Neurotensin Enhances GABAergic Activity in Rat Hippocampus CA1 Region by Modulating L-Type Calcium Channels

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Li S, Geiger JD, Lei S. Neurotensin enhances GABAergic activity in rat hippocampus CA1 region by modulating L-type calcium channels. J Neurophysiol 99: 2134–2143, 2008. First published March 12, 2008; doi:10.1152/jn.00890.2007. Neurotensin (NT) is a tridecapeptide that interacts with three NT receptors; NTS1, NTS2, and NTS3. Although NT has been reported to modulate GABAergic transmission and the involved cellular and signaling mechanisms of NT in the hippocampus. Application of NT dose-dependently increased the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from CA1 pyramidal neurons with no effects on the amplitude of sIPSCs. NT did not change either the frequency or the amplitude of miniature (m)IPSCs recorded in the presence of tetrodotoxin. GABAergic interneurons via phospholipase C–protein kinase C (PLC–PKC) pathway-mediated modulation of L-type calcium channels.

INTRODUCTION

Neurotensin (NT) is a 13 amino acid peptide originally isolated from bovine hypothalamus (Carraway and Leeman 1973). This neuropeptide is widely expressed not only in the CNS but also in peripheral tissues. NT interacts with three subtypes of NT receptors; NTS1, NTS2, and NTS3. NTS1 and NTS2 are G-protein–coupled receptors, whereas NTS3 is a single transmembrane domain receptor (Dicou et al. 2004; Martin et al. 2003). NTS1 has high affinity for NT and its effects are usually blocked by the selective nonpeptide antagonist SR48692 (Pelaprat 2006). NTS1 is functionally and typically coupled to phospholipase C (PLC) and the inositol phosphate (IP) signaling cascade via Gα proteins (Chabry et al. 1994; Najimi et al. 2002); however, other possible signaling could occur through activation of cyclic GMP, cyclic AMP, arachidonic acid production (Canonicco et al. 1985; Najimi et al. 2002), MAP kinase phosphorylation (Ehlers et al. 2000; Poinot-Chazel et al. 1996), and inhibition of Akt activity (Liu et al. 2004). NTS2 shows lower affinity for NT and the function of NTS2 is largely unclear. To date, very little is known about the physiological role of NTS3 receptors (Chalon et al. 1996).

γ-Aminobutyric acid (GABA), as the major inhibitory transmitter in brain, is important for the development of neuronal circuits and functions. GABA dysfunction has been linked to a variety of pathophysiological conditions including schizophrenia, anxiety disorders, drug addiction, and Parkinson’s disease (Crestani et al. 1999; Ferraro et al. 1998; Tiihonen et al. 1997). NT-mediated increases in GABA release have been demonstrated in many brain regions including prefrontal cortex, striatum, globus pallidus, and hippocampus (Canonicco et al. 1985; Ferraro et al. 1997; O’Connor et al. 1992; Petrie et al. 2005; Rakovska et al. 1998). An involvement of NTS1 in the effects of NT on GABA release is supported by findings that NT-induced increases in GABA release were blocked by the NTS1 antagonist SR48692. However, to date the detailed underlying mechanisms whereby NT regulates GABA release in rat hippocampus, a major region regulating learning and memory, are largely unknown.

In the present study, we studied the effects of NT on GABAergic transmission in the hippocampus. Our results demonstrate that NT transiently increases GABA release in the CA1 subregion via activation of NTS1 receptors on GABAergic interneurons. We also found that NT increases the excitability of interneurons via phospholipase C–protein kinase C (PLC–PKC) pathway-mediated modulation of L-type calcium channels.

METHODS

Brain slice preparation

Sprague–Dawley rats (12- to 22-day-old) were anesthetized with isoflurane and killed by decapitation. Brains were removed and horizontal brain slices (400 μm) were cut in oxygenated ice-cold dissection solution using a Vibratome (Leica VT1000S) and incubated at 34°C for 30 min before performing electrophysiological measurements. Dissection solution contained the following (in mM): 130 NaCl, 24 NaHCO3, 3.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, 5.0 MgCl2, and 10 glucose, and was saturated with 95% O2-5% CO2. All animal procedures conformed to the guidelines approved by the University of North Dakota Animal Care and Use Committee, which is in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
dance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Electrophysiology in brain slices

Whole cell recordings in voltage- or current-clamp were performed from hippocampal CA1 pyramidal neurons or interneurons visually identified by characteristic morphology and regional distribution with infrared video microscopy (Olympus BX51WI). For recordings of spontaneous inhibitory postsynaptic currents (sIPSCs), slices were maintained at room temperature and were gravity perfused with artificial cerebrospinal fluid (ACSF) solution containing (in mM): 130 NaCl, 24 NaHCO3, 3.5 KCl, 1.25 NaH2PO4, 1.5 MgCl2, 2.5 CaCl2, and 10 glucose. d-(-)-2-Amino-5-phosphonopentanoic acid (d-AP5, 50 μM) and 6,7-dimtroquinoxaline-2,3-dione (DNQX, 10 μM) were added to ACSF to block glutamatergic responses mediated through N-methyl-d-aspartate (NMDA) and non-NMDA ionotropic glutamate receptors, respectively. The tip resistance of the recording pipettes was 4–6 MΩ after being filled with the following intracellular solution (in mM): 100 K-glucuronate, 0.6 EGTA, 5 MgCl2, 8 NaCl, 2 Na-ATP, 0.3 Na-GTP, 40 HEPES, and 1 QX-314 [N-(2,6-dimethylphenyl-carbamoylmethyl)triethylammonium bromide], a quaternary derivative of lidocaine. The pH was adjusted to 7.35 with CsOH. sIPSCs were recorded at a holding potential of +30 mV using whole cell voltage clamp (Lei and McBain 2003).

Whole cell recordings in current-clamp mode were used to record action potentials from hippocampal CA1 interneurons. Slices were maintained at room temperature and were gravity perfused with the ACSF solution described earlier. The patch pipettes were filled with intracellular solution containing (in mM): 100 K+-glucuronate, 0.6 EGTA, 5 MgCl2, 8 NaCl, 2 Na-ATP, 0.3 Na-GTP, 40 HEPES, and 1 QX-314 [N-(2,6-dimethylphenyl-carbamoylmethyl)triethylammonium bromide], a quaternary derivative of lidocaine. The pH was adjusted to 7.35 with CsOH. sIPSCs were recorded at a holding potential of +30 mV using whole cell voltage clamp (Lei and McBain 2003).

Western blot

Sprague-Dawley rats (12- to 22-day-old) were anesthetized with isoflurane, perfused with phosphate-buffered saline, and killed by decapitation. Hippocampi were removed and put immediately into lysis buffer [10 mM HEPES, 320 mM sucrose, 2 mM EDTA, 2 mM EGTA, 0.1 mM PMSF, 1 mM DTG, 5 mM NaF, 100 μM Na3VO4, and a protease inhibitor cocktail (1:100; Sigma–Aldrich, St. Louis, MO)] and homogenized. Insoluble material was removed by centrifugation (14,000 g, 10 min, 4°C). The protein concentration was quantified by the Bradford method (Bradford 1976). The protein sample was fractioned by 10% SDS-PAGE and transferred to polyvinylidene fluoride membrane. Membranes were incubated with anti-NTS1 antibody (0.4 μg/ml, 1:500) or the preabsorbed NTS1 antibody (1:500) overnight at 4°C, followed by incubation with HRP-conjugated anti-goat secondary antibody. The preabsorbed NTS1 was made by incubating the NTS1 antibody with an equal amount of blocking peptide (0.4 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 h. Antibody binding was detected via enhanced chemiluminescence (Pierce, Rockford, IL).

Immunohistochemistry

Frozen horizontal brain sections (14 μm) were cut from 12- to 22-day-old Sprague–Dawley rats. Sections were air-dried, fixed in ice-cold acetone for 10 min, and treated with 0.1% hydrogen peroxide in methanol for 30 min. Sections were incubated with polyclonal goat anti-NTS1 (4 μg/ml, 1:50) antibody (Santa Cruz Biotechnology) overnight at 4°C. After incubation with biotinylated secondary antibody (1:500; Vector Laboratories, Burlingame, CA), sections were incubated with extravidin-peroxidase (Sigma–Aldrich), visualized with diaminobenzidine/hydrogen peroxide (Vector Laboratories), and counterstained lightly with Gill’s hematoxylin (Vector Laboratories). Sections on slides were dehydrated sequentially using 70, 95, and 100% alcohol; cleared in xylene; and mounted with Vector mounting medium. All procedures were carried out at room temperature unless otherwise indicated.

For immunohistochemical characterization of patched cells, cells were labeled with biocytin during recordings and hippocampal slices were fixed in 4% paraformaldehyde overnight at 4°C. Sections were incubated with goat anti-NTS1 antibody (4 μg/ml) and monoclonal anti-GAD65 antibody (1:100; Santa Cruz Biotechnology) or anti-GABA antibody (1:1,000; Chemicon, Temecula, CA) for 72 h at 4°C. Slices were incubated in FITC-conjugated donkey anti-goat secondary antibody (1:500), Cy3-conjugated donkey anti-mouse secondary antibody (1:500), or Texas red-conjugated donkey anti-rabbit secondary antibody (1:500) overnight. Biocytin-filled cells were identified using AMCA streptavidin (1:100; Vector Laboratories) or Alexa 647 streptavidin (1:200; Molecular Probes, Burlington, CA). Images were acquired using an LSM 510 Meta confocal microscope (Zeiss, Thornwood, NY).

Pharmacological agents

Neurotensin was purchased from American Peptide Company (Sunnyvale, CA). d-(-)-2-Amino-5-phosphonopentanoic acid (d-AP5, 6,7-dimtroquinoxaline-2,3-dione (DNQX), U73122 [1-[6-[[17β]-3-methoxyoestra-1,3,5(10)-tri-en-17-yl][amino]hexyl]-1H-pyrrrole-2,5-dione], and U73343 [1-[[6-[[17β]-3-methoxyoestra-1,3,5(10)-tri-en-17-yl]amino]hexyl]-2,5-pyrolidimedine] were purchased from Tocris Bioscience (Ellisville, MO). GF109203X (3-[1-[(dimethylamino)prop-2-enyl]-1H-indol-3-yl]-1H-pyrrrole-2,5-dione), 2-amino-4-phosphonobutyric acid (2-APB), leovastabine, nifedipine, and nimodipine were purchased from Sigma–Aldrich. o-Conotoxin GVIA (o-CTX) was bought from Biomol International (Plymouth Meeting, PA). SR48962 (2-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxy-phenyl)pyrazol-3-yl)carbamoylethyl]tricyclo[3.3.1.15,7]decan-2-carboxylic acid) was kindly provided by Sanofi-Aventis (Bridgewater, NJ).
All data are expressed as means and SE. *N* numbers in the text represent the number of cells examined. Statistical significance was established using a two-tailed Student’s *t*-test, with *P* < 0.05 considered statistically significant.

**RESULTS**

*NT increased sIPSC frequency of CA1 pyramidal neurons, but did not change EPSCs*

NT receptor mRNA is expressed in the CA1 subregion of rat hippocampus (Mendez et al. 1997). To understand the specific role of these NT receptors in modulating glutamate and GABA release, we recorded both spontaneous inhibitory postsynaptic currents (sIPSCs) and excitatory postsynaptic currents (EPSCs) from CA1 pyramidal neurons. Although there was no change in EPSCs (data not shown), bath application of NT (0.25 μM) significantly increased sIPSC frequency to 185.3 ± 17.3% of control (*n* = 11, *P* < 0.01; Fig. 1, A and B). The sIPSC frequency usually started to increase 1 min after NT application, maximal increases were reached in about 3 min, and peak levels were maintained for approximately 4 min before returning to baseline by 10 min. There was no statistically significant change in sIPSC amplitude between control and NT treatment.

**Data analysis**

FIG. 1. Neurotensin (NT) increases spontaneous inhibitory postsynaptic current (sIPSC) frequency of CA1 pyramidal neurons. A: sIPSCs recorded from a CA1 pyramidal neuron in the absence and presence of NT. Holding potential was +30 mV. *B*: time course of NT-induced changes in sIPSC frequency of CA1 pyramidal neurons. sIPSC frequency was averaged for each minute and then normalized to the last minute of control. *C*: cumulative probability and bar graph of sIPSC amplitude. *D*: dose–response curve of NT-induced changes in sIPSC in pyramidal cells in CA1. The numbers show the number of cells recorded in each concentration. *E*: different ages of rats have consistent responses to NT. The average sIPSC frequency increased to 241 ± 50% of control, from 508 ± 92/min in control to 1,045 ± 126 after exposure to NT in postnatal day 12 (P12) rats (*n* = 5, *P* < 0.05). The average sIPSC frequency changed from 580 ± 276 in control to 952 ± 236 after application of NT and increased to 243 ± 72% of control (*n* = 5, *P* < 0.05) in P17 rats. The average sIPSC frequency changed from 611 ± 192 in control to 894 ± 161 after application of NT and increased to 174 ± 32% of control (*n* = 5, *P* < 0.05) in P22 rats. *F*: application of NT1 antagonist SR48692 (5 μM, *n* = 5), but not vehicle (0.004% DMSO, *n* = 4), abolished NT-induced increases in sIPSCs. *G*: application of levocabastine (1 μM, *n* = 4), a NT52 ligand, and vehicle (0.02% ethanol, *n* = 6) failed to block the NT-induced increases in sIPSC.
The CA1 subregion of the hippocampus (Fig. 3, using immunohistochemistry. NTS1 receptors were expressed in expression of NTS1 in the CA1 subregion of the hippocampus interneurons should express NT receptors. We next examined the NT increased the excitability of CA1 interneurons (Fig. 3, C). To test whether activation of NTS1 modulates the potential firing rates after application of NT (Fig. 3K). Therefore in the following action potential recording experiments only NTS1-positive GABAergic neurons were included in the statistics. NT-induced changes in the time course of action potential firing rates (Fig. 3E) were similar to NT-induced changes in the time course of sIPSC frequency in pyramidal neurons (Fig. 1B), suggesting that NT increases GABA release by facilitating action potential generation in interneurons. To examine the effects of NT on the shape of action potential, we compared the averaged action potentials prior to and during the application of NT after normalization of action potential baselines to 0 mV. The afterhyperpolarization (AHP) amplitudes were decreased significantly fol-

NT-induced increases in sIPSC frequency were action potential dependent

We recorded miniature IPSCs (mIPSCs) in the presence of tetrodotoxin (TTX). Before we added TTX to the normal ACSF, there was a significant increase in sIPSC frequency after exposure to NT as shown earlier in Fig. 1. Application of NT (0.25 μM) in the presence of TTX (0.5 μM) failed to change either the frequency or the amplitude of mIPSCs (n = 5, P > 0.05; Fig. 2). Because presynaptic action potentials are involved in GABA release onto pyramidal neurons, these results suggest that the effects of NT were action potential dependent and that the site of action was on GABAergic interneurons and not on pyramidal neurons.

NT increased the excitability of CA1 interneurons

If the action site of NT is on GABAergic interneurons, CA1 interneurons should express NT receptors. We next examined the expression of NTS1 in the CA1 subregion of the hippocampus using immunohistochemistry. NTS1 receptors were expressed in the CA1 subregion of the hippocampus (Fig. 3, A and B). To determine the specificity of the goat anti-NTS1 antibody, we performed Western blots. The antibody specifically detected a unique band of approximately 60-kDa molecular mass, which is consistent with the molecular mass of rat NTS1 (Martorana et al. 2006). This specific band was blocked by preadsorbing the antibody using an equal amount of the blocking peptide (Fig. 3C). To test whether activation of NTS1 modulates the excitability of the interneurons, we first identified CA1 radia-

FIG. 2. NT fails to change miniature inhibitory postsynaptic current (mIPSC) recorded from CA1 pyramidal neuron. A: mIPSCs recorded from a CA1 pyramidal neuron in the presence of tetrodotoxin (TTX, 0.5 μM) before and during the application of NT. B: time course of mIPSC frequency in the absence or presence of NT. C: cumulative probability and bar graph of mIPSC amplitude. D: frequency-amplitude graph of mIPSC of TTX in the absence or presence of NT.
lowing NT application (control: $-6.2 \pm 1.2$ mV, NT: $-3.3 \pm 0.8$ mV, $n = 19$, $P < 0.05$; Fig. 3, F and G).

To exclude the possibility that NT-induced increases in action potential firing rates of interneurons were due to membrane depolarization, membrane potentials were held at $-50$ mV under whole cell voltage-clamp conditions to monitor current changes after application of NT in the presence of $0.5 \mu M$ TTX. There were no significant changes in holding currents after NT application ($n = 7$, $P > 0.05$; Fig. 3H).

\[ L\text{-type calcium channel blockers prevented NT-induced increases in action potential firing} \]

We then tested the roles of $\text{Ca}^{2+}$ in NT-induced increases in action potential firing rate of NTS1-immunopositive GABAergic neurons. NT-induced increases in action potential firing were abolished when experiments were conducted in nominally calcium free extracellular solution ($n = 5$, $P > 0.05$; Fig. 4, A and I), suggesting that $\text{Ca}^{2+}$ influx is essential for the effects of NT. To test the roles of voltage-gated $\text{Ca}^{2+}$ channels, we included 50$\text{M}$$\text{T}

\[ \text{Fig. 3. NT increases the excitability of CA1 interneurons.} \]

A: immunostaining demonstrated the presence of NTS1 receptors in rat hippocampus CA1 region ($\times 10$). B: higher magnification of A; black arrows indicate NTS1 immunopositive cells ($\times 40$). C: Western blot shows that anti-NTR1 antibody recognizes a specific band at the molecular mass of about 60 kDa, but not preadsorbed NTR1 antibody. Anti-actin was used as loading control. D: action potentials (APs) recorded from a CA1 interneuron before and during the application of NT (0.25 $\mu M$). E: time course of NT-induced changes in CA1 interneuron firing rates. F: average AP traces from a single cell before and after application of NT. G: quantitative AP afterhyperpolarization (AHP) amplitude before and after application of NT ($^{*}P < 0.05$). AP baseline was adjusted to 0 mV. H: the effects of NT on holding current changes in the presence of TTX (0.5 $\mu M$). Holding potential was $-50$ mV under voltage-clamp conditions. I: the expression of NTS1 (green) and GAD65 (red) on biocytin-filled neurons (blue). J, K: the expression of NTS1 (green) and $\gamma$-aminobutyric acid (GABA) (red) on biocytin-filled neurons (blue). The biocytin-filled neurons were labeled by Alexa647-conjugated streptavidin and pseudocolored with blue.
μM gadolinium (Gd³⁺), a high-voltage-gated calcium channel blocker, in the extracellular solution to block Ca²⁺ channels. Application of Gd³⁺ blocked NT-induced increases in action potential firing frequency (n = 6, P > 0.05; Fig. 4, B and I), suggesting that Ca²⁺ influx via voltage-gated Ca²⁺ channels is required. We then tested the roles of the subtypes of voltage-gated Ca²⁺ channels in NT-induced increase in action potential firing. Application of nifedipine (20 μM), the L-type calcium channel blocker, blocked NT-induced increase in action potential firing frequency of interneurons (n = 5, P > 0.05; Fig. 4, C and I), suggesting the requirement of L-type Ca²⁺ channels. Furthermore, application of nimodipine (10 μM) alone increased the action potential firing rates by 17.9 ± 6.3% (n = 7, P < 0.05) and blocked NT-induced increases in action potential firing frequency (n = 7, P > 0.05; Fig. 4, D and I). Together, these results suggest that L-type Ca²⁺ channels are required for NT-induced increases in action potential firing in interneurons. We also examined the role of N-type Ca²⁺ channels using ω-conotoxin GVIA, an N-type calcium channel blocker. Although application of ω-conotoxin GVIA (3 μM) alone increased interneuron firing rates by 34.5 ± 0.7% (n = 4, P < 0.05), subsequent application of NT still significantly increased the action potential firing rates by 65.0 ± 23.0% (n = 4, P < 0.05; Fig. 4, E and I), suggesting that N-type Ca²⁺ channels are not involved in NT-induced increases in action potential firing. We also examined the effects of the Ca²⁺ channel blockers on NT-induced reduction of AHP amplitude. NT failed to alter AHP amplitude in the presence of nifedipine (n = 4, P > 0.05; Fig. 4H) or nimodipine (n = 7, P > 0.05; Fig. 4H). However, NT in the presence of ω-conotoxin decreased AHP amplitude from −8.9 ± 1.1 to −5.8 ± 2.8 mV, although they were not statistically different (n = 4, P > 0.05; Fig. 4, G and H). These findings suggest that NT-mediated changes in action potential firing rate and AHP amplitude in interneurons were modulated by L- but not N-type calcium channels.

**NT-induced decreases in postburst AHP recorded from interneurons were blocked by nimodipine**

Because calcium channels are coupled to calcium-activated potassium channels (K₉Ca), we determined next whether changing L-type calcium channels activity with NT could also change K₉Ca activity and AHP amplitude. To record postburst AHP in interneurons, 0.1- to 0.2-nA currents were injected for 200 ms under current-clamp condition to induce a burst of five to six action potentials (Fig. 5A). AHP peak amplitude was calculated as the maximal negative voltage deflection from baseline during the first 500 ms after the current offset. NT application significantly decreased postburst AHP amplitude (n = 4, P < 0.05; Fig. 5B) and nimodipine blocked this decrease. Nimodipine treatment alone changed the AHP amplitude from −3.9 ± 1.9 to −1.3 ± 0.3 mV (n = 6, P < 0.05; Fig. 5C) and NT further reduced AHP amplitude to −0.9 ± 0.5 mV (n = 6, P > 0.05). These results suggest that NT acts on L-type calcium channels to delay activation of calcium-activated potassium channels, decrease the AHP peak, and increase action potential firing rates of CA1 interneurons.

**NT-induced increases in pyramidal neuron sIPSCs were blocked by L-type calcium channel blockers**

We determined next whether nifedipine and nimodipine blocked NT-induced increases in sIPSC frequency recorded from CA1 pyramidal neurons (Fig. 6, A, B, and E). NT increased pyramidal neuron sIPSC frequency (as shown earlier on June 27, 2017 http://jn.physiology.org/ Downloaded from
in Fig. 1) and both nifedipine and nimodipine blocked the NT-induced changes in sIPSC frequency (see Fig. 3, P < 0.05). These results suggest that NT-mediated inhibition of L-type Ca\(^{2+}\) channels on GABAergic interneurons leads to an increase in the frequency of sIPSCs recorded from pyramidal neurons.

**Charybdotoxin and apamin failed to block NT-induced increases in sIPSCs**

To further investigate the extent to which the effects of NT were mediated through calcium-activated charybdotoxin-sensitive, apamin-sensitive, and charybdotoxin- or apamin-insensitive potassium channels we tested the possible effects of charybdotoxin (50 nM; n = 4) and apamin (100 nM; n = 9) on sIPSCs. Neither compound blocked NT-induced increases in the frequency of sIPSCs recorded from CA1 pyramidal neurons (Fig. 6, C–E). These results suggest that NT’s modulation of GABAergic activity in CA1 hippocampus is not dependent on either charybdotoxin- or apamin-sensitive K\(_{Ca}\) channels. Together, these results indicate that NT regulates both AHP amplitude and spontaneous firing rate of interneurons by regulating L-type calcium channels, subsequently altering charybdotoxin- and apamin-insensitive K\(_{Ca}\) channel(s) activities, and AHP amplitude.

**NT increased GABAergic activity via PLC and PKC**

We then probed the signal transduction mechanisms whereby NT increases GABA release. Activation of NTS1 increases the activity of PLC, which hydrolyzes PIP\(_2\) to produce inositol triphosphate (IP\(_3\)) to facilitate intracellular Ca\(^{2+}\) release and diacylglycerol to activate PKC. We next tested the roles of this pathway in NT-induced increases in GABA release. Preincubation of slices for 60 min with the PLC inhibitor U73122 (10 \(\mu\)M) blocked NT-induced increases in sIPSC frequency (n = 4, P > 0.05; Fig. 7A), whereas treatment of slices with the inactive analog U73433 (10 \(\mu\)M) had no effects on NT-induced increases in sIPSC frequency (n = 4, P < 0.05; Fig. 7A). We further tested the roles of the two downstream targets; IP\(_3\) receptors and PKC. NT-mediated increases in sIPSC frequency were blocked by treatment of slices with the intracellular IP\(_3\) receptor blocker 2-APB (50 \(\mu\)M; n = 4, P > 0.05; Fig. 7B) and the PKC inhibitor GF109203X (100 nM; n = 4, P > 0.05; Fig. 7C). These results suggest that NT binds to high-affinity NTS1 receptors and activates the PLC–IP\(_3\) pathway as well as PKC.

**DISCUSSION**

NT is an important neuropeptide known to affect neuronal signaling in general and GABAergic signaling more specifically. Here we addressed mechanisms underlying NT-mediated increases in GABAergic activity in a specific population of neurons of rat hippocampus. Our results demonstrate that NT increases the excitability of CA1 GABAergic interneurons by
modulating L-type calcium channels to decrease AHP amplitude and that NT-induced increases in GABAergic interneuron excitability was achieved by activating NTS1 receptors coupled to PLC, IP₃, and PKC.

**NT and ion channels**

Several ionic mechanisms could be proposed to explain NT-mediated increases in GABA release. First, NT could inhibit resting K⁺ channels to depolarize GABAergic interneurons, resulting in increases in action potential firing and GABA release. Although NT has been reported to depolarize neurons via inhibition of K⁺ channels in globus pallidus (Chen et al. 2004) and the nucleus of the solitary tract (Ogawa et al. 2005), our results indicated that NT did not depolarize CA1 interneurons because NT failed to significantly change holding currents when the cells were held at a potential close to the resting membrane potential (Fig. 3H). Second, NT could reduce AHP to increase action potential firing frequency without generating membrane depolarization. Our data support this mechanism because NT significantly reduced action potential afterhyperpolarization (Fig. 3G). NT-induced reduction in afterhyperpolarization was further demonstrated by recording the postburst AHP from interneurons.

KCa and calcium channels regulate AHP amplitude (Pedarzani et al. 2005; Shah and Haylett 2000; Villalobos et al. 2004). Of the three types of KCa channels, the large conductance calcium-activated potassium channels (BK) are mainly sensitive to charybdoxin, except for BK channels that contain β4 subunits (Meera et al. 2000). The small-conductance calcium-activated potassium channels (SKs) are sensitive to apamin (Bond et al. 2004; Stocker et al. 1999), whereas a third type is insensitive to either charybdoxin or apamin. In our study, we studied the roles of KCa in NT-induced increases in GABA release. There are two potential mechanisms whereby NT could modulate KCa to reduce AHP. First, NT could directly inhibit KCa channels. Our results do not provide any evidence for this mechanism because application of two KCa inhibitors, charybdoxin or apamin (Bond et al. 2004; Stocker et al. 1999), failed to block NT-induced increases in sIPSC frequency. This suggests that the actions of NT are not mediated through apamin- or charybdoxin-sensitive KCa. Second, calcium channels could also regulate AHP amplitude mainly because different types of calcium channels (L, N, P/Q, T type) are tightly coupled to KCa channels (Berkefeld et al. 2006; Bowden et al. 2001; Empson and Jefferys 2001; Hallworth et al. 2003; Wolfrt and Roeper 2002; Womack et al. 2004). Therefore NT-mediated decreases in interneuron AHP amplitude may be explained by NT-mediated modulation of certain subtypes of calcium channels. We examined the roles of Ca²⁺ channels in NT-induced increases in GABA release. Our results support a role for L-type Ca²⁺ channels in NT-mediated increases in GABA release because application of L-type Ca²⁺ channel blockers blocked NT-induced increases in sIPSC frequency and action potential firing rate as well as NT-mediated reduction in AHP. Consistent with our results, it has been shown that KCa and calcium channels regulate AHP amplitude (Pedarzani et al. 2005; Shah and Haylett 2000; Villalobos et al. 2004).

Our findings are consistent with the results of previous studies showing that NT could modulate calcium channels. NT was previously shown to inhibit N-type calcium channels in rat pallidal neurons, but the study did not discuss the effects of NT on GABAergic or glutamatergic activities, even though NT receptors were expressed on both parvalbumin-positive and -negative neurons (Martorana et al. 2006). NT has also been shown to inhibit both low-voltage–activated calcium channels such as T-type calcium channels and high-voltage–activated calcium channels such as L-type or N-type calcium channels in nucleus basalis neurons and diagonal band of Broca of rats (Jassar et al. 1999; Margeta-Mitrovic et al. 1997). A considerable number of cholinergic neurons reside in diagonal band of Broca and nucleus basalis. The cholinergic neurons in this region express more than one type of calcium channels. This may explain the differences in the identities of Ca²⁺ channels that NT interacts with from different studies. It seems that NT could couple to different ion channels depending on the types of channels expressed on individual cell types. In our study, we detected that NT modulated L-type calcium channels and reduced AHP to increase GABAergic activities in juvenile rat hippocampus. To our knowledge, this is the first study to address the underlying mechanism of NT-induced increases in GABAergic neuron excitability by modulating L-type calcium channels in rat hippocampus.

**NT receptors and their intracellular signaling pathway**

NT receptors can interact with three subtypes of NT receptors, but we generated two lines of evidence that indicate that NT increases GABA release in hippocampus via activation of only one subtype, NTS1. First, application of SR48962, the specific NTS1 receptor blocker, completely blocked NT-induced increases in sIPSC frequency, whereas application of the ligand for NTS2, levocabastine, failed to change NT-mediated increases in sIPSC frequency. Second, NTS1 receptors were expressed on GABAergic interneurons in the CA1 region and NT increased action potential firing frequency in GABAergic interneurons expressing NTS1. Consistent with our results, NTS1 has been shown to be required for NT-induced increases in GABA release in prefrontal cortex (Petrie et al. 2005).

Activation of NTS1 is coupled to PLC and subsequent activation of PKC. We have also demonstrated that PLC,
intracellular Ca\(^{2+}\) release, and PKC are required for NT-induced increases in GABA release because application of the inhibitors for PLC and \(\mathrm{IP_3}\) receptors as well as PKC blocked NT-induced increases in sIPSC frequency. The results that both intracellular Ca\(^{2+}\) release and PKC are involved in NT-induced increases in sIPSC frequency suggest that members of the Ca\(^{2+}\)-dependent conventional protein kinase C (PKC) family (PKC\(\alpha\), PKC\(\beta\), or PKC\(\gamma\)) are involved in the effects of NT in the hippocampus. Our results support a scenario in which NT interacts with NTS1 and then activates the PLC–PKC pathway. The activated PKC potentially belonging to the Ca\(^{2+}\)-dependent, conventional PKC family phosphorylates L-type Ca\(^{2+}\) channels or their associated proteins to reduce AHP and increase GABA release. Consistent with this scenario, the activity of L-type calcium channel is subject to modulation by PKC (Belmeguenai et al. 2002).

**NT and juvenile rats’ hippocampus**

In the hippocampus, the expression of NTS1 changes with the development of CNS. It is expressed early in development (before birth) and, after postnatal day 21, the expression level progressively decreases thereafter until adulthood (Boudin et al. 2000; Kiyama et al. 1987; Schotte and Laduron 1987). The selective temporary expression of NTS1 early in development suggests that it plays a specific role in the establishment of neuronal circuitry in these regions. In our study, we found that in the specific development period (12–22 days), NT consistently increased GABAergic interneuron excitability by modulating L-type calcium channels. Since both the GABergic system and the L-type calcium channel have been reported to be important for the development of neuron circuits (Costa et al. 2004; Striessnig et al. 2006), our findings may provide a potential explanation for the phenomena and function of this transiently expressed NTS1 in rat hippocampus.

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