NTS2 expression in cerebellar granule cells

Pharmacology and functional properties of NTS2 neurotensin receptors in cerebellar granule cells

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ABSTRACT

The binding and signaling properties of neuronal NTS2 neurotensin (NT) receptors were examined in cultured rat cerebellar granule cells. As shown by RT-PCR, receptor autoradiography, and confocal microscopic localization of fluorescent NT, these cells selectively express the NTS2 receptor sub-type. Accordingly, a single apparent class of $^{125}$I-NT binding sites, with an affinity of 3.1 nM, was detected in cerebellar granule cell cultures. This binding was competed for with high affinity (IC$_{50}$ = 5.7 nM) by the NTS2 ligand, levocabastine, and with low affinity (IC$_{50}$ = 203 nM) by the NTS1 antagonist, SR48692. Hypertonic acid stripping of surface-bound ligand and hyperosmolar sucrose treatment revealed that 64% of specifically bound $^{125}$I-NT was internalized at equilibrium via a clathrin-dependent pathway. In cells loaded with the Ca$^{2+}$-sensitive fluorescent dye Fluo4, SR48692, but neither NT nor levocabastine, triggered a marked increase in cytosolic [Ca$^{2+}$]. By contrast, both NT and levocabastine, but not SR48692, induced a sustained (> 60 min) activation of the mitogen-activated protein kinases, p42/p44, indicating functional coupling of NTS2 receptors. Complementary experiments carried out on synaptosomes from adult rat cerebellum demonstrated the presence of presynaptic NTS2 receptors. However, in contrast to perikaryal NTS2 sites, these presynaptic receptors did not internalize in response to NT stimulation. Taken together, the present results demonstrate that NTS2 receptors are present both pre- and postsynaptically in central neurons and that NT and levocabastine act as agonists on these receptors.

Key words: Neurotensin, NTS2, endocytosis, confocal microscopy, intracellular calcium, signaling.
INTRODUCTION

Neurotensin (NT) is a tridecapeptide documented to act as a neurotransmitter/neuromodulator in the central nervous system (CNS) and as a local hormone in the periphery (for review, see 1). In the CNS, NT has been shown to modulate dopamine transmission in nigrostriatal and mesolimbic pathways (2) and to play a role in the regulation of pain, temperature, appetite, and pituitary hormone secretion (for review, see 3).

NT exerts its central and peripheral effects through interaction with specific membrane receptors. Three different sub-types of NT receptors, referred to as NTS1, NTS2 and NTS3, have been cloned (for review see 4). Although most of the documented effects of NT appear to be exerted through the high affinity, NTS1 receptor, recent studies suggest that the levocabastine-sensitive, low affinity NTS2 sub-type may be responsible for the mediation of NT’s antinociceptive actions (5).

Originally referred to as an acceptor site (6), NTS2 has since been demonstrated to correspond to a bona fide, 7 trans-membrane domain, G protein-coupled receptor (7). Autoradiographic (6, 8, 9) and in situ hybridization (10, 11) studies revealed that NTS2 is widely expressed throughout the rodent CNS although it appears late during ontogeny (~ 1 month after birth in the rat) (10, 12, 13). NTS2 mRNA was detected in both neurons (10) and reactive astrocytes (14). However, whereas the binding and internalization properties of NTS2 have been characterized in astrocytes (14), nothing is known of its pharmacological and functional properties in neurons.

NT, levocabastine, and the NTS1 antagonist SR48692 were all found to induce an inward calcium-activated chloride current in Xenopus oocytes transfected with cDNA encoding the mouse NTS2 receptor (7, 15). By contrast, SR48692, but neither NT nor levocabastine, were
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capable of activating classical second messenger systems, including Ca^{2+} mobilization, inositol phosphate production (InsPs), or MAP kinase phosphorylation in mammalian cells transfected with human NTS2 (16). Furthermore, the effects of SR48692 were blocked by NT and levocabastine, suggesting that these drugs could act as competitive antagonists at NTS2 sites (16). Yet, mouse NTS2 receptors expressed in transfected epithelial cells were reported to internalize upon NT binding (17), a property usually associated with activation by agonists.

The aim of the present study was to characterize the binding, internalization and signaling properties of NTS2 receptors in central neurons and to determine whether NT is agonist or antagonist at these sites. These properties were tested on rat cerebellar granule cells, as these cells express among the highest concentrations of NTS2 mRNA in the brain (10) and can be maintained for several weeks in culture (18), a feature required by the late ontogenetic appearance of the receptor. We also examined the pharmacological properties of NTS2 receptors in synaptosomes from adult rat cerebellum, to discriminate putative presynaptic from postsynaptic features.
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MATERIAL and METHODS

All animal-related procedures were approved by McGill and Sherbrooke University Animal Care Committees and carried out according to the regulations of the Canadian Council on animal care.

Autoradiographic localization of NTS2 binding sites

Adult male Sprague-Dawley rats (220-250 g; Charles Rivers, St. Constant, Quebec, Canada) were killed by decapitation and their brains were rapidly frozen by immersion in liquid isopentane at -40°C for 30 sec. The brains were then deposited on dry ice for 10 min and stored at -80°C until use. Sagittal sections (20 µm-thick) were cut on a cryostat at -18°C, mounted onto polylysine-coated slides, and stored at -20°C for at least 24 h before further processing. For $^{125}$I-Tyr$^3$-neurotensin ($^{125}$I-NT) labeling, sections were equilibrated at 4°C for 5 min in ice-cold binding buffer (50 mM Tris-HCl, 5 mM MgCl$_2$, pH 7.4 and 0.2% bovine serum albumin (BSA)). They were then incubated with 8 nM $^{125}$I-NT (100 Ci/mmol) (kindly provided by Dr. Jean Mazella, Sophia-Antipolis, France), with or without 1 µM levocabastine, for 60 min at 4°C in 125 µl of binding buffer supplemented with 0.8 mM 1,10-phenanthroline. Additional sections were incubated in the presence of $10^{-3}$ M unlabeled NT for the determination of non-specific binding. After incubation, sections were washed twice for 2 min at 4°C in 50 mM Tris-HCl, pH 7.4, containing 0.2% BSA and again twice for 2 min at 4°C in 50 mM Tris-HCl, pH 7.4. Autoradiographs were obtained by apposition of $^{125}$I-labeled sections to $\beta$max-hyperfilm (Amersham) for 2 weeks at room temperature in a light-proof X-ray cassette. Films were scanned using an AGFA Duoscan T1200 scanner at 1200 ppi resolution. The resulting TIFF files were processed using adobe photoshop 6.0 and Deneba’s Canvas 7 imaging software on an Apple PowerBook G3.
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**Cerebellar granule cell cultures**

Primary cultures of mixed cerebellar cells were prepared as described by Cambray-Deakin (19), with the following modifications. Cerebelli (4-6 per culture) from 7-9 days old Long Evans rats were isolated and minced with fine scissors. Tissue was transferred to buffer A (0.25 mg/ml trypsin and 200 µl solution 4 (BSA fraction V (150 mg/ml), 0.7 M glucose, 75 mM MgSO₄·7H₂O) in 10 ml Earle’s Balanced Salt Solution, (EBSS)) and incubated for 15 min in a shaking water-bath at 37°C. Buffer B (0.4 mg/ml soybean trypsin inhibitor, 200 µl solution 4, 25 U DNAse I, and 6 mM MgSO₄·7H₂O in 10 ml EBSS) was then added to the tissue mixture and the suspension centrifuged for 10 sec at 180 g to pellet the tissue chunks. The cells were then dispersed mechanically by repeated gentle pipetting through a sterile siliconized Pasteur pipette in 1.5 ml of buffer C (0.4 mg/ml soybean trypsin inhibitor, 200 µl solution 4, 75 U DNAse I, and 6 mM MgSO₄·7H₂O in 10 ml EBSS). After 1-2 min settling time, the supernatant was passed through a 4% BSA gradient and then centrifuged at 180 g for 5 min at room temperature. The cell pellet was suspended in culture medium [Minimum Essential Medium containing 10% horse serum, 0.4 mM L-glutamine, 1.2 mg/ml glucose, 0.36 mg/ml KCl and 0.25% penicillin-streptomycin (GIBCO, Burlington, Ont, Canada)] and seeded into petri dishes pre-coated with poly-D-Lysine (0.1 mg/ml) at an initial plating density of 1000 cells/mm². Cells were grown in a humidified atmosphere of 95% air, 5% CO₂, at 37°C and used for experiments after 20 days in culture.

**RNA extraction and Reverse Transcription-Polymerase Chain Reaction analysis**

Total RNAs were extracted from cultured cerebellar granule cells as well as from adult rat brain using the method of Chomczynski and Sacchi (20). These total RNAs (2 µg) were then reverse-transcribed at 42°C for 1 h using 1 µg of oligo(dt)₁₅ primer (reverse transcription system
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kit, Promega) and 30 U of AMV reverse transcriptase (H.C.) in a total volume of 20 µl of the supplied buffer. The reaction was stopped by heating at 95°C for 5 min. First strand cDNAs were subjected to 35 cycles of PCR in 25 µl of a final reaction volume containing 50 mM KCl, 10 mM Tris pH 9, 1.5 mM MgCl2, 0.1% Triton X-100, 0.02% BSA, 200 µM dNTPs, 0.5 U Taq DNA polymerase, and 100 ng of either one of the following three pairs of sense and antisense primers. The first pair (5’-ACACCCATTGTGGACACAGCC-3’ and 5’-TTCATCCGAGATATAGCAGAA-3’) allowed the amplification of a fragment of NTS1 receptor cDNA from bases 676-1011 in the sequence reported previously (21). The predicted size of the amplified fragment was 335 bp. The second pair (5’-GAATGTGCTGGTGTCCTTCGC-3’ and 5’-ACTTGTATTTCTCCCAGGCTG-3’) allowed amplification of a fragment of NTS2 receptor cDNA from bases 667-1287 in the sequence reported previously (22). The predicted sizes of the amplified fragments were 620 bp for NTS2 and 439 bp for the splice variant form of this receptor (23). The third pair (5’-TCCCGAGAACTCTGGAAAGGT-3’ and 5’-CACAGAGCGAAGAGGAAACG-3’) allowed amplification of a fragment of NTS3 receptor cDNA from bases 255-681 in the sequence reported previously (24). The predicted size of the amplified fragment was 426 bp. Amplification was carried out with a first cycle at 94°C for 3 min, 52°C for 2 min, 72°C for 1.5 min, followed by 34 cycles at 94°C for 45 sec, 52°C for 40 sec, 72°C for 1 min, and a final extension step at 72°C for 8 min. PCR products were analyzed on a 2% agarose gel. In all reverse transcription experiments, 2 types of controls were performed: (1) each total RNA sample was subjected to reverse transcription (RT) in the absence of enzyme to control for intrinsic contamination by genomic DNA and (2) the reaction was performed on the RT mixture without RNA added to control for contamination during the experiment.

Binding of 125I-NT to cerebellar granule cells
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To determine the binding parameters of $^{125}\text{I}$-NT to cultured granule cells and to investigate whether specifically bound $^{125}\text{I}$-NT was internalized in these cells, three sets of binding experiments were carried out.

For saturation studies, the culture medium was discarded from 12 mm dishes containing $2 \times 10^5$ cells and the cells were equilibrated for 10 min at 37°C in Earle's buffer (140 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 0.9 mM MgCl$_2$ and 25 mM HEPES, pH 7.4) supplemented with 0.1% glucose and 0.1% BSA. Equilibration buffer was then replaced by 250 µl of Earle’s buffer containing 0.1 to 16 nM of $^{125}\text{I}$-Tyr$^3$-NT isotopically diluted with unlabeled NT in the presence of 0.8 mM 1,10-phenanthroline for 30 min at 37°C. At the end of the incubation, cells were washed twice with 1 ml of equilibration buffer and harvested with 1 ml of 0.1 M NaOH. The associated radioactivity was then counted in a $\gamma$ counter. Dissociation constant ($K_d$) and maximal binding capacity ($B_{\text{max}}$) were derived from Scatchard analysis of the data.

Competition experiments were carried out on membranes freshly prepared from cerebellar granule cells. For this purpose, cells were scraped off the culture dishes with ice cold phosphate-buffered saline and centrifuged in microcentrifuge tubes at 15,000 g for 5 min at 4°C. The pellet was homogenized by incubation in hypotonic TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and sonicated. Membrane homogenates were then recovered by centrifugation at 15,000 g for 30 min at 4°C. Cell membranes (50 µg) were incubated with 2 nM $^{125}\text{I}$-NT (100 Ci/mmol) for 30 min at 25°C in 250 µl of binding buffer (50 mM Tris-HCl, pH 7.5, containing 0.2% BSA and 0.8 mM 1-10 phenanthroline) in the presence of increasing concentrations (from $10^{-11}$ to $10^{-5}$ M) of non-radioactive NT, N$\alpha$-Bodipy-NT (2-13), levocabastine (kindly provided by Janssen Research, Beerse, Belgium ; 6), or SR48692 (kindly provided by Sanofi Research, Toulouse, France ; 25). Binding experiments were terminated by addition of 3 ml of ice-cold buffer followed by filtration through cellulose acetate filters (Sartorius). Filters were then washed twice with 3 ml of ice-cold
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buffer and the radioactivity retained in them was counted in a γ counter. IC₅₀ values were determined from inhibition curves as the unlabeled ligand concentration inhibiting 50% of ¹²⁵I-Tyr³-NT specific binding.

To investigate ¹²⁵I-NT internalization, whole cells were incubated with 0.4 nM ¹²⁵I-NT in Earle’s buffer, with or without 0.45 M sucrose, for 45 min at 37°C. At the end of the incubation, cells were washed twice with either 1 ml of Earle’s buffer, or with 1 ml of a hypertonic acid buffer (Earle’s buffer containing 0.2 M acetic acid and 0.5 M NaCl, pH 4) for 3 min to strip off surface-bound radioactivity. Cells were then harvested with 1 ml of 0.1 M NaOH and associated radioactivity was counted in a γ counter. Nonspecific binding, as measured in the presence of 1 µM unlabeled NT, represented less than 5% of the total binding. All binding/internalization data were calculated and plotted using Prism 3.02 (Graph Pad Software) and represent the mean ± standard deviation (S.D.) of n determinations (as indicated in Results).

Binding of Nα-Bodipy-NT (2-13) (fluo-NT) to cerebellar granule cells

For fluo-NT labeling, cerebellar granule cells were grown on 12-mm polylysine-treated glass coverslips in 18 mm Petri dishes. Cells were equilibrated for 10 min at 37°C in Earle’s buffer containing 0.2% BSA and 0.1% glucose and then incubated for 10 or 40 min in the same buffer with 20 nM fluo-NT (kindly provided by PerkinElmer Life Sciences), in the presence or absence of 10⁻⁵ M non-fluorescent NT, 10⁻³ M levocabastine, or 10⁻³ M SR48692.

To determine whether fluo-NT internalization proceeded through clathrin-coated pits, a second set of experiments was performed on whole cells by adding or not 0.45 M sucrose, 10 µM phenylarsine oxide (PAO. Sigma, Ontario, Canada) or 1 µM monodansylcadaverine (MDC, Sigma, Ontario, Canada) to both the equilibration and binding buffers.
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At the end of the incubation, cells were washed twice with either binding buffer or with hypertonic acid buffer (pH 4) for 3 min. The cells were then either air-dried and mounted on glass slides with Aquamount, or further processed for immunocytochemistry as described below.

Double immunofluorescence labeling

To identify cell types and intracellular compartments in which fluo-NT had been internalized, fluo-NT-incubated granule cells were fixed with 4% paraformaldehyde (PFA) containing the cross-linking agent BS3 (Bis(sulfosuccinimidyl) suberate ; 5 mM ; Pierce) for 15 min at room temperature. The cells were then washed twice with Earle’s buffer, rinsed twice with phosphate-buffered-saline (PBS), pre-incubated with PBS containing 10% normal goat serum (NGS), 0.05% Triton X-100, and 2% BSA for 20 min, rinsed again with PBS, and incubated for 60 min at room temperature with one of the following mouse antibodies : anti-glial fibrillary acidic protein (GFAP ; 1:200 ; Sigma) ; anti-neurofilament (N52 ; 1:200 ; Sigma) ; anti-syntaxin 6 (3 µg/ml ; Transduction Laboratories). All primary antibodies were diluted in PBS containing 0.5% normal goat serum (NGS) and 0.02% Triton X-100. After rinsing three times (5 min each) with PBS, bound primary antibodies were revealed with goat anti-mouse antibodies conjugated to either fluorescein isothiocyanate (FITC) (I.C.N ; diluted 1:50 in PBS) or to Alexa 488 (Molecular Probes ; diluted 1:500 in PBS) for 60 min at room temperature. After washing, the coverslips were mounted on glass slides using Aquamount and examined with either a Nikon Eclipse TE300 microscope equipped for epifluorescence using a B-1E FITC filter set (Nikon, Mississauga, Ontarion, Canada) or a Zeiss laser scanning microscope (CLSM 410) equipped with an Axiovert 100 inverted microscope and an argon/Krypton laser. To determine the proportion of neurons endowed with NTS2 receptors, the percentage of N52-immunoreactive
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neurons exhibiting fluo-NT labeling was determined in 12 wells from 3 different experiments (total of 425 neurons counted) and the results were expressed as a mean ± S.E.M.

**Intracellular calcium measurements**

For intracellular calcium ([Ca\(^{2+}\)]\(_i\)) measurements, cells were cultured for 21 days on plastic coverslips (Sartstedt, St-Laurent, Qué, Canada) and incubated in serum-free Dulbecco’s Modified Eagle’s Medium (Invitrogen, Burlington, Canada) supplemented with 5 µM fluo-4/AM (Molecular Probes, Eugene, OR, Canada) at 37°C for 30 min. Cells were then washed three times with 0.5% BSA and further incubated in PBS-HEPES (140 mM NaCl, 5.4 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\).6H\(_2\)O, 10 mM HEPES, pH 7.35) at 37°C for 30 min to allow for hydrolysis of the AM form. The coverslips were then mounted on the stage of Nikon Eclipse TE300 inverted microscope (Nikon, Mississauga, Ontario, Canada).

Cells were maintained at 37°C throughout the experiments with a heating Peltier element. NT, levocabastine or SR48692, diluted in 1 ml of fresh PBS-HEPES were added to the incubation medium, individually or in combination, at concentrations of 10\(^{-8}\)-10\(^{-6}\) M. Images were acquired each 10 s using a CoolSnap f\(_x\) digital CCD camera (Roper Scientific, Tucson, AZ) cooled at -35°C. Band pass filters (450-490 nm) and (520-560 nm) were used for excitation and emission, respectively. Average intensity for each cell was measured on line using the Metafluor software package (Universal Imaging Corp, West Chester, PA). Further analyses were performed with the Metafluor software on images stocked on the hard disc of an IBM-compatible computer. Each Ca\(^{2+}\) curve represents the average response (± SD) of n cells as indicated in figure legends. Statistical significance was verified using Student’ t-test.
 measurement of inositol phosphate (InsPs) accumulation

Experiments were performed as described previously (26). Briefly, cells were grown for 21 days in MEM culture medium containing 10% horse serum and labeled for 18 hours with fresh medium containing 5 μCi/ml of myo-[³H]-inositol. The radioactive medium was then discarded and the cells were incubated in isotope-free culture medium. After 1 hour, cultures were equilibrated for 15 min in HBS(Hank’s Balanced Salts Solution) -glucose/LiCl (10 mM). The stimulation was performed for 15 min using NT (10⁻⁷ M and 10⁻⁶ M), levocabastine (10⁻⁷ M and 10⁻⁶ M), SR48692 (10⁻⁷ M and 10⁻⁶ M) or fluoroaluminate (AlF₄⁻). Incubation was stopped by aspiration of the medium and rapid addition of 1 ml of 5% (v/v) HClO₄ and 200 μl of BSA (20 mg/ml). InsPs were separated by ion exchange chromatography on Dowex 1 X 8 columns. Radioactivity incorporated in the InsPs fractions was determined by scintillation counting in gel phase in a Beckman beta counter, with a counting efficiency of 18%. Values correspond to the mean ± S.E.M. of three independent experiments, each performed in triplicate.

Western blotting analyses of Mitogen-activated protein kinases, p42/p44

Cultured granule cells (21 days post-plating) were incubated for various time intervals (from 1 to 120 min) at 37°C in culture medium in the presence of NT (10⁻⁵ M and 10⁻⁷ M), levocabastine (10⁻⁶ M), or SR48692 (10⁻⁷ M and 10⁻⁶ M). The reaction was stopped by aspiration of the medium and addition of HBS-glucose (Hank's Balanced Salts Solution) containing 0.1 μM staurosporine and 1 mM sodium orthovanadate (Na₃VO₄). Cells were then lysed at 4°C in 50 mM HEPES, pH 7.8 containing 1% Triton X-100, 0.1 μM staurosporine, 1 mM Na₃VO₄, and Complete™ protease inhibitor (Roche Diagnostic Laboratories, Montreal, Qué, Canada). Cell extracts were centrifuged at 8,000 g for 15 min at 4°C and the supernatants were stored at -20°C until use.
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Equal amounts of proteins (30 µg) were separated on 10% sodium dodecyl sulfate (SDS)-
polyacrylamide gels. Western blots were performed as previously described (27). Briefly,
polyvinylidene difluoride (PVDF) membranes (Roche Diagnostic Laboratories) containing
proteins were incubated for 2 hours at room temperature with anti-phosphorylated p42/p44
\text{mapk} (dilution 1:1,000, New England Biolabs, Missisauga, Ont, Canada), anti p42/p44
\text{mapk} (dilution 1:1,000, New England Biolabs, Missisauga, Ont, Canada) antibodies, followed by four washes
with TBS-Tween 20 buffer. Detection was accomplished using horseradish peroxidase-conjugated
anti-rabbit or anti-mouse antibodies (1:2,000, Amersham Pharmacia) and an enhanced
chemiluminescence (ECL) detection system (Roche Diagnostics Laboratories).

To quantitate the effects of NT and SR48692 on p42/p44\text{mapk} phosphorylation, the ratios of
phosphorylated p42/p44\text{mapk} over total p42/p44\text{mapk} levels were determined by densitometry, using
Molecular Dynamics’ Image Quant Imaging software. Statistical significance was verified using
Barlett’s test and P values were obtained from Dunett’s tables. Calculations and statistical
analyses were performed using excel 2000 (Microsoft) and Prism 3.02 (Graph Pad Software).

Binding of $^3$H-NT to rat cerebellar synaptosomes

To determine whether cerebellar granule cells did target NTS2 receptor proteins
presynaptically, synaptosomes were prepared from the cerebells of rat. Rats were killed by
decapitation and their brains were rapidly removed. The cerebells from ten rats were pooled and
homogenized with a hand glass homogenizer using a Teflon-coated pestle rotating at 900 rpm in
ice-cold HEPES, pH 7.4 containing 0.32 M sucrose and peptidase inhibitors. The homogenate
was centrifuged at 750 g for 5 min at 4°C and the resulting supernatant was recuperated and
further centrifuged at 12,000 g for 15 min at 4°C. The synaptosomal P2 pellet was equilibrated in
0.32 M sucrose and centrifuged at 14,500 g for 15 min at 4°C and then resuspended in modified
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Earle’s buffer (150 mM choline chloride, 5 mM KCl, 1.8 mM CaCl₂, and 0.9 mM MgCl₂) for experimental use.

To assess morphological preservation, synaptosomes were fixed with 2% acrolein-2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). To test the effects of experimental manipulations on morphological integrity, synaptosomes were pre-incubated for 25 min in Earle’s buffer, rinsed or not with a hypertonic solution (pH 4) containing 0.5 M NaCl and fixed as above. Synaptosomes were post-fixed with 2% osmium tetroxide for 30 min and rinsed with 0.1 M PB. They were then dehydrated in graded alcohols, embedded in Epon, pelleted in a plastic mold by centrifugation (11,000 g) and polymerized for 4 days at 60°C. Ultrathin sections (80 nm) were cut on an ultramicrotome and counterstained with uranyl acetate and lead citrate prior to examination with a JEOL 100CX electron microscope (Peabody, MA).

For saturation binding experiments, 100 µg of synaptosomal proteins were incubated in 250 µl of Earle’s buffer containing increasing concentrations (from 0.5 to 10 nM) of ³H-NT (108 ci/mmol, NEN) and 0.8 mM 1,10-phenanthroline for 25 min at 37°C. For determination of non-specific binding, additional samples were incubated in the presence of 1 µM non-radioactive NT. Binding was terminated by two successive additions of 12 ml ice-cold Earle’s buffer and filtering under vacuum through GF/B filters presoaked for 1-2 hours at 4°C in Earle’s buffer containing 0.3% polyethylenimine. After incubation for > 5 hours with 10 ml of Safe liquid Scintillation cocktail (ICN Biomedicals), the radioactivity was measured in a Beckman beta counter with a counting efficiency of 30%.

For competition experiments, synaptosomes were incubated for 20 min at 37°C in Earle’s buffer containing 3 nM ³H-NT and increasing concentrations (from 10⁻¹¹ to 10⁻³ M) of non-radioactive NT, levocabastine, or SR48692. The binding was terminated and the radioactivity counted as described above.
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To determine the proportion of internalized $^3$H-NT, synaptosomes were incubated at 37°C in Earle’s buffer containing 3 nM $^3$H-NT for intervals ranging between 2 and 20 min. They were then rinsed for 3 min with either ice-cold Earle’s buffer or with a hypertonic acid solution (pH 4) and the radioactivity was counted as above. Binding data were calculated and plotted using Prism 3.02 (Graph Pad Software) and represent the mean ± standard deviation from two experiments performed in triplicate.
RESULTS

**Autoradiographic distribution of \(^{125}\text{I}-\text{NT}-\text{labeled NTS2 binding sites in rat cerebellum}\)**

In order to determine whether the distribution of NTS2 binding sites correlated with the documented expression (10) of NTS2 mRNA in rodent cerebellar cortex, sagittal sections from the adult rat brain were incubated with 8 nM \(^{125}\text{I}-\text{Tyr}^3\)-NT in the presence or absence of an excess of the NTS2 ligand, levocabastine. In the absence of levocabastine, high levels of specific (i.e. NT-displaceable) \(^{125}\text{I}-\text{NT} binding were observed throughout the cerebellar cortex (Fig. 1A, n = 2). Cerebellar \(^{125}\text{I}-\text{NT} labeling was totally abolished in the presence of levocabastine indicating that it concerned exclusively NTS2 sites (Fig. 1B).

**NT receptor expression in cultured cerebellar granule cells**

After 21 days in culture, cerebellar granule cells exhibited highly refringent, rounded perikarya and formed a dense network of highly ramified processes superimposed over a monolayer of glial cells (Fig. 2).

In order to determine which of the cloned NT receptors were expressed in these cultures, cell homogenates were subjected to Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis of their mRNA content using three different pairs of primers designed to selectively recognize the cloned rat NTS1 (21), NTS2 (22), and NTS3/gp95 sortilin (24) receptors. As shown in Fig 3, PCR amplification of mRNA with NTS2 probes yielded two bands of respectively 620 bp and 439 bp in size. These two bands were of the same molecular weight as the unspliced and spliced variants of the NTS2 receptor amplified from rat brain extracts (23). Probing amplified mRNA with NTS3 receptor primers yielded a single band of 426 bp, corresponding to the molecular weight of the cloned NTS3 receptor (24). A fragment of identical size was also amplified...
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from mRNA prepared from rat brain extracts. No hybridization signal was detected in cerebellar culture extracts probed with NTS1 primers. However, NTS1 fragments of appropriate size (335 bp) were amplified in rat brain extracts using the same pair of primers, demonstrating the efficacy of the probes. Finally, no signal was detected when transcribed products from cultured cells were amplified with either one of the sense or antisense primers alone (not shown).

**Binding and internalization of 125I-NT in cerebellar granule cell cultures**

Incubation of 21 day-old granule cell cultures with increasing doses of 125I-NT for 30 min at 37°C revealed the presence of specific (i.e. NT-displaceable) and saturable 125I-NT binding (Fig. 4A). Scatchard analysis of the data yielded a linear plot, indicating that the binding involved a single apparent population of sites (Fig. 4A, insert). The deducted dissociation constant (Kd) of 125I-NT binding to these sites was 3.1 ± 0.1 nM and the maximal binding capacity (B_max) was 50.5 ± 1.7 fmol/mg (n = 5).

The ability of various unlabeled peptide and nonpeptide compounds to compete with 125I-NT binding in membrane preparations from cultured granule cells is illustrated in Fig 4B and Table 1. Levocabastine (IC_{50} = 5.7 ± 0.5 nM) was almost as potent as unlabeled NT (IC_{50} = 3.2 ± 0.1 nM) in inhibiting 125I-NT binding (Fig. 4B, n = 2). The fluorescent analog Nα-Bodipy-NT (2-13) (fluo-NT) also competed with specific 125I-NT binding, but with less potency than unlabeled NT (IC_{50} = 9.2 ± 0.3 nM). The nonpeptide NTS1 antagonist, SR48692, was the least potent of all drugs tested with an IC_{50} value of 203 ± 21 nM (Fig. 4B).

To determine whether 125I-NT internalized through NTS2, association kinetics of 125I-NT binding were performed on whole cells at 37°C and the proportion of sequestered radioactivity was determined after hypertonic acid-wash of surface bound molecules. In the absence of any pretreatment, 125I-NT bound specifically to intact cultures in a time-dependent manner (Fig. 4C, n = 4). Specific 125I-NT binding reached a plateau within 20 min. At that time, removal of surface-bound
radioactivity by acid-NaCl wash revealed that 64.4 ± 0.7% of total bound $^{125}$I-NT was intracellular (Fig. 4C). After preincubation and labeling of the cells in the presence of 0.45 M sucrose, which inhibits internalization via clathrin coated-pits (28), the amount of radioactivity specifically associated with the cells at equilibrium was about 75.5 ± 0.9% of that measured in the absence of treatment (Fig. 4C). This bound radioactivity was almost entirely washed off by hypertonic acid buffer treatment, indicating that the binding was confined to the cell surface (Fig. 4C). Taken together, these data suggest that in cerebellar granule cell cultures, NT internalizes in an NTS2-mediated, clathrin-dependent manner.

**Binding and internalization of Fluo-NT in cerebellar granule cell cultures**

To visualize NT binding and internalization, 21 day-old cerebellar granule cell cultures were incubated at 37°C with 20 nM fluo-NT. Following 10 min of incubation, punctate fluorescent labeling was evident over both perikarya and processes of neuronal cells (Fig 5A, B). This labeling was specific in that it was entirely competed for by an excess of non-fluorescent NT (not shown). Hypertonic acid wash confirmed that the bulk of this fluorescent signal was intracellular (Fig. 5B). In keeping with the results of radioligand binding studies, fluo-NT labeling was totally abolished when the incubation was carried out in the presence of an excess of levocabastine or of SR48692 (not shown). When the incubation was carried out in the presence of 0.45 M sucrose, phenylarsine oxide, or monodansylcadaverine, specifically bound fluo-NT molecules remained clustered on the cell surface (Fig. 5C). This peripheral labeling was exclusively surface-bound since it was entirely strippable by hypertonic acid wash (Fig. 5D).

Between 10 and 40 min of incubation, hot spots of internalized fluo-NT hot spots coalesced into a single juxta-nuclear fluorescent cluster (Fig. 6A, B). Immunostaining of fluo-NT-labeled cells with an antibody against syntaxin-6, which selectively labels the recycling endosome/Trans Golgi
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Network (TGN) complex (29), revealed that this juxta-nuclear compartment corresponded to the TGN (Fig. 6C).

To confirm the identity of fluo-NT-labeled cells, fluo-NT labeling was combined with immunocytochemical detection of selective neuronal or glial markers. Combined epifluorescence visualization of internalized fluo-NT (Fig. 6D) with the glial marker GFAP (Fig. 6E) showed a complete lack of fluo-NT uptake by GFAP-positive glial cells (Fig. 6F). In contrast, dual localization of fluo-NT (Fig. 6G) and of the neuronal marker N52 (Fig. 6H) showed that all cells labeled with fluo-NT were N52-positive and thus corresponded to neurons (Fig. 6I). Conversely, 59.6 ± 0.5% of N52-immunoreactive neurons exhibited fluo-NT labeling suggesting that only a sub-population of cultured granule cells express NTS2 receptors.

Measurements of intracellular calcium

To determine whether NTS2 receptor stimulation resulted in intracellular [Ca²⁺] mobilization, cultured cerebellar granule cells were loaded with the calcium sensitive fluorescent dye, Fluo4 and stimulated with a variety of purported NTS2 agonists. As shown in Fig. 7, neither 1 µM NT (Fig. 7A) nor 1 µM levocabastine (Fig. 7B) were able to stimulate cytosolic [Ca²⁺] mobilization. Yet, intracellular Ca²⁺ stores were readily releaseable in unsuccessfully stimulated neurons since application of the drug thapsigargin (TG), a known inhibitor of the sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) pumps, induced a robust Ca²⁺ signal (Fig. 7A, B).

In accordance with data on heterologous transfection systems (15, 16, 30), perfusion of cerebellar granule cells with the NTS1 antagonist SR48692 (1 µM) caused a marked elevation of free intracellular calcium (Fig. 8A), with a stimulation ratio of 4 to 6 fold over basal values, a level similar to that observed with TG. This increase varied from cell to cell, but consistently showed a biphasic profile with rapid ascending and slow descending slopes (Fig. 8A). This stimulatory
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effect of SR48692 was antagonized neither by prior (Fig. 8B) nor concomitant (Fig. 8C) application of 1 \( \mu \)M NT. Likewise, the SR48692 response was unaffected by concomitant application of 10 \( \mu \)M levocabastine (Fig. 8D). In cells preincubated for 5 min in a Ca\(^{2+}\)-free medium, stimulation with 1 \( \mu \)M SR48692 induced a transient Ca\(^{2+}\) increase (Fig. 8E). Furthermore, the sustained phase of the Ca\(^{2+}\) response to SR48692 was markedly shorted by the addition of 5 mM EGTA (a Ca\(^{2+}\) chelator) to the extracellular medium (Fig. 8F). These responses revealed that the increase in [Ca\(^{2+}\)] was the consequence of both the release of Ca\(^{2+}\) from intracellular stores and the entry of Ca\(^{2+}\) from the external medium.

**Measurements of inositol phosphate accumulation**

To further investigate the putative involvement of the Ca\(^{2+}\)/phosphoinositide pathways in the mechanism of action of the NTS2, the capacity of NT, levocabastine and SR48692 to modulate InsPs accumulation was also assayed. As shown in Fig. 9, neither NT (10\(^{-7}\) or 10\(^{-6}\) M) nor levocabastine (10\(^{-7}\) or 10\(^{-6}\) M) stimulation significantly altered the basal level of InsPs present in control cells. Similarly, SR48692 (10\(^{-7}\) or 10\(^{-6}\) M) application did not modify phosphoinositide turnover. By contrast, after 15 min of stimulation with AlF\(_4\)\(^-\), a non specific activator of all heterotrimeric G-proteins, there was a 3-fold increase in InsPs accumulation, indicating that granule cells in culture were able to produce this class of second messengers.

**NTS2-induced p42/p44\(^{mapk}\) phosphorylation**

Stimulation of granule cells in culture with 0.1 \( \mu \)M NT for periods ranging between 1 and 60 min induced a sustained increase in p42/p44\(^{mapk}\) phosphorylation. This increase was first detectable 5 min after NT application and was maintained for over 60 min thereafter (Fig. 10A; \( n = 4 \)). This effect on MAPK activation was also observed at lower doses of NT (10\(^{-8}\) M) (Fig. 10B, \( n = 3 \)).
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Densitometric analyses of phosphorylated p42/p44\textsuperscript{mapk} over total p42/p44\textsuperscript{mapk} ratios indicated that NT induced a robust increase in MAPK phosphorylation that reached a plateau at approximately 30 min of incubation (Fig. 10F). Similar p42/p44\textsuperscript{mapk} activation was observed following 10 and 30 min of stimulation with 1 µM levocabastine, confirming the implication of NTS2 in the MAPK activation (Fig. 10C; n = 3). By contrast, under the same conditions, stimulation with 0.1 µM (Fig. 10D, F; n = 3) or 1 µM (Fig. 10E, F; n = 2) SR48692 failed to modify the level of p42/p44\textsuperscript{mapk} phosphorylation.

\textit{3H-NT binding to cerebellar synaptosomes}

In order to determine whether NTS2 binding sites were also associated with the terminal arbor of cerebellar granule cells, synaptosomal preparations from adult rat cerebella were incubated with increasing concentrations of \textit{3H-NT} for 25 min at 37°C. Electron microscopy confirmed that these synaptosomes were structurally well preserved and were largely comprised of synaptic terminals endowed with small clear vesicles (Fig. 11A). The affinity (Kd = 2.4 ± 0.5 nM; n = 2) and maximal capacity (B\text{max} = 60.1 ± 2.7 fmol/mg) of \textit{3H-NT} binding to these synaptosomes were similar to those measured in cultured granule cells (compare Figs. 11B and 4A). Furthermore, competition experiments showed that the NTS2 ligand, levocabastine, displaced this specific \textit{3H-NT} binding with an IC\text{50} of 16.5 ± 1.3 nM (Fig. 11C; n = 2), i.e. with the same affinity as in cultured granule cells (Table 1). NT and SR48692 also competed for \textit{3H-NT} binding with IC\text{50} values (6.5 ± 0.2 nM and 142 ± 7.8 nM, respectively) similar to those obtained on membranes from cultured cerebellar granule cells (Fig. 11C; Table 1).

To determine whether specifically bound \textit{3H-NT} was internalized within cerebellar synaptosomes, synaptosomal preparations were incubated for 2-20 min at 37°C with 3 nM \textit{3H-NT} and the proportion of internalized \textit{3H-NT} was assessed following dissociation of surface-bound ligand by hypertonic acid wash (Fig. 7D, n = 2). Association kinetics of \textit{3H-NT} to whole
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synaptosomes at 37°C reached a plateau value between 5 and 10 min (Fig. 11D). As opposed to what was observed on cultured granule cells (Fig. 4C), specific $^3$H-NT binding was entirely strippable by hypertonic acid wash, indicating that the bound molecules were confined to the cell surface (Fig. 11D).
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DISCUSSION

The present study reveals that NTS2 neurotensin receptors are associated with somatodendritic and terminal arbors of rat cerebellar granule cells. It also provides the first demonstration that NTS2 is a functional receptor in neurons and that both NT and levocabastine may act as agonists at this site.

Selective expression of NTS2 receptors by cerebellar granule cells

Receptor autoradiography demonstrated high concentrations of $^{125}$I-NT binding sites in sections of rat cerebellum. Virtually no residual $^{125}$I-NT-labeling was observed in the presence of levocabastine, confirming the lack of NTS1 receptors in this structure (6, 8, 31). Radiolabeled NTS2 receptors were most conspicuous in the granule cell layer, in keeping with earlier in situ hybridization data which demonstrated high expression of NTS2 mRNA in granule cells of mouse cerebellar cortex (10). Relatively dense autoradiographic labeling was also observed in the molecular layer. This labeling most likely corresponds to presynaptic NTS2 receptors associated with granule cell axons (parallel fibers) since neither $^{125}$I-NT-labeling (present study) nor NTS2 mRNA (10) were detected over Purkinje cells, the dendrites of which form the bulk of the molecular layer. This interpretation is also supported by the demonstration of presynaptic NTS2 receptors in synaptosomes prepared from adult rat cerebellum (vide infra). Nonetheless, the possibility that a small proportion of NTS2 receptors labeled in this layer might be associated with stellate or basket cells cannot be completely excluded since sparse interneurons were reported to express NTS2 mRNA in mouse cerebellar cortex (10).

RT-PCR analysis of cerebellar granule cell cultures revealed the presence of mRNA for the two NTS2 receptor variants previously detected in whole brain extracts (23). Although the present


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Experiments did not allow to determine whether NTS2 mRNA was of neuronal or glial origin, the fact that only the larger form of NTS2 receptor mRNA was detected in astrocytes cultured from rat cerebral cortex (14) suggests that the shorter form of the NTS2 receptor may be selectively neuronal. In any event, fluorescence imaging experiments using fluo-NT and specific neuronal or glial markers demonstrated that in our culture preparations, NTS2 receptor proteins were only present in neurons.

In keeping with the results of autoradiographic binding data, no NTS1 mRNA was detected by RT-PCR in mixed cerebellar cultures. However, NTS3/sortilin mRNA was detected in this preparation suggesting that NTS3 receptors are present in cerebellar granule and/or glial cells. The paucity of NTS3 receptor mRNA detected by in situ hybridization over granule cells in sections of adult rat cerebellum (Sarret et al., unpublished data) suggests that the NTS3 message detected here by RT-PCR is of glial origin.

Binding experiments carried out on cerebellar granule cell cultures demonstrated saturable $^{125}\text{I}$-NT binding to a single apparent population of sites. The affinity of these sites for the radiolabeled ligand was very close to that reported previously for NTS2 receptors transfected in either COS-7 (7, 22), HEK 293 (17), or CHO (16) cells. It also conformed to that of levocabastine-sensitive NT binding measured in rat brain (6, 32). Specific $^{125}\text{I}$-NT binding was entirely competed for by the NTS2 ligand, levocabastine, suggesting that if translated in granule cell cultures, the NTS3 receptor was not recognized by the radioactive ligand. This lack of recognition by NTS3 is consistent with the reported low-affinity of this site for NT in heterologous transfection systems (40 nM; 33). The IC$_{50}$ of levocabastine in granule cell cultures (5.7 ± 0.5 nM) was similar to that reported in epithelial cells transfected with either rat (22) or mouse (7, 17) NTS2. However, it was markedly higher than that reported in CHO cells transfected with human NTS2 (100 nM; 16). $^{125}\text{I}$-NT binding to cerebellar granule cells was also competed for by the NTS1 antagonist
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SR48692, with an affinity comparable to that observed on recombinant NTS2 receptors (7, 16, 17, 22). Taken together, these results indicate that the sole NT receptor recognized by exogenous NT in mixed cerebellar cultures corresponds to the NTS2 sub-type and that this receptor is selectively expressed by granule cells. Not all granule cells appear to express NTS2 receptors, however, since only 60% of cultured neurons, identified using the neuronal marker N52, were found to bind fluo-NT in dual labeling experiments.

Ligand-induced internalization of neuronal NTS2 receptors

Following incubation of cultured cerebellar granule cells with either ¹²⁵I-NT or fluo-NT, approximately 65% of specifically bound ligand was resistant to hypertonic acid wash, indicating that it had been internalized. These results are congruent with those obtained in cells transfected with cDNA encoding the mouse NTS2 receptor (17) but differ from those obtained on cortical astrocytes endogeneously expressing the rat receptor (34). NT internalization was blocked by hypertonic sucrose, PAO and MDC, suggesting that it proceeded via clathrin-coated pits (for review see, 28). Accordingly, confocal microscopy revealed that the internalized ligand was first concentrated within small endosome-like organelles, a pattern consistent with earlier reports of receptor-mediated internalization of fluorescent peptides in neurons (eg. 34, 35, 36). At longer time intervals, internalized fluo-NT was clustered deeper within the cell, in the juxta-nuclear region. Double labeling experiments identified this late targeting compartment as being syntaxin 6-positive, i.e. as corresponding to the Trans-Golgi network (TGN)/pericentriolar recycling endosome (29, 37, 38). Although trafficking of internalized ligand to the TGN has previously been demonstrated in cells transfected with the NTS1 receptor subtype (29), the present data provide the first demonstration that an internalized neuropeptide may be targeted to the TGN in
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neurons. More broadly, the finding that NT internalizes via native NTS2 receptors suggests that NT acts as an agonist on this receptor.

**Functional coupling of neuronal NTS2 receptors**

Despite their good affinity for the NTS2 receptor, neither NT nor levocabastine were able to induce Ca\(^{2+}\) mobilization in cerebellar granule cells loaded with the Ca\(^{2+}\)-sensitive fluorescent dye, Fluo-4. A similar lack of Ca\(^{2+}\) response had been observed following application of the same drugs onto cells transfected with the human NTS2 receptor (16). By contrast, both NT and levocabastine were reported to elicit Ca\(^{2+}\) currents in frog oocytes transfected with the mouse NTS2 receptor, or in CHO cells transfected with the rat NTS2 receptor (7, 15, 30). The present results therefore suggest that endogenously and ectopically expressed NTS2 receptors are differentially coupled to Ca\(^{2+}\)-activating systems.

As previously reported in cells transfected with either mouse (15), rat (30), or human (16) NTS2 receptors, stimulation with SR48692 induced a dose-dependent Ca\(^{2+}\) mobilization in cerebellar granule cells. However, in contrast to what had been reported in heterologous transfection systems, this Ca\(^{2+}\) response was blocked by neither NT nor levocabastine, suggesting that in cerebellar granule cells, the effect of SR48692 on Ca\(^{2+}\) mobilization may not be mediated through NTS2 receptors. The mechanisms by which SR48692 exerts these Ca\(^{2+}\) mobilizing effects remain to be elucidated. Our results demonstrate that they involve Ca\(^{2+}\) mobilization from both intracellular stores and extracellular medium and therefore that they implicate more than one effector. The nature of the mobilized intracellular stores is unknown, although the similarity in the Ca\(^{2+}\) release profiles elicited by stimulation with thapsigargin and with SR48692 in the absence of extracellular Ca\(^{2+}\) suggests that it may correspond to the thapsigargin-sensitive pool previously described in cerebellar granule cells (39, 40). Clearly, intracellularly released Ca\(^{2+}\)
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does not originate from an InsPs-sensitive pool, since none of the NTS2 ligands tested (NT, levocabastine or SR48692) elicited an increase in the concentration of InsPs in cerebellar granule cells. This result differs from those obtained in epithelial cells transfected with the human NTS2, in which stimulation with SR48692, but neither with NT nor levocabastine, induced an increase in inositol phosphate turnover (16).

By contrast, stimulation of cultured cerebellar granule cells with both NT and levocabastine, but not with SR48692, induced a sustained activation of the mitogen-activated kinase p42/p44. Again, this result is at odds with the reported effects of human NTS2 receptor stimulation in transfected CHO cells in which stimulation with SR48692, but with neither NT nor levocabastine, was found to activate the p42/p44\textsuperscript{mapk} cascade (16). These differential effects may be due to differences in the structure of the C-terminal tail of the rat versus the human NTS2 receptor or to differential coupling of endogenously versus ectopically-expressed NTS2 receptors. In any event, the present results provide the first demonstration that neuronal NTS2 receptors are functional and, together with our internalization data, indicate that NT is an agonist on these receptors.

Presynaptic localization of central NTS2 receptors

To determine whether, as suggested by our autoradiographic binding data, cerebellar NTS2 receptors were expressed presynaptically and to characterize the pharmacological properties of these putative presynaptic sites, binding and internalization assays were carried out on synaptosomes freshly prepared from adult rat cerebellar cortex. Binding experiments demonstrated that in these preparations, radiolabeled NT bound to the same apparent population of sites as in cerebellar granule cells in culture. The NT affinity and drug sensitivity of these sites were also similar to those observed in cerebellar granule cells, indicating that they correspond to NTS2 receptors. However, contrary to our observations in cultured granule cells, specific $^3$H-NT
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binding was entirely strippable by hypertonic acid wash, indicating that surface-bound ligand was not internalized. This result cannot be attributed to the type of preparation used, since synaptosomes similarly prepared from rat neostriatum were found to internalize \(^{3}\text{H}\)-NT in an NTS1-dependent manner (41). Therefore, it appears that presynaptic NTS2 receptors, in contrast to somatodendritic NTS2 ones, do not internalize in response to ligand binding. This difference may be due to interaction of NTS2 receptors with distinct internalization-related proteins (e.g. receptor kinases, \(\beta\)-arrestins, amphiphysins, etc) in somatodendritic versus axonal domains.

In conclusion, the present results demonstrate that, in rat cerebellum, NTS2 receptors are localized in granule cells and are functionally coupled to the p42/p44\(^{\text{mapk}}\) cascade and not to the phospholipase C/Ca\(^{2+}\) pathway. Recent studies have shown that stimulation of the MAPK pathway could involve binding of \(\beta\)-arrestins to the GRK-phosphorylated receptor and hence be linked to ligand-induced receptor internalization (for review, see 42). Further studies will be needed to determine whether the NTS2-induced activation of the MAPK pathway reported here occurs in response to \(\beta\)-arrestin binding or as a result of G-protein activation. In any event, the protracted time course of the NTS2 response suggests that cerebellar NTS2 receptors are associated with long term metabolic effects rather than with rapid synaptic-like actions.
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REFERENCES


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1042-1047.


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FIGURE LEGENDS

**Figure 1.**

Autoradiographic distribution of $^{125}$I-Tyr$^3$-NT binding in rat cerebellum. Sagittal sections were incubated with 8 nM $^{125}$I-Tyr$^3$-NT (100 ci/mmol), (A) in the absence or (B) in the presence of 1 μM levocabastine. (A) In the absence of levocabastine, labeled $^{125}$I-NT binding sites are evident in both granule cell (Gc) and molecular cell (ml) layers of the cerebellar cortex. (B) This labeling is reduced to background levels in the presence of levocabastine. Scale bar, 2.5 mm.

**Figure 2**

Representative phase contrast micrograph of a rat cerebellar cell culture, 21 days post-plating. Several small, rounded cell bodies of granular cells (arrows) and numerous neuritic processes (arrowheads) are visible, growing on a layer of glial cells. The morphological appearance of granule cells indicate extensive differentiation. Scale bar, 20 μm.

**Figure 3.**

Expression of NT receptor mRNAs in 21 day-old cerebellar granule cells as compared to the adult rat brain. PCR reactions were performed on mRNAs reverse-transcribed using specific primers for rat NTS1, NTS2 or NTS3. Two bands of 620 bp and 439 bp in size are detected in cerebellar granule cells with NTS2 primers. The large band corresponds to the unspliced form of the rat NTS2 receptor and the 439 bp form to the spliced variant (vNTS2). These bands are of the same molecular weight as in rat brain extracts. No band is detected in cerebellar granule cells by using the NTS1 primers, although a signal migrating as 335 bp, consistent with the size of rat NTS1, is present in whole rat brain extracts. Finally, a single band of 426 bp is observed in...
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cerebellar granule cells using NTS3 primers. This band migrates at the same molecular weight mark as in rat brain extracts.

Figure 4.
Specific binding of $^{125}$I-Tyr$^3$-NT to cerebellar granule cells in culture. (A) Saturation binding experiments performed on whole cells for 30 min at 37°C with increasing concentrations of labeled NT. Scatchard analysis (insert) indicates binding to a single apparent population of sites, with a Kd of 3.1 ± 0.1 nM and a maximal binding capacity ($B_{\text{max}}$) of 50.5 ± 1.7 fmol/mg. Values correspond to the mean ± S.D. of five independent experiments performed in duplicate. (B) Competition inhibition of $^{125}$I-Tyr$^3$-NT (2 nM) binding to membranes of cultured granule cells by non-radioactive NT (open squares), Nα-Bodipy-NT (2-13) (closed circles), levocabastine (closed squares) or SR48692 (open circles). Each point represents the mean of two separate experiments performed in triplicate (means ± S.D.). IC$_{50}$ values are listed in Table 1. (C) Binding kinetics of $^{125}$I-Tyr$^3$-NT to whole cerebellar cells at 37°C. Experiments were performed in the absence (squares) or in the presence (circles) of 0.45 M sucrose. At the indicated times, cells were either washed twice with 500 µl of Earle’s/HEPES/Tris buffer (open symbols) or treated with 500 µl of acid-NaCl buffer (pH4) for 3 min (closed symbols). Values are the means ± S.D. of four independent experiments carried out in duplicate.

Figure 5.
Confocal microscopic images of fluo-NT-labeled cerebellar granule cells in culture. (A) After 10 min incubation with 20 nM Nα-Bodipy-NT (2-13) at 37°C, small fluorescent hot spots are visible throughout the cytoplasm. Note the presence of labeling in both the perikaryon and processes (arrowheads). (B) Hypertonic acid wash of cell surface binding reveals that fluo-NT labeling is
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mainly intracellular (arrowheads). (C) When the incubation is carried out in the presence of 0.45 M sucrose, bound fluorescent molecules remain confined to the periphery of the cell (arrowheads). (D) This peripheral labeling disappears after hypertonic acid wash, confirming that it is associated with the cell surface. N: nucleus. Scale bar, 5 µm.

**Figure 6.**

Time-course of fluo-NT binding internalization in cultured cerebellar granule cells. (A-C) Confocal microscopic images of cells incubated with 20 nM Nα-Bodipy-NT (2-13). (A) After 10 min incubation with fluo-NT at 37°C, the internalized ligand forms numerous hot spots distributed throughout the cytoplasm of the cell (arrows). (B) By 40 min, the bulk of intracellular fluorescence is concentrated in the perinuclear region (arrow). (C) Immunocytochemical labeling of the cell in (B) with antibodies against syntaxin-6 identifies this juxtanuclear compartment as being part of the Trans Golgi Network/pericentriolar recycling endosome complex. (D-I) Epifluorescence localization of internalized fluo-NT (D) and of GFAP (E) in doubled-labeled cells; merged images in F demonstrate that cells internalizing the fluorescent ligand are strictly GFAP-negative (F, arrows); by contrast, co-labeling of Fluo-NT (G, arrowheads) with N52 (H, arrowheads) shows that fluo-NT internalizes in N52-positive, i.e. neuronal cells (I, merged image; arrowheads). Scale bar, 10 µm.

**Figure 7.**

Intracellular Ca$$^{2+}$$ mobilization in cultured cerebellar granule cells. (A) Application of 1 µM NT does not stimulate cytosolic [Ca$$^{2+}$$] mobilisation in fluo4-loaded neurons (n = 18) (successful loading demonstrated by thapsigargin (TG) depletion of intracellular Ca$$^{2+}$$ stores). (B) Similary,
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no Ca\(^{2+}\) response is induced by stimulation with 1 µM levocabastine (n = 15). Each curve represents the mean ± S.D. of n responding cells.

Figure 8.
Intracellular Ca\(^{2+}\) mobilization in fluo4-loaded cultured cerebellar granule cells. (A) Application of 1 µM of SR48692 induces a transient increase in Ca\(^{2+}\) followed by a plateau (n = 50). (B, C) This response is not antagonized by prior (B, n = 42) or concomitant (C, n = 25) application of 1 µM neurotensin (NT). (D) Similarly, the Ca\(^{2+}\) response to SR48692 is unaffected by concomitant application of 10 µM levocabastine (levo, n = 18). (E, F) Ca\(^{2+}\) response to application of 1 µM SR48692 in the absence of extracellular Ca\(^{2+}\) (E, n = 8) or in the presence of 5 mM EGTA (F, n = 15). Curves represent the mean ± S.D. of n responding cells. The peak values from Ca\(^{2+}\) increase in panels, A, B, C, and are not statistically different from one another at P • 0.05 level.

Figure 9.
Inositol phosphate (InsPs) accumulation in 21 day-old cerebellar granule cells. Irrespective of the concentrations used, NT, levocabastine, and SR48692 do not modify the intracellular levels of InsPs in cerebellar granule cells. The ability of these cells to produce InsPs is confirmed by stimulation with AlF\(_3\). Values corresponded to the mean ± S.E.M. of three independent experiments, each performed in triplicate.

Figure 10.
Mitogen-activated protein kinase (p42/p44\(\text{mapk}\)) phosphorylation following activation of NTS2 receptors endogenously expressed in cultured cerebellar granule cells. (A) NT (0.1 µM) induces a time-dependent and sustained increase in MAPK phosphorylation (upper panel), which appears
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after 5 min of incubation and is maintained for over 60 min after NT application. The lower panel corresponds to Western blotting of total p42/p44 mapk in the same samples. (B) The same effect on MAPK activity is observed at lower doses of NT (10^{-8} M). (C) Similar effects are observed following 10 and 30 min stimulation with 1 µM levocabastine, suggesting that p42/p44 mapk are activated through stimulation of NTS2 receptors. (D) Under the same conditions, SR48692 (10^{-7} M) fails to modify the basal level of MAPK phosphorylation. (E) Similarly, no modification of the MAPK phosphorylation pattern is observed following stimulation with 1 µM SR48692. (F) Comparative densitometric analysis of the ratio of phosphorylated p42/p44 mapk over total p42/p44 mapk levels following incubation with 0.1 µM NT (circles, n = 4), 0.1 µM SR48692 (squares, n = 3), or 1 µM SR48692 (triangles, n = 2). Values are the means ± S.E.M. of n experiments as indicated above. * P • 0.05 and ** P • 0.02 as compared to controls (C).

Figure 11.

Pharmacological profile of ^3^H-NT binding to synaptosomes from adult rat cerebellum. (A) Electron microscopic assessment of synaptosomal integrity. Three well preserved axon terminals (AT) show numerous clear synaptic vesicles as well as several large dense cored vesicles (arrows). Scale bar, 0.6 µm. (B) Saturation experiments. Incubations were carried out at 37°C for 25 min with increasing concentrations of ^3^H-NT (0.5 to 10 nM). (C) Competition of ^3^H-NT binding to rat cerebellar synaptosomes by unlabeled NT (open squares), levocabastine (closed squares) and SR48692 (open circles). IC_{50} values are listed in Table 1. (D) Association kinetics of ^3^H-NT binding to synaptosomal preparations. Synaptosomes were incubated with 3 nM ^3^H-NT for increasing time intervals at 37°C and non-specific binding was determined in the presence of an excess of 1 µM unlabeled NT. The proportion of internalized ligand was obtained by comparing association curves before (open squares) and after (closed squares) hypertonic acid treatment.
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Values corresponded to the mean ± S.D. from two independent experiments performed in triplicate.
Table 1. IC\textsubscript{50} values of NT and its related compounds in competition experiments on membrane preparations from cerebellar granule cells\textsuperscript{1} and rat cerebellar synaptosomes\textsuperscript{2}.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC\textsubscript{50} Values\textsuperscript{1} (nM)</th>
<th>IC\textsubscript{50} Values\textsuperscript{2} (nM)</th>
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<tr>
<td>NT</td>
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<td>8.5 ± 0.2</td>
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<td>NT-fluo</td>
<td>-</td>
<td>9.2 ± 0.3</td>
<td>-</td>
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<tr>
<td>Levocabastine</td>
<td>Nonpeptidic</td>
<td>5.7 ± 0.5</td>
<td>16.5 ± 1.3</td>
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<td>SR48992</td>
<td>Nonpeptidic</td>
<td>203 ± 21</td>
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Data are means ± S.D. of triplicate determinations from two independent experiments.
Pharmacology and functional properties of NTS2 neurotensin receptors in cerebellar granule cells
Philippe Sarret, Louis Gendron, Peter Kilian, Ha Minh Ky Nguyen, Nicole Gallo-Payet, Marcel Daniel Payet and Alain Beaudet

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