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

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 μM) and AM251 (4 and 10 μM) were obtained from Ascent Scientific (Bristol, UK). Tetrahydrolipstatin (2 μM; intra-pipette) and U-73122 (10 μM; intra-pipette) were from Calbiochem (San Diego, CA, USA). Neurotensin 8-13 (50 nM), BAPTA (20 mM; intra-pipette) and GDP-βS (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% O$_2$ and 5% CO$_2$) cutting-solution (glycerol-containing artificial cerebrospinal fluid (G-ACSF)) containing (in mM): Glycerol (250), KCl (3.5), MgCl$_2$ (1.3), CaCl$_2$ (2.0), NaHPO$_4$ (1.2), glucose (11), NaHCO$_3$ (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 μm) 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), MgCl$_2$ (1.3), CaCl$_2$ (2), NaHPO$_4$ (1.2), glucose (11), NaHCO$_3$ (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 ± 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, Bodoin, ME, USA) was placed in the rostral VTA and recordings were started after an equilibration period of 20 min. Recording electrodes (4–6 MΩ) 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), phospho-creatine (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 μM) 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 multiclamp 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 μm) 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× 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 μm 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 μm^2 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