibility that activation of glutamatergic inputs to VTA DA neurons may promote concurrent release of NT, an effect that is likely to be of increasing significance with age. Furthermore, repetitive stimulation of glutamatergic fibers during protocols used to induce LTP is likely to be a condition favoring NT co-release.

In the present study, we report that NT causes a long-lasting depression of glutamate-mediated synapses onto VTA DA neurons. Our findings stand in relative contrast to several studies that have found that NT can increase glutamatergic transmission in the striatum (Ferraro et al., 1995; Ferraro et al., 1998) and cortex (Ferraro et al., 2000). However, it is important to note that in these earlier studies, microdialysis was used to measure global extracellular glutamate levels, a technique that cannot provide a direct evaluation of glutamate release at specific synapses. More recently, it was shown that bath-application of NT can induce an EC-

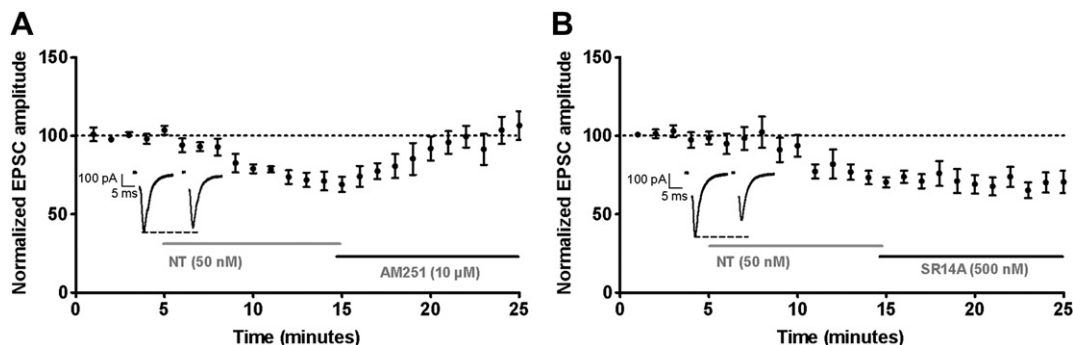


Fig. 4. Neurotensin induce protracted release of endocannabinoids. **A.** The CB1 receptor antagonist AM251 (10 μ M), applied immediately after NT (50 nM), reversed the synaptic depression induced by NT. **B.** The long-lasting EPSC depression was not due to incomplete washout of NT, as revealed by the inability of the broad spectrum NT receptor antagonist SR142948A (500 nM) to reverse the decrease in EPSC amplitude induced by prior application of NT. The insets show averaged example traces of evoked EPSCs before and during washout following NT application.

mediated decrease of EPSC amplitude in the dorsolateral striatum (Yin et al., 2008). This decrease was dependent on a rise in intracellular calcium, a stimulus well-known to cause the production of ECs (Piomelli, 2003; Chevalyere et al., 2006).

We found that bath application of NT causes a decrease of evoked EPSCs in VTA DA neurons through a mechanism implicating the NTS1 receptor, 2-AG synthesis and CB1 receptor activation. However, we found this mechanism to be calcium-independent, a relative surprise considering that NT is known to cause an increase in intracellular calcium in DA neurons (St-Gelais et al., 2004). The signal leading to 2-AG production is unlikely to have been a rise in intracellular calcium in other, surrounding neurons, as selectively blocking G-protein signaling, PLC activation and 2-AG synthesis in the recorded neuron fully blocked the

ability of NT to decrease evoked glutamate-mediated EPSCs. The Gq-coupled NTS1 receptor could activate other calcium-independent signaling pathways subsequent to stimulation of PLC. In addition, it is known that ECs can also be released via activation of G-protein coupled receptors, without elevation in intracellular calcium (for review, see (Gyombolai et al., 2011)). In fact, in 5-HT neurons of the dorsal raphé, the neuropeptide orexin-B can induce calcium-independent EC release that inhibits glutamatergic transmission (Haj-Dahmane and Shen, 2005). In this report, it was found that orexin-B acted by direct activation of PLC and diacylglycerol (DAG)-lipase. In the present study, we found that a very similar mechanism occurs in VTA DA neurons in response to NT, as shown by the block of NT-induced EC release by the G-protein inhibitor GDP- β S and the PLC inhibitor U-73122. The experiments with the DAG-lipase inhibitor THL furthermore suggest that 2-AG is the main EC mediating the NT-induced synaptic depression in the VTA. A summary diagram of the mechanism of NT induced EC release suggested by the present results is presented in Fig. 6.

In addition to inducing EPSC depression, we found that NT also caused a distinct increase in membrane current variance. This finding is compatible with previous reports showing that NT receptors also activate non-selective cationic channels with a large permeability to sodium (Jiang et al., 1994). We found that this elevation of membrane current variance was still present in the presence of a NTS1 receptor antagonist, but not in the presence of a broad spectrum NT receptor antagonist known to block both NTS1 and NTS2 (Betancur et al., 1998). This suggests that cationic channel activation induced by NT is mediated by the NTS2 receptor, the second G-protein coupled NT receptor. Our finding that the increase in membrane variance is absent when G-proteins are inhibited also argues in favor of the implication of NTS2, since the type 3 NT receptor is not part of the G-protein coupled receptor family (Mazella et al., 1998). Our results are thus compatible with the presence of NTS2 on DA neurons, as suggested by immunolabeling studies showing the presence of this receptor in the VTA (Sarret et al., 2003). Surprisingly, chelation of intracellular calcium caused a large increase in NT-induced membrane current variance. One interpretation of this observation is that under basal conditions, calcium actually acts to inhibit the cationic channels activated by NT. However, this conclusion is not readily compatible with the previous finding that activation of cationic channels by NT in acutely-dissociated substantia nigra compacta DA neurons is blocked by intracellular BAPTA (Wu et al., 1995). An alternative interpretation is that in the presence of BAPTA, the activation of other calcium-dependent channels, such as calcium-activated potassium channels, was blocked, thus

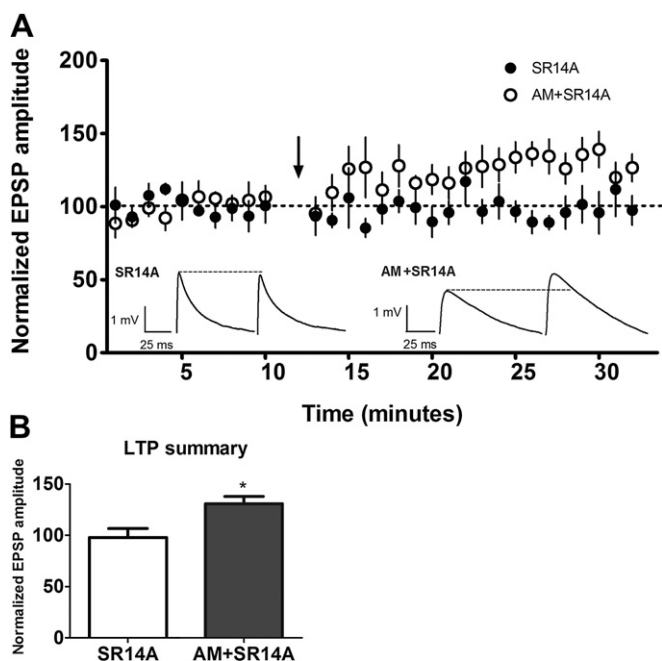


Fig. 5. Lack of evidence for NT-mediated EC release during an LTP-induction protocol. **A.** The broad spectrum NT receptor antagonist SR142948A (500 nM), applied continuously before, during and after a spike-time dependent pairing protocol did not unmask LTP in VTA DA neurons, whereas a combination of SR142948A and AM251 (4 μ M) did. The arrow represents the moment at which the pairing protocol was initiated. The insets show averaged example traces of evoked EPSPs before and after the pairing protocol. **B.** Summary graph of the magnitude of LTP in both groups. * $p < 0.05$.

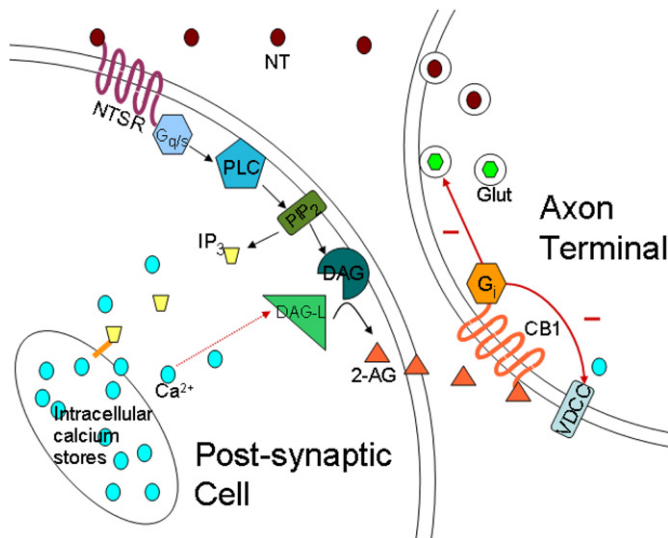


Fig. 6. Schematic diagram of the proposed mechanism of NT-mediated EC release. Post-synaptic NT receptor activation leads to G-protein induced activation of phospholipase C (PLC), which in turn cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol trisphosphate (IP₃) and diacylglycerol (DAG). DAG can be hydrolyzed by diacylglycerol-lipase (DAG-L) to create 2-AG, which then leaves the post-synaptic neuron to act on CB1 receptors located on axon terminals, where the associated G-protein reduces glutamate (Glut) release through inhibition of voltage-dependent calcium channels (VDCCs) or of the exocytosis mechanism. Concurrently, IP₃ cleaved from PIP₂ acts to release calcium (Ca²⁺) from intracellular stores. Increased intracellular calcium can play an important role in the production of endocannabinoids like 2-arachidonoylglycerol (2-AG) by increasing PLC and DAG-L activity, although in our model it seems to play a negligible role.

increasing cationic channel currents secondarily to an increase in membrane resistance. Further experiments will be required to examine this possibility.

An intriguing result from the present study is that NT appears to trigger protracted EC release, leading to sustained EPSC depression. This conclusion is based on our observation that EPSC depression did not recover significantly during the 10 min NT washout period, even in the presence of an NT receptor antagonist, while post-NT perfusion with a CB1 receptor antagonist readily reversed the depression. This finding can be contrasted with previous work showing that EC-mediated long term depression (LTD) in the brain does not seem to require continuous CB1 receptor activation (for review, see Chevalyere et al., 2006). In the VTA, such an EC-mediated LTD of GABAergic transmission occurs after cocaine application (Pan et al., 2008a, b). Our findings are nonetheless compatible with the results of (Yin et al., 2008) who showed that in the striatum, the CB1 antagonist AM251 is able to reverse NT-induced EPSC depression if applied directly after NT. Further studies will be required to extend our findings and determine exactly how long NT-induced EPSC depression lasts in VTA DA neurons and whether it represents an LTD-like phenomenon.

Several classes of drugs of abuse cause an increase of the AMPA to NMDA synaptic current ratio in VTA DA neurons (Ungless et al., 2001; Saal et al., 2003). This ratio is widely believed to reflect the induction of an LTP-like phenomenon *in vivo* in response to drugs of abuse. Such an increase in AMPA/NMDA ratio indeed occludes subsequent spike-time pairing dependent LTP in VTA DA neurons (Argilli et al., 2008). We have recently shown that 2-AG and CB1 receptor activation can negatively gate LTP induced in VTA DA neurons by spike-time dependent pairing (Kortleven et al., 2011). With the demonstration that (1) NT is expressed in VGLUT-positive terminals in the VTA and could therefore be released during stimulation of such fibers and (2) NT induces a distinct EC release, we hypothesized that conditions that lead to significant release of NT

in the VTA could negatively regulate glutamate inputs to DA neurons and LTP induction. However, in the present study, we found no evidence of an implication of endogenously-activated NT receptors in regulating LTP induction with the spike-time pairing protocol used. Further studies will be required to examine whether direct activation of the main NT-containing axonal fibers in the VTA, perhaps using optogenetics, would reveal plasticity of VTA glutamate inputs by endogenous NT.

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The authors declare to have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.neuropharm.2012.07.037>.

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