Identification and Functional Characterization of a 5-Transmembrane Domain Variant Isoform of the NTS2 Neurotensin Receptor in Rat Central Nervous System*

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The present study demonstrated that alternative splicing of the rat nts2 receptor gene generates a 5-transmembrane domain variant isoform (vNTS2) that is co-expressed with the full-length NTS2 receptor throughout the brain and spinal cord, as evidenced by reverse transcription-PCR. The vNTS2 polypeptide is 281 amino acids in length, which is 135 amino acids shorter than the full-length isoform. Immunohistochemical and radioligand binding studies revealed that the HA-tagged recombinant vNTS2 receptor is poorly targeted to plasma membranes in transfected COS-7 cells. Binding studies also showed that the truncated receptor displayed a 5000-fold lower affinity for neurotensin (NT) than its full-length counterpart (IC50 of 10 μM and 2 nM, respectively). Yet NT binding induced efficient internalization of receptor-ligand complexes in vNTS2-transfected cells. Furthermore, it produced a rapid (<5 min) activation of the mitogen-activated protein kinases (ERK1/2) pathway, indicating functional coupling of the variant receptor. This activation is sustained (>1 h) and is also produced by the NTS2 agonist levocabastine. Western blotting experiments suggested that vNTS2 is not expressed in monomeric form in the rat central nervous system. However, it does appear to form a variety of multimeric complexes, including homodimers and heterodimers, with the full-length NTS2. Indeed, co-immunoprecipitation studies in dually transfected cells demonstrated that the two receptor isoforms can form stable associations. Taken together, the present results indicated that the rat vNTS2 is a functional receptor that may play a role in NT signaling in mammalian central nervous system.

The tridecapeptide neurotensin (NT)1 produces a wide array of biological responses when administered peripherally or in the central nervous system (CNS). NT effects include analgesia (1, 2), hypothermia (3), antipsychosis (4), catalepsy (5), and change in blood pressure (6). NT is also known for its regulatory role on midbrain dopaminergic and basal forebrain cholinergic neurons (7, 8), and cumulative evidence has implicated the NT system in the pathophysiology of schizophrenia (9).

NT signaling is mediated by interaction of the peptide with either one of three different receptor subtypes, referred to as NTS1, NTS2, and NTS3. NTS1 and NTS2 belong to the family of seven transmembrane-spanning, G protein-coupled receptors (GPCRs) and exhibit high and low affinity for NT, respectively (10, 11). The NTS3 receptor is a single transmembrane domain sorting receptor with 100% homology to gp95/sortilin (12, 13). NTS1 is predominantly coupled to Gαq11 (14, 15) and activates phospholipase C (16, 17). Pharmacological and biochemical studies have indicated that NTS1 is also involved in the modulation of intracellular levels of cGMP (18), cAMP (19, 20), inositol phosphates (21), and extracellular signal-regulated kinases (ERK1/2) (22). Much less is known about the signaling pathways of NTS2. Stimulation of NTS2 was found to induce Ca2+-dependent chloride currents in Xenopus oocytes expressing the mouse receptor (22). More recent studies have shown that stimulation of NTS2 with either NT or the selective NTS2 ligand, levocabastine, activates the ERK1/2 cascade both in CHO cells stably transfected with cDNA encoding rat or human NTS2 (24) or in cultured rat cerebellar granule cells (25).

The cDNA sequence of the mouse NTS2 receptor is composed of four exons separated by three introns (26). The first exon encodes the region containing TM domains 1–4, whereas exons 2–4 encode the region containing TM 5–6, TM 6, and TM 7, respectively. The existence of a deletion-type NTS2 mRNA encoding a C-terminally truncated form of the receptor, which lacks an internal 181-bp sequence, has been reported in the mouse (27). Both mouse NTS2 mRNA isoforms are derived from a single nts2 gene, the short form resulting from alternative splicing of the primary NTS2 transcript at intron 2a (26). The corresponding truncated NTS2 mRNA encodes a 282-amino acid protein (27).

Other GPCRs encoding sequences have been shown to similarly generate truncated receptor isoforms through alternative splicing, exon skipping, or intron retention (28). Many of these splice variants, such as the truncated forms of the prostanoid hamster ovary cells; COS-7 cells, green African monkey kidney cells; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinases 1/2; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; HRP, horseradish peroxidase; HA, hemagglutinin; RT, room temperature; CNS, central nervous system; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEK, MAPK/ERK kinase; fluo-NT, Nα-BODIPY-neurotensin (2–13).

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1 The abbreviations used are: NT, neurotensin; GPCR(s), G protein-coupled receptor(s); TM, transmembrane domain; CHO cells, Chinese hamster ovary cells; COS-7 cells, green African monkey kidney cells; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinases 1/2; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; HRP, horseradish peroxidase; HA, hemagglutinin; RT, room temperature; CNS, central nervous system; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEK, MAPK/ERK kinase; fluo-NT, Nα-BODIPY-neurotensin (2–13).
receptor EP₃ and endothelin B receptor (39, 30), differ from their full-length counterparts in their intracellular C-terminal tails. Others show a disparity in the third intracellular loop (e.g., the D₃ dopamine receptor variant (31)) or in the transmembrane (TM) domains (e.g., the D₃ dopamine receptor isoform (32)). Yet others differ from the full-length receptor in their extracellular N-terminal loop as exemplified by the truncated form of the angiotensin II receptor (33).

Splice variations may have little or no effect on ligand binding properties (34). However, some deletions, particularly in TM domains, were shown to have significant impact on ligand recognition. For example, a short variant of the 5-hydroxytryptamine 2C receptor lacking TM domains 6 and 7 was reported to be totally devoid of serotoninergic binding activity (35). Similarly, the 5-TM domain isoforms of the D₃ and endothelin A receptors exhibit no ligand binding (32, 36). Shortened receptor isoforms may also display aberrant or impaired coupling, even in the face of normal ligand binding. For instance, the four alternatively spliced isoforms of the EP₂ receptor, which vary only in their C-terminal tails, couple to different G proteins and activate diverse second messenger signaling (29).

The generation of alternatively spliced GPCRs may also affect the function of their full-length counterparts. Thus, co-expression of the full-length gonadotropin-releasing hormone receptor together with that of its C-terminally truncated isoform, which is incapable of ligand binding and signal transduction, was found to impair targeting of the full-length receptor to the plasma membrane (37). Finally, alternative splicing of GPCRs has been associated with a number of genetic disorders (38). For instance, splice variants of the growth hormone-releasing hormone receptors have been documented in primary human prostate carcinomas and diverse human cancer cell lines (39).

This study was initiated to determine whether the splice variant form of the NTS2 receptor originally identified in mouse brain extracts was also expressed in rat brain and to investigate the binding, internalization, and signaling properties of this receptor isoform, as compared with those of the full-length NTS2 receptor, in mammalian cells. Our results demonstrate the existence of a functional 5-TM domain variant form of NTS2 (vNTS2) in rat brain and suggest that this truncated receptor may play a role in the modulation of NT effects in the CNS.

### EXPERIMENTAL PROCEDURES

#### Expression of Rat vNTS2 mRNA—

In order to assess the expression of vNTS2 mRNA in rat CNS and spinal cord, adult male Sprague Dawley rats (200–250 g; Charles River Breeding Laboratories, St-Constant, Quebec, Canada) were killed by decapitation. The brain and subcaudal cord were rapidly removed, and the areas of interest were dissected on ice. Samples were solubilized in lysis buffer (4 M guanidinium thiocyanate, 0.01 M Tris-HCl, pH 7.5, 0.97% sodium dodecyl sulfate (SDS), 0.025 mg/ml chlороdoxin, 0.01 M Tris-HCl, pH 7.5, 0.97% β-mercaptoethanol), and total RNA was extracted using the SV RNA Isolation System Kit (Promega, Madison, WI), according to the manufacturer’s instructions. These total mRNAs (2 μg) were then reverse-transcribed at 42 °C for 1 h using the Reverse Transcription System kit (Promega, Madison, WI). First strand cDNAs were subjected to 35 cycles of PCR in a final volume of 50 μl of reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.02% bovine serum albumin (BSA), 200 μM dNTPs, 0.5 unit of TaqDNA polymerase) using a set of primers (5′-GAATGTGCCTGGTGTCCTTCGC-3′ and 5′-ACTTGGATTTTCTCCAGCGGTG-3′) derived from bases 667–1287 in the sequence reported previously (11) for the rat NTS2 receptor. The oligonucleotides used are flanking the region where the deletion occurs in the mouse NTS2 receptor (27) and allow the amplification of fragments of predicted sizes of 620 and 439 bp, as demonstrated previously (25) in rat cerebellar granule cell cultures. As internal standards for semi-quantitative analysis, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was concurrently amplified using primers 5′-CAAGATTGTCTGAACATCTGATCC-3′ (sense, nucleotides 511–530) and 5′-CTTGGATGTCTCATATCTTGGCC-3′ (antisense, nucleotides 856 to 836), which target a 346-bp sequence in the rat GAPDH gene.

The ratios of NTS2 over GAPDH mRNAs and between the NTS2 receptor isoforms were determined by densitometry, using NIH Scion Image Software. Calculations and statistical analyses were performed using Excel 2000 (Microsoft) and Prism 3.02 (Graph Pad Software). Statistical analyses were performed using a one-way analysis of variance (Bonferroni’s multiple comparison test). Total RNA samples were subjected to reverse transcription in the absence of the enzyme to control for intrinsinc contamination by genomic DNA, and the reaction was performed without RNA to control for contamination during the experiment.

#### Gene Constructs—

The HA-tagged cDNA encoding the rat variant NTS2 receptor was obtained through reverse transcription of vNTS2 mRNA isolated from rat brain by PCR using nucleotides (5′-ACAGAGTGTCTCATACGACAGCTGACAGTCACTGTCG-3′) and (5′-TCATACATTCTTCACCGACAGTCGTC-3′) as sense and antisense primers, respectively. The former contains the HA epitope followed by the 44-31-bp sequence of the rat receptor mRNA (11), whereas the latter corresponds to the sequence 1268–1291 bp of the open reading frame of the rat nts2 receptor gene. The predicted sizes of the amplified fragments were 1.6 kb for the full-length NTS2 and 1.4 kb for the spliced variant form of the receptor. Fidelity of PCR amplification was confirmed by DNA sequence analysis using the ABI PRISM® 3100 Genetic Analyzer in the MOBIX laboratory (McMaster University, Hamilton, Ontario, Canada). A product corresponding to vNTS2 was purified from a 1% low melting agarose gel and subcloned into the pTargeT expression vector (Promega, Madison, WI).

#### Cell Culture and Transfections—

For MAPK kinase activity and radioligand binding experiments, CHO and COS-7 cells were stably transfected with the HA-vNTS2 construct. Briefly, cells were maintained at 37 °C in a 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) F-12 and DMEM with high glucose, respectively, supplemented with 5% fetal bovine serum in the presence of 100 units/ml penicillin/streptomycin (Invitrogen). Cells were grown in 100-mm dishes to 70–80% confluence and transfected with 4 μg of the HA-vNTS2-pTargeT plasmid by using the Lipofectamine™ transfection reagent (Invitrogen) according to the manufacturer’s instructions. After 72 h at 37 °C, positive cells selected in the presence of 1 mg/ml G418 (for MAPK kinase activity) and COS-7 (for radioligand binding experiments) transfectants were selected in the presence of 500 μg/ml G418. To block mitogen-activated protein (MAPK) kinase activity, cells were serum-depleted medium for 1 h at 37 °C. Cells were then washed again with PBS prior to examination.

For dual immunolocalization of vNTS2 and NTS2, COS-7 cells expressing the HA-tagged vNTS2 receptor were stained using the Alexa 488-conjugated goat anti-rabbit and Alexa 488-conjugated goat anti-mouse antibodies (1/750; Molecular Probes, Eugene, OR). For specificity controls, cells were incubated with antibodies preabsorbed with the corresponding antigenic peptide.

For cell surface immunolabeling experiments, COS-7 cells expressing the HA-tagged vNTS2 receptor were stained using the Alexa 488-conjugated monoclonal anti-HA IgG (1/500; Molecular Probes, Eugene, OR) in serum-depleted medium for 1 h at 37 °C. Cells were then washed with PBS, fixed with 10% paraformaldehyde for 30 min at RT, and washed again with PBS prior to examination.

For dual immunolocalization of vNTS2 and NTS2, COS-7 cells co-
expressing HA-tagged vNTS2 and the untagged NTS2 were incubated overnight at 4°C with mouse monoclonal anti-HA antibody in concert with rabbit NTS2 peptide antiserum (1/10,000) in PBS containing 1% vNTS2 and 0.05% Tween X-100. This second NTS2 antiserum is directed toward a synthetic peptide (YSFLRWSPRNPSLG) corresponding to the 397–412 predicted amino acid sequence in the C-terminal tail of the rat NTS2 receptor (custom-raised by Affinity BioReagents, ABR, Golden, CO) that specifically recognizes the full-length receptor. Cells were then incubated for 1 h at RT with a mixture of Alexa 488-conjugated goat anti-mouse and Alexa 594-conjugated goat anti-rabbit antibodies (1/50; Molecular Probes, Eugene, OR).

Cells were examined with a Zeiss 510 laser-scanning confocal microscope equipped with argon2 (488 nm) and HeNe1 (543 nm) lasers (Carl Zeiss Micro Imaging Inc., Thornwood, NY). Images were processed using the Zeiss 510 laser-scanning microscope software and Adobe Photoshop 6.0.

Expression of NTS2 Receptor mRNAs in the CNS—In order to assess expression patterns of NTS2 mRNAs in rat brain and spinal cord, a set of oligonucleotide primers designed to selectively recognize the region flanking the deletion yielding vNTS2 in the mouse (27) was used for reverse transcription-PCR. As shown in Fig. 1, PCR amplification of total mRNAs yielded two bands of 620 and 439 bp, corresponding to the expected sizes of NTS2 and vNTS2 receptor fragments, respectively. Semi-quantitative analyses performed using GAPDH as an internal control standard indicated that mRNAs levels for either of the two isoforms, and hence the ratio of vNTS2 to NTS2, were not statistically different between the various regions examined (Table I).
of the amplification was verified by agarose gel electrophoresis, which revealed two bands of the expected size (full-length of the amplification was verified by agarose gel electrophoresis, A immunoreactive receptors, visualized using either HA (Fig. 3, Torchemical analysis revealed that within these cells, the bulk of tagged vNTS2 receptor, using cDNA transfection. Immunohis-

cated that vNTS2 is a 5-transmembrane domain receptor with cysteine and methionine residues.

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unique C-terminal amino acids. B, schematic representation of the secondary structure of NTS2 receptors. Common amino acids are shown in black. Unique amino acid residues in the sequence of NTS2 and vNTS2 are represented in hatched and gray, respectively.

Fig. 2. Comparison of rat full-length and variant NTS2 isoform sequences. A, alignment of rat NTS2 isoform sequences. Identical sequences found in NTS2 receptor isoforms are shaded, and the putative transmembrane segments are boxed. Gaps for alignment are indicated by dots. The short form of NTS2 contains 281 amino acids, instead of 416 for the long form. The resulting protein is devoid of the last two transmembrane domains and contains 37 unique C-terminal amino acids. B, schematic representation of the secondary structure of NTS2 receptors. Common amino acids are shown in black. Unique amino acid residues in the sequence of NTS2 and vNTS2 are represented in hatched and gray, respectively.

Fig. 1. Reverse transcription-PCR analysis of NTS2 mRNAs. Amplification of NTS2 and vNTS2 mRNAs was from various brain regions. PCRs were performed on mRNAs reverse-transcribed using primers flanking the truncated portion of NTS2. The expected sizes of the reverse transcription-PCR products were 620 and 439 bp for the full-length and spliced form of NTS2, respectively. The housekeeping gene GAPDH was also amplified and used as an internal control for semi-quantitative analyses.

| Structure          | Receptor to GAPDH ratio | vNTS2/NTS2 |
|--------------------|-------------------------|------------|
|                    | vNTS2 | NTS2 | vNTS2/NTS2 |
| arbitrary units    |       |      |            |
| Neocortex          | 0.91 ± 0.15 | 0.99 ± 0.07 | 0.92 ± 0.15 |
| Spinal cord        | 0.93 ± 0.10 | 1.06 ± 0.01 | 0.88 ± 0.11 |
| Medulla            | 0.93 ± 0.10 | 1.03 ± 0.05 | 0.91 ± 0.13 |
| Olfactory bulb     | 0.98 ± 0.10 | 1.15 ± 0.08 | 0.88 ± 0.14 |
| Hippocampus        | 0.91 ± 0.12 | 1.21 ± 0.13 | 0.78 ± 0.16 |
| Hypothalamus       | 1.09 ± 0.14 | 1.28 ± 0.17 | 0.89 ± 0.14 |
| Cerebellum         | 0.72 ± 0.12 | 0.57 ± 0.05 | 0.83 ± 0.15 |
| Thalamus           | 0.90 ± 0.09 | 1.17 ± 0.12 | 0.81 ± 0.14 |
Binding and Internalization Properties of vNTS2—To determine the binding properties of the short NTS2 isoform, COS-7 cells stably expressing vNTS2 were incubated with 0.4 nM $^{125}$I-labeled NT for 30 min at 37 °C with increasing concentrations of nonradioactive NT. As shown in Fig. 4A, unlabelled NT inhibited specific $^{125}$I-labeled NT binding with an IC$_{50}$ value of 10 μM (Fig. 4A). No specific $^{125}$I-labeled NT binding was observed in nontransfected cells (data not shown).

In order to visualize NT binding and internalization, COS-7 cells expressing vNTS2 were incubated for various periods of time with 50 nM Fluo-NT at 37 °C and were examined by confocal microscopy (Fig. 4, B–D). Following 30 min of incubation with the fluorescent ligand, punctuate fluorescent labeling was evident throughout the cytoplasm of transfected cells, sparing the nucleus (Fig. 4, B and C). No fluorescent labeling was visible in nontransfected cells (not shown) or in transfected cells incubated with an excess of NT (Fig. 4D). When the incubation was carried out in the presence of the endocytosis inhibitor phenylarsine oxide, bound fluorescent molecules remained clustered on the cell surface (data not shown).

Signaling Properties of vNTS2—We then investigated whether the truncated form of the rat NTS2 receptor retained the MAPK activation (ERK1/2 pathway) properties exhibited by the long form of the receptor (24). Stimulation with NT of CHO cells stably expressing the rat vNTS2 induced the phosphorylation of ERK1/2 (p42/44$^{MAPK}$) starting at concentrations of 1 μM (Fig. 5A). Time course studies in which the cells were stimulated for 1–60 min with 1 μM NT showed this effect to be rapid (<5 min) and sustained (over 1 h) (Fig. 5B). Densitometric analysis of the ratio of phosphorylated ERK1/2 over total ERK1/2 levels indicated that the NT-induced increase in MAPK phosphorylation was 1.4 ± 0.1-fold and reached a plateau after 15 min of stimulation (Fig. 5C, ●). This effect was vNTS2-mediated, as it was not observed in nontransfected cells (Fig. 5C, ○). A similar activation was observed following 10 min of stimulation with the NTS2 agonist levocabastine (1 μM; Fig. 5D). Pretreatment with selective MAP kinase kinase (MEK) inhibitors (PD98059 or U0126) significantly inhibited NT-mediated ERK1/2 phosphorylation in these cells (Fig. 5E).

Heterodimerization of NTS2 Receptors—In rat spinal cord membrane preparations immunoblotted with the N-terminally directed NTS2 antiserum (which recognizes both NTS2 isoforms; Fig. 6A), a prominent band was evident at 46 kDa, corresponding to the molecular weight of the monomeric form of the full-length NTS2 receptor detected in transfected COS-7 cells (compare Fig. 6A with Fig. 3E, lane 2). Surprisingly, no band was visible at 32 kDa, i.e. at the size expected for the monomeric form of the truncated receptor (e.g. Fig. 3E, lane 1). However, as in COS-7 transfected with the HA-vNTS2 (Fig. 3E, lane 1), an immunoreactive band was detected at 60 kDa, corresponding to the size of putative vNTS2 homodimers (Fig. 6A). An additional band was also observed at 75–85 kDa (Fig. 6A, asterisk), which might correspond to vNTS2/vNTS2 heterodimers. All of these bands were absent when the antibody was pre-saturated with the immunizing peptide (not shown).

To investigate whether the species of NTS2 receptors detected at the 75–85-kDa molecular weight mark could correspond to vNTS2/NTS2 heterodimers, we co-expressed HA-tagged vNTS2 together with untagged NTS2 receptors in COS-7 cells and subjected cell lysates to Western blotting analysis. As shown in Fig. 6B, both monomeric and putative homodimeric forms of variant (32 and ~60 kDa, arrows) and full-length (46 and ~85 kDa, arrowheads) NTS2 receptors were detected using the N-terminally directed NTS2 peptide anti-
serum. An immunoreactive band was also observed at the ~75-kDa mark (asterisk), consistent with the theoretical molecular weight of a vNTS2/NTS2 heterodimer.

Cell lysates were then subjected to immunoprecipitation with the rat anti-HA antibody. Immunoblotting of these immunoprecipitates with the N-terminally directed NTS2 antiserum

FIG. 4. Binding and internalization of NT in COS-7 cells expressing vNTS2. A, competition inhibition of 125I-labeled NT binding to whole COS-7 cells stably expressing vNTS2. Cells were incubated with 0.4 nM 125I-labeled NT for 30 min at 37 °C with increasing concentrations of nonradioactive NT. Binding IC50 = 10 μM. The results are representative of three independent experiments. B and C, confocal microscopic imaging of fluo-NT internalization in COS-7 cells expressing vNTS2. Cells were incubated for 30 min at 37 °C with 50 nM fluo-NT and air-dried. Internalized fluorescent ligand molecules are detected in the form of small endosome-like particles distributed throughout the cytoplasm. D, fluo-NT labeling is prevented when the incubation is performed in the presence of an excess of nonfluorescent NT. Scale bar, 10 μm in B; 5 μm in C and D.

FIG. 5. MAPK kinase signaling in CHO cells heterologously expressing vNTS2. A, dose-dependent effect of NT on MAPK (ERK1/2) phosphorylation. Cells were treated for 5 min with the indicated concentrations of NT. Phosphorylation levels of MAPK were detected by immunoblotting as described under “Experimental Procedures.” Upper panels of A, B, D, and E, phosphorylated ERK1/2; lower panels, total ERK1/2. B, time course of NT-stimulated MAPK phosphorylation in CHO cells stably expressing vNTS2. C, densitometric analysis of the ratio of phosphorylated ERK1/2 over total ERK1/2 levels following incubation with 1 μM of NT. Untransfected (○) and vNTS2-expressing (●) CHO cells were incubated at 37 °C for the indicated times. Values represent the means ± S.E. of five independent experiments. Transfected cell values are significantly different from nontransfected cell values at all time points as follows: *, p ≤ 0.05; **, p ≤ 0.01; and ***, p ≤ 0.001. D, ERK1/2 phosphorylation levels in vNTS2-expressing CHO cells stimulated with levocabastine for 10 min at 37 °C. E, MEK inhibitors prevent ERK1/2 activation by NT in CHO cells expressing vNTS2. Cells were pretreated with PD98059 (50 μM) or U0126 (10 μM) and treated with NT (1 μM) for 5–15 min at 37 °C. The results are representative of three individual experiments, each with duplicate determinations.
shown that they contained HA-tagged vNTS2 as well as untagged NTS2 receptors, suggesting that long and short forms of NTS2 receptors physically interact (Fig. 6C, lane 1). Indeed, immunoreactive bands were detected at both 32 and 46 kDa, i.e., at the molecular weights of the monomeric forms of the variant and full-length receptors, as well as at ~60 kDa, corresponding to the presumptive homodimeric isoform of vNTS2 (Fig. 6C, lane 1). Higher molecular weight bands (~75 and ~110 kDa) were also evident, which may represent heteromultimeric forms of NTS2 receptors. Immunoreactive bands corresponding to monomeric and putative homodimeric forms of vNTS2 (32 and 65 kDa, respectively) were also detected in COS-7 cells transfected with the HA-vNTS2 cDNA alone (Fig. 6C, lane 3). However, no specific bands were observed in cells expressing the full-length NTS2 alone (Fig. 6C, lane 2).

Immunoprecipitates were also subjected to Western blotting analysis using the C-terminally directed NTS2 antiserum, which selectively recognizes the full-length NTS2. This antibody revealed the presence of the full-length isoform in cotransfected cells subjected to immunoprecipitation with the HA antibody, confirming that NTS2 interacts physically with vNTS2 (Fig. 6D, lane 1). No bands were detected with the C-terminally directed NTS2 peptide antiserum in immunoprecipitates from COS-7 cells expressing either NTS2 (Fig. 6D, lane 2) or HA-vNTS2 (Fig. 6D, lane 3) alone, confirming the specificity of the interaction.

To investigate whether vNTS2/NTS2 heterodimerization influences trafficking of the truncated receptor to the cell surface, COS-7 cells co-expressing the HA epitope-tagged vNTS2 and the untagged full-length NTS2 were incubated for 2 h with the N-terminally directed NTS2 peptide antiserum, and antibody-bound cell surface receptors were separated from unbound cytoplasmic receptors by using protein A-Sepharose beads. Immunoblotting was then performed using the mouse anti-HA antibody. As seen in Table II, the density of both low (monomers) and high (dimers and multimers) molecular weight forms detected in the cell surface fraction (22 ± 2, 26 ± 3, and 52 ± 4%, respectively) was the same as in the cell fraction from cells transfected with vNTS2 alone (21 ± 2, 28 ± 4, and 51 ± 6%, correspondingly). Confocal microscopy confirmed that as in COS-7 cells transfected with vNTS2 alone (Fig. 3), the bulk of vNTS2 immunoreactivity in COS-7 co-expressing NTS2 and HA-vNTS2 was intracellular (Fig. 7B). Predictably, vNTS2 and NTS2 immunoreactive intracellular stores closely overlapped, in keeping with their demonstrated heterodimerization (Fig. 7, A–C).

### Discussion

In this study, we have demonstrated the presence of an alternatively spliced form of the NTS2 receptor mRNA in rat brain, comparable with the one previously identified in the mouse (27). Most importantly, we have shown that this 5-transmembrane domain truncated receptor protein is functional, in that it specifically binds and internalizes NT and is coupled to the activation of the ERK1/2 pathway.

Alternative splicing is a frequent occurrence in mammalian gene expression, contributing both to proteome diversity and to functional complexity of genomes by generating structurally distinct isoforms from a single gene (43). Nucleic acid sequence analysis demonstrated that the vNTS2 isoform results from a 181-bp deletion in the full-length cDNA that leads to a frameshift in the reading frame, introducing a premature stop codon. The sequence of the cDNA fragment isolated showed partial sequence overlap with the previously published rat full-length NTS2 sequence (11), indicating that vNTS2 was indeed generated by alternative splicing from a donor-acceptor splice site as suggested by Sun et al. (26). This type of processing does not appear to be regionally selective because mRNA levels and ratios of the two isoforms did not vary significantly between the different regions examined.

Western blotting analysis of COS-7 cells transfected with cDNA encoding an HA-tagged rat vNTS2 revealed the presence of distinct translation products of ~32 and 60 kDa. The former corresponds to the molecular weight of the monomeric form of vNTS2 as deduced from its cDNA sequence, whereas the latter likely represents a homodimeric form of the receptor. Immunocytochemistry revealed that the bulk of these vNTS2 proteins were intracellular. This observation is in agreement with the results of subprograms executed by the Psort II server (Kenta Nakai, Human Genome Center, Institute for Medical Science, University of Tokyo, Japan), which predict the subcellular localization of vNTS2 from its amino acid sequence in the endoplasmic reticulum (44.4%), intracellular vacuoles (22.2%), Golgi apparatus (11.1%), plasma membrane (11.1%), and mitochondrial compartments (11.1%). Both immunoblotting and immunocytochemical experiments suggest that the vNTS2 receptor is poorly targeted to plasma membranes. However, this restricted targeting does not appear to be linked to the mole-
ular species recruited to the membrane because both low (monomeric) and high (putative dimeric) molecular weight forms were detected at the cell surface.

Despite its short transmembrane span, the rat vNTS2 still specifically binds $^{125}$I-labeled NT, albeit with a considerably lower affinity than the full-length receptor (IC$_{50}$ of 10 $\mu$m versus 2 nM, for the full-length isoform). This result differs from those of Botto et al. (27), who found no specific binding of $^{125}$I-labeled NT to mouse vNTS2 transiently expressed in COS-7 cells. This discrepancy may be explained by species differences, by variations in the sensitivity of the methods employed, or by the nature of the expression system. Indeed, stable transfectants might express higher levels and/or recruit more efficiently vNTS2 to the membrane than transiently transfected cells. Previous studies have shown that NT binding to rat NTS1 and human NTS2 receptors involved residues located in TM 6 and in the third intracellular loop (44, 45). These residues are lost in the variant isoform, due to the splicing of the last two TM domains of NTS2, which may explain the lower affinity of vNTS2 for NT as compared with the full-length receptor.

Confocal microscopic experiments demonstrated specific, receptor-mediated internalization of fluorescent NT. The internalized ligand was concentrated within small endosome-like organelles, a pattern consistent with earlier reports (24, 46) on internalization via the full-length NTS2 receptor. This finding suggested to us that the NTS2 variant isoform was functionally responsive to NT, as confirmed by MAPK activation experiments.

Stimulation with NT induced a rapid and sustained increase in ERK1/2 phosphorylation in CHO cells transfected with vNTS2, indicating functional coupling of the truncated receptor to the MAPK signaling pathway. Stimulation of these cells with the NTS2-specific agonist levocabastine also resulted in ERK1/2 activation. The time course of ERK1/2 activation corresponded to that observed following stimulation of the full-length receptor in a similar transfection system (24). Activation of ERK1/2 was already apparent at concentrations of NT lower than the IC$_{50}$ (1 $\mu$m), in keeping with the detection of ligand-induced internalization at concentrations of fluo-NT of 50 nM. However, phosphorylation levels obtained following stimulation with 1 $\mu$m NT were lower in cells transfected with the vNTS2 than with the full-length receptor (1.4 versus 2.9-fold increase over control (24)). They were also lower than those produced in cultured rat cerebellar granule cells, which endogenously express the two NTS2 isoforms (25). These results suggest that the third intracellular loop and the C-terminal tail of the full-length NTS2 are not essential for but may play an accessory role in MAPK activation. To our knowledge, the vNTS2 is the first TM-spliced GPCR variant cloned to date that was found to maintain signaling properties. Indeed, other TM splice variants of GPCRs, such as the corticotrophin-releasing factor receptor 2, have been reported to retain their agonist binding properties but to totally lose their functional coupling (47).

Western blotting studies using an N-terminally directed NTS2 peptide antiserum revealed that the 32-kDa monomeric form of vNTS2 is not expressed in the spinal cord, whereas a band twice the size of vNTS2 is present, suggesting that the NTS2 receptor variant may exist in homodimeric form in the rat CNS. By contrast, a specific band was detected at 46 kDa, i.e. at the molecular weight of the full-length isoform of the receptor, indicating that in contrast to its variant isoform, the full-length receptor exists in monomeric form in rat CNS. These results are similar to those previously reported for membranes prepared from rat brain and cerebellum (40). In addition, specific bands were detected at molecular weight marks higher than the putative vNTS2 dimers. One of these was approximately twice the size of the monomeric form of the full-length receptor and was therefore interpreted as a putative NTS2 homodimer. Another migrated slightly lower, as would be expected from a vNTS2/NTS2 heterodimer. Indeed, recent biochemical, biophysical, and functional studies (48, 49) have shown that GPCR can assemble as hetero- as well as homodimeric complexes. To test whether the vNTS2 could actually associate with its full-length counterpart, we carried out immunoprecipitation experiments on COS-7 cells co-expressing HA-tagged vNTS2 and native NTS2. These experiments demonstrated that the two NTS2 isoforms did associate when co-expressed in COS-7 cells, because both receptors were pulled down using an HA antibody. Furthermore, the presence in these dually transfected cells of a band at the theoretical molecular weight of vNTS2/NTS2 heterodimers indicated that the band detected at the same level in spinal cord membranes might indeed have corresponded to a vNTS2-NTS2 heterodimer. This vNTS2/NTS2 heterodimer was stable during cell lysis and reducing Tris-glycine gel electrophoresis, suggesting that the interaction involves a noncovalent hydrophobic interface between the receptor proteins.

To investigate whether heterodimerization of NTS2 and vNTS2 receptors affected targeting of the truncated receptor to the plasma membrane, we examined by Western blot the expression of vNTS2 on the cell surface of singly transfected versus dually transfected cells, and we compared the immunocytochemical distribution of NTS2 and vNTS2 in vNTS2 dually transfected cells. By using either technique, we found that co-expression of the full-length NTS2 did not noticeably increase the cell surface density of the truncated form over that seen in cells expressing HA-vNTS2 alone, suggesting that the full-length NTS2 does not act as a chaperone protein for its shorter isoform. However, intracellular vNTS2 stores closely overlapped with those of NTS2, supporting the notion that the two receptors heterodimerize.

In summary, our results indicate that the rat vNTS2 is a functional receptor that is expressed in conjunction with the full-length NTS2 receptor throughout the CNS. Our data also indicate that this truncated 5-TM receptor does not exist in monomeric form in the rat CNS. Rather, it associates both with itself and with the full-length 7-TM receptor to form large molecular weight homo- and heterodimer species. Therefore, it is likely that these associations provide for subtle regulation of...
the NT signal, as demonstrated previously (50) for the splice variant isoform of the growth hormone-releasing hormone receptor.

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