The Effects of Deleting the Mouse Neurotensin Receptor NTR1 on Central and Peripheral Responses to Neurotensin

DOUGLAS J. PETTIBONE, J. FRED HESS, PATRICIA J. HEY, MARLENE A. JACOBSON, MICHAEL LEVITEN, EDWARD V. LIS, PIERRE J. MALLORGA, DANETTE M. PASCARELLA, MELISSA A. SNYDER, JACINTA B. WILLIAMS, and ZHIZHEN ZENG


Received August 22, 2001; accepted October 1, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

Mice deficient in the neurotensin (NT)-1 receptor (NTR1) were developed to characterize the NT receptor subtypes that mediate various in vivo responses to NT. F2 generation (C57BL/6/Sv129J) NTR1 knockout (−/−) mice were viable, and showed normal growth and overt behavior. The −/− mice lacked detectable NTR1 radioligand binding in brain, whereas NTR2 receptor binding density appeared normal compared with wild-type (+/+) mice. The gene deletion also resulted in the loss of NTR1 expression as determined by reverse transcription-polymerase chain reaction and in situ hybridization. Intracerebroventricular injection of NT (1 μg) to +/+ mice caused a robust hypothermic response (5–6°C) and a significant increase in hot-plate latency. These effects were absent in the −/− mice. Similar results were obtained with i.p. injections of the brain-penetrant NT analog NMe-Arg-Lys-Pro-Trp-Tie-Leu (NT-2, 1 mg/kg i.p.). NT-2 administration also impaired rotaor performance in wild-type mice, but had no effect on motor coordination in knockout mice. In vitro, NT and NT-2 at 30 nM caused predominantly contraction and relaxation in isolated distal colon and proximal ileum, respectively, from +/+ mice, but no responses were observed with tissues from −/− mice. A similar loss of the contractile effects of NT was observed in the isolated stomach fundus from the knockout mice. In vivo, NT-2 administration reduced colonic propulsion substantially in wild-type mice. In contrast, NT-2 had no effect in NTR1 null mice, whereas the hypomotility effect of clonidine was intact. These data indicate that NTR1 mediates several of the central and peripheral effects of NT.

Neurotensin (NT) is a tridecapeptide that was initially discovered and sequenced almost 30 years ago (Carraway and Leeman, 1973; Brown and Miller, 1982). NT is synthesized within neurons throughout the CNS and within endocrine-like cells of the gastrointestinal tract (Carraway and Leeman, 1976; Kitabgi et al., 1976) and is known to exert several effects at these sites, including hypothermia (Bissette et al., 1976; Nemeroff et al., 1977), analgesia (Clineschmidt et al., 1979), and effects on gastrointestinal motility (Kitabgi and Freychet, 1978). Two G protein-coupled receptors for NT have recently been cloned, NTR1 (or NTS1) (Tanaka et al., 1990; Vita et al., 1993) and NTR2 (or NTS2) (Chalon et al., 1996; Mazella et al., 1996; Botto et al., 1998). These two receptor subtypes are 41% identical at the amino acid level. A third receptor or binding site, NTR3 (or sortlin or gp95), has recently been described as a non-G protein-coupled receptor that binds NT with high affinity and appears to function in hormone trafficking and/or neurotensin uptake (Navarro et al., 2001). Despite the extensive study of NT over the years, the precise functions mediated by the various NT receptor subtypes are still unclear (Vincent et al., 1999). Two NT antagonists, 2-[(1-(-7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazole-3-carbonyl]amino}adamantane-2-carboxylic acid (SR48692) and 2-[(5-(2,6-dimethoxyphenyl)-1-(4-(N-(3-dimethylaminopropyl))-N-methylcarbamoyl)-2-isopropylphenyl)-1H-pyrazole-3-carbonyl]amino}adamantane-2-carboxylic acid (SR142948A), have recently been described (Gully et al., 1993, 1997) that have contributed much to the understanding of NT physiology. However, although SR48692 exhibits some selectivity (10–30×) for NTR1 versus NTR2 (Gully et al., 1993; Chalon et al., 1996; Botto et al., 1998; Vita et al., 1998), neither compound has the selectivity to be truly useful for defining the roles of the various NT receptor subtypes. Leovobastine is known to have selective, high affinity for the NTR2 (versus NTR1) receptor (Chalon et al., 1996; Botto et al., 1998); however, it is unclear whether it possesses primarily NTR2 agonist or antagonist activity (Botto et al., 1997, 1998; Vita et al., 1998; Yamada et al., 1998; Dubuc
et al., 1999a), and with its high affinity for histamine H1 activity, has also not been very useful in defining the roles of these receptor subtypes in vivo.

Accordingly, we have produced and characterized mutant mice deficient in NTR1 to begin to more clearly define the roles of the NT receptor subtypes, and report herein that the hypothermic, analgesic, impaired motor coordination, and gastrointestinal tissue motility effects of NT agonism are dependent on this receptor subtype.

Materials and Methods

Generation of NTR1 Null Mice

A construct for the targeted disruption of the mouse NTR1 gene (GenBank accession no. AB017027) was prepared by Deltagen, Inc. (Menlo Park, CA). Homologous recombination with this construct was predicted to result in the deletion of 457 nucleotides of the mouse NTR1 gene, which included the initiator methionine codon at nucleotide 8, and the introduction of a neomycin resistance gene. The deletion removes the first 150 amino acids of the 424 amino acid NTR1 protein. The targeting construct was introduced into the R1 embryonic stem (ES) cell line. Neomycin resistant ES cell colonies were screened for targeted integration of the construct by PCR and Southern blot analysis by using external probes. High-percentage chimeric NTR1 mice were bred with C57BL/6 mice at Deltagen, Inc. and tested for transmission of the mutant allele by using a PCR assay. Heterozygous F1 progeny were then interbred to generate F2 hybrid mice.

The R1 ES cell line used to generate the NTR1 mice is a hybrid derived from strains 129X1/SvJ and 129S/SvImJ. Genotype analysis (Research Genetics, Huntsville, AL) of heterozygous and wild-type NTR1 mice by using the polymorphic marker D2 Mit346, located approximately 15 cM centromeric of the Ntr1 gene, is consistent with the targeted chromosome being derived from 129X1/SvJ, which is nongenomic to the construct that was generated from a 129S/SvImJ genomic library. The mice used in these experiments weighed 25 to 30 g. All studies reported herein were conducted in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Mouse NT Receptor Cell Lines

Mouse NTR1 and NTR2 cDNAs (Mazella et al., 1996) encoding the full-length receptors were isolated by PCR and subcloned into the mammalian expression vectors pcDNA3 (Invitrogen, Carlsbad, CA) and pIRESpuro (CLONTECH, Palo Alto, CA), respectively. The expression constructs were transfected into CHO NFAT-ß-lactamase cells (Aurora Biotechnology, San Diego, CA) with LipofectAMINe 2000 (Invitrogen) and the appropriate antibiotic was used for selection. Cells were sorted by fluorescence-activated cell sorting analysis into pools of approximately 20,000 to 40,000 cells to develop a population of NT receptor expressing cells and single cells into a 96-well plate to generate clonal cell lines. Cell populations and the clonal cell lines were expanded and assayed for functional response to the appropriate agonist and for expression level by saturation binding with radiolabeled neurotransin.

NT Receptor Expression Studies

RT-PCR Analysis of NTR1 mRNA Expression. Total brain Poly (A⁺) mRNA was isolated from wild-type, heterozygous, and homozygous NTR1 knockout mice by using the QIAGEN Oligotex Direct mRNA Isolation kit (QIAGEN, Valencia, CA) according to the manufacturer’s recommendations. RT-PCR was performed using Superscript One-Step RT-PCR kit from Invitrogen, with 400 ng of poly (A⁺) RNA as template and 1 pmol of 5’ PCR primer specific for the mouse NTR1 sequence deleted in the targeting construct (CAGACT- CATTACCACCTGG) together with 1 pmol of the 3’ primer AGAGGACGCTTGGTCAGC. PCR was carried out with the following parameters: denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min for 40 cycles. The mouse glyceraldehyde 3-phosphate dehydrogenase gene was amplified using the CLONTECH 0.45-kb control amplifier set. Products were analyzed on a 0.8% agarose gel. The specificity of the NTR1-amplified products was confirmed by DNA sequence analysis.

Northern Blot Analysis of mNTR2 Expression. Poly (A)⁺ brain RNA, 7.5 µg, from wild-type, heterozygous, and homozygous NTR1 knockout mice was fractionated on a 1% agarose/formaldehyde gel, transferred to Hybond-N, cross-linked, and hybridized in Rapid-hyb solution (Amersham Biosciences, Inc., Piscataway, NJ) containing 2 × 10⁶ cpm/ml of radiolabeled probe for 3 h at 65°C. The mouse NTR2 receptor cDNA probe corresponds to a 1.3-kb fragment containing the complete reading frame of the mouse NTR2 receptor. Filters were washed at 65°C in a solution containing 0.1× SSC and 0.1% SDS and analyzed by autoradiography. Filters were stripped and reprobed for β-actin.

In Situ Hybridization. Mouse NTR1 RNA probes were synthesized in an in vitro transcription reaction with 1 µg of the template DNA by using a kit from Roche Molecular Biochemicals (Indianapolis, IN) (catalog no. 999644). The probes were labeled with 32P-UTP (≥2500 Ci/mmol, 20 mCi/ml; Amersham Pharmacia, Inc.). A 1300-bp cDNA fragment encoding the mouse NTR1 receptor was used as a template for synthesis of RNA probes. The labeled RNA probes were purified by passing through a quick spin column (catalog no. 1274015; Roche Molecular Biochemicals), and followed by probe size reduction in a carbonate buffer (120 mM Na₂CO₃, 80 mM NaHCO₃, pH 10.4) at 60°C. After alkaline hydrolysis neutralization buffer (1: 28, v/v) was added, the probes were precipitated in ethanol, and the pellets were resuspended in diethyl pyrocarbonate-treated water.

The brains were removed from NTR1 knockout mice and wild-type mice after CO₂ inhalation-induced anesthesia/euthanasia, frozen on dry ice, and stored at −80°C until use. A series of coronal sections of the brains, 10 µm of each, were made with a Leica cryostat (model CM3050), fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4, at 4°C for 30 min. The sections were dehydrated through graded ethanol solutions. Slides were then hybridized in Hybridization Cocktail from AMRESCO (catalog no. 0975; Solon, OH), at a probe concentration of 1 × 10⁶ cpm/ml hybridization buffer, overnight at 60°C in a humid chamber. After hybridization, slides were treated with RNase A (20 µg/ml) for 30 min, 37°C, washed three times in 0.1 × SSC at 65°C, 30 min each, once in 0.1 × SSC at 70°C for 10 min, rinsed briefly in deionized H₂O, and air-dried. Autoradiography was carried out with Kodak Biomax MR film.

Radioligand Binding Studies. CHO NFAT-ß-lactamase cells transfected with mouse NTR1 (CHO-NTR1) and NTR2 (CHO-NTR2) receptors were grown to confluence and dissociated using Hanks’ based dissociation solution (S-004-B; Specialty Media, Phillipsburg, NJ) (10 ml/T162 flask) and centrifuged to precipitate. The pellet was resuspended in homogenization buffer (50 mM NaCl, 80 mM NaHCO₃, pH 7.4) in a cold storage buffer (50 mM Tris, pH 7.4, 40 mM bacitracin), harvested, and aliquoted (0.5 or 1 ml/vial) for storage at −80°C until the day of the binding experiment. Whole forebrain, duodenum, colon, and ileum were dissected from mice after CO₂ anesthesia/euthanasia, frozen immediately in liquid nitrogen, and stored at −70°C until the day of the binding experiment. The tissues were chopped into small pieces, homogenized using a polytron in ice-cold Tris homogenization buffer, and followed by a series of washes as described above for the cells. The preparation of membranes from peripheral tissues included a filtration through four layers of cheesecloth before the last centrifugation. The final resuspension was done in storage buffer at tissue concentration, 1 g/15 ml for the brain and 1 g/5 ml for the other tissues. The membrane suspensions were used immediately in the binding experiments. For both cell and tissue preparations, protein concentration
was measured using SDS (2%) solubilized membranes with the Micro BCA protein kit (23235; Pierce Chemical, Rockford, IL).

Competition binding studies were performed by incubating membranes at the appropriate concentration with 200 pM (CHO-NTR1) or 1 nM (CHO-NTR2, tissues) 125I-NT (NEX 198; PerkinElmer Life Sciences, Boston, MA) for 30 min at room temperature in a final volume of 250 μl, in the presence of 40 μg/ml bacitracin, 0.8 mM o-phenanthroline, 0.1% bovine serum albumin, and the concentration of the compound to be studied. Saturation binding studies were performed using 125I-NT over a concentration range of 50 pM to 4 nM. Nonspecific binding was determined using 1 μM NT, which caused the same level of maximal inhibition of binding as NT-2 and other reference agents such as SR48692. In some incubations with mouse peripheral tissue membranes, levocabastine was present at 10 μM to mask possible binding to NTR2. The assay was terminated by rapid filtration on Filtermat A (1204-401, GF/C filter; PerkinElmer Wallac, Gaithersburg, MD) pretreated for 90 min with 0.2% polyethyleneimine followed by three washes with cold 10 mM Tris, pH 7.4, containing 100 mM NaCl by using a Skatron Micro 96 harvester (Molecular Devices, Sunnyvale, CA). Dried filters were counted in a PerkinElmer Wallac betaplate 1205 scintillation counter.

Analysis of the data for determination of IC50 values was performed using the GraphPad Prism software (GraphPad, San Diego, CA) for one-site (cloned mouse NTR binding) and two-site (mouse brain NTR binding) analysis. Kd values were then calculated for the individual sites using the equation $K_d = IC50/(1 + c/Kd).$

**Isolated Tissue Bath Studies**

Two 1-cm segments of both proximal ileum and distal colon were removed from C57BL6/129J hybrid mice of each genotype and gender after CO2 anesthesia/euthanasia. The tissue segments were attached to a Gould-Statham force-displacement transducer and placed under 200-mg tension into 10-ml heated-jacket glass organ baths filled with physiological Tyrode’s solution, pH 7.4 (37°C), and bubbled continuously with 95% O2, 5% CO2. After 60-min equilibration, the tissues were contracted once to acetylcholine (1 μM) to assess tissue viability, washed repeatedly over the next 60 min, and then exposed to either NT or NT-2 at 30 nM. These concentrations were determined in separate experiments to exert maximal effects. Contractile responses (mg of tension) were recorded and calculated as group means ± S.E.M.

**Measurement of Core Temperature and Hot-Plate Analgesia**

The effects of NT given i.v. (1 μg in 5 μl) or NT-2 given i.p. (1 mg/kg) on core temperature and hot-plate analgesia were evaluated in C57BL6/129J hybrid mice of each genotype (approximately 20 weeks old) and gender, weighing 20 to 30 g. These doses of NT and NT-2 were determined in pilot experiments to produce maximal effects in control animals. Injection of NT (in phosphate buffer, pH 7.4) into the lateral ventricle was done free-hand in conscious mice by using a 26-gauge needle with a 2.5-mm depth stop at a location in the head 2 mm unilateral to the midline and just caudal to the ocular orbit. Placement of the injection was verified in some cases by using methylene blue as a marker in the dosing solution. At various times after the i.v. or i.p. injections, core temperature was measured by inserting a thermometer probe 2 cm into the rectum for about 5 s and the temperature was recorded. At different times, the same animals were tested for hot-plate reaction times by placing them gently into a 5-inch-diameter glass cylinder atop a metal plate maintained at 55°C and recording the length of time for the animal to respond (i.e., flinch, lick a paw, or jump). The hot-plate test has been a test used by several investigators to describe the analgesic actions of NT (Dubuc et al., 1985a; Tyler et al., 1985b; Clineschmidt et al., 1979). Data were analyzed and plotted as group means ± S.E.M.

**Motor Performance (Rotarod)**

C57BL6/129J hybrid mice of each genotype and gender were trained on the morning of the test to remain on a Ugo Basile (Comerio, Italy) rotarod apparatus for 2 min rotating at 12 rpm. Pretest (baseline) measurements were taken for the time the mice were able to remain on the rotarod accelerating from 4 to 40 rpm over a period of 5 min. The mice were then injected i.p. with NT-2 (1 mg/kg) and the accelerating rotarod test was repeated at 45 and 90 min later. Data were analyzed and represented as mean percentage ± S.E.M. pretest values.

**Measurement of Colonic Propulsion**

Colonial motility or propulsion was measured according to the method of Pendleton et al. (1986). In these studies, C57BL6/129J hybrid mice of each genotype and gender were injected i.p. with either NT-2 (1 mg/kg) or clonidine (100 μg/kg) by using 0.9% NaCl as vehicle. Five minutes later, a 3-mm glass bead was inserted 2 cm rectally into mice by using a glass syringe plunger. The number of mice in each group (n = 20) expelling their beads at various time points were recorded and expressed as a percentage. The doses of NT-2 or clonidine were determined in pilot studies to be maximal doses. All mice treated with vehicle consistently expelled their bead within 20 min.

**Compounds**

NT (neurotensin 1-13) was obtained from Peninsula Laboratories (Belmont, CA) and NT-2 was purchased from Neosystem, Group SNPE (Strasbourg, France). Clonidine was purchased from Sigma Chemical (St. Louis, MO).

**Statistics**

Significant differences (P value < 0.05) between groups for all studies except the glass bead test were determined using one-way analysis of variance followed by a Global F test and pairwise comparisons with the least significant difference method of Fisher (1958). For the glass bead test, significant differences between groups at individual time points were determined using a two-sided Fisher’s exact test (Agresti, 1990).

**Results**

**General Phenotype of NTR1 Knockout Mice**

F2 generation C57BL6/129J hybrid +/-, -/+ , and --/-- NTR1 mice were viable at birth and lived through at least 1 year of age. The mutant genotypes matured with very similar, if not identical growth rates, to the wild-type mice and had no obvious physical abnormalities. Autopsy of three mice of each gender and genotype at 7 weeks of age indicated no important effects of gene deletion on organ development (gross and histological), serum chemistry, hematology (red blood cell count, white blood cell count, hematocrit) (data not shown).

**Expression of Neurotensin Receptor Subtypes in NTR1 Knockout Mice**

RT-PCR and Northern Blot Analysis. In preliminary experiments, it was determined that NTR1 mRNA expression in whole brain was too low for acceptable Northern blot analysis. Therefore, RT-PCR was used to determine the mRNA expression in wild-type, heterozygous, and knockout mice. A PCR primer pair was designed to preferentially detect the NTR1 coding sequences that were deleted in the targeting construct used to generate the knockout mice. The 779-bp product resulting from primers against the deleted sequence was amplified from both wild-type and heterozy-
gous mouse mRNA, and was absent from knockout mice mRNA (Fig. 1A). No products were detected in the absence of reverse transcriptase or in the absence of template for all three genotypes. The identity of the amplified PCR products was confirmed to be NTR1 by DNA sequence analysis.

To determine whether expression of NTR2 was affected by the ablation of NTR1 expression, mRNA levels were determined by Northern blot analysis. A 1.8-kb NTR2 transcript was detected in +/+ , +/− , and −/− mice and no difference in the level of expression was observed among the three genotypes (data not shown).

**In Situ Hybridization.** Adjacent brain coronal sections were selected across the brains from wild-type and knockout mice (A-P coordinates: bregma 1.00 mm and −3.4 mm) and hybridized with either [35P]UTP-labeled antisense or sense NTR1 RNA probes. In three pairs of sections from wild-type mice, the NTR1 mRNA expression was revealed by in situ hybridization with an antisense RNA probe. High intensity of hybridization signal was shown in several brain regions, including the hippocampus, amygdala, hypothalamus, and cerebral cortex, particularly layers 5 and 6 (see Fig. 1B for representative image). In addition, moderate intensity of hybridization signal was also shown in the thalamic region (data not shown). In contrast, no hybridization signal was detected in any of the sections from the knockout mice (Fig. 1B). The sense RNA probe did not show any significant hybridization signal in all adjacent brain sections from either genotype (Fig. 1B).

**Radioligand Binding Studies.** 125I-NT saturation studies indicated the presence of two populations of binding sites in the wild-type brain: a high-affinity site (presumptive NTR1) with a $K_d = 290 \pm 50 \mu M$, and $B_{max} = 22 \pm 4 \text{ fmol/mg}$ of protein, and a low-affinity site (presumptive NTR2) with $K_d = 1900 \pm 220 \mu M$ and $B_{max} = 42 \pm 11 \text{ fmol/mg}$ of protein ($n = 3$) (see Fig. 2 for representative experiment). Competition of 1 nM 125I-NT binding by the NTR2-selective drug levocabastine confirmed the existence of the two sites in the brains of the +/+ and the +/− mice (Fig. 3). However, in the −/− NTR1 mice, only one site was revealed (Figs. 2 and 3) with a $K_i$ for levocabastine of 22 nM, consistent with its affinity for mouse NTR2 (Table 1). The experiment was repeated three times and comparable results were obtained with the proportion of the high-affinity site for levocabastine (NTR2) varying from 53 to 68% in the wild-type and 60 to 75% in the heterozygous mice.

In the peripheral tissues, binding at only one concentration of 125I-NT (1 nM) was determined, a concentration high enough to label NTR2 sites (Fig. 2). Only NTR1 was clearly detected in the +/+ mouse tissues (fmol/mg of protein at 1 nM 125I-NT: duodenum, 3.2; colon, 4.1; ileum, 2.5) by using levocabastine at 10 μM as a tool to prevent 125I-NT binding to NTR2 binding sites, if present. In the −/− mouse tissues, no 125I-NT specific binding was detected in the presence or absence of levocabastine.

**Isolated Tissue Studies**

The exposure of ileal and colonic segments from wild-type mice to a single, maximal dose of NT at 30 nM caused transient, biphasic relaxation/contraction responses. Cumulative dose-response curves could not be conducted with NT because of tachyphylaxis. The predominant, and most consistent, responses for the colon and ileum from +/+ mice were contraction and relaxation, respectively, and the magnitude of these effects is shown in Fig. 4. In contrast, tissues from knockout mice did not respond to NT (Fig. 4), whereas +/− tissues responded like +/+ tissues. The relaxation of colon and contraction of ileum were relatively weak and variable and could not be quantitated reliably, but qualitatively these responses were also absent in the −/− mice (data not shown). Qualitatively similar results were obtained with NT-2 (30 nM) in ileum and colon and for contractile responses to NT.
Like NT, NT-2 shows high affinity for both mouse NTR1 and NTR2 with about a 30-fold higher affinity for NTR1 (Table 1).

**Behavioral Studies**

**Core Body Temperature and Hot-Plate Analgesia Studies.** The i.c.v. administration of NT to wild-type mice caused a large drop in body temperature of about 5–6°C at 45 to 90 min after a dose of 1 μg (Fig. 5A). Heterozygous mice responded in a similar manner to wild-type mice, but knockout mice exhibited no hypothermia over this time course (Fig. 5A). A similar profile of responses was observed at 45 min after peripheral dosing of NT-2 (1 mg/kg i.p.) where a 5–6°C drop in body temperature was obtained in wild-type and heterozygous mice, but not in knockout mice (Fig. 5B). The doses of NT and NT-2 used here were maximal, based on preliminary dose-response studies in control mice (data not shown). Hot-plate (55°C) reaction times were measured in the same animals as for core temperature, but at different time points. Both NT (1 μg i.c.v.) and NT-2 (1 mg/kg i.p.) caused significant increases in response latency after placement on the heated surface at 30 and 60 min after dosing of wild-type mice (Fig. 6, A and B). A similar analgesic response was seen after NT or NT-2 dosing of heterozygous mice, whereas no increase in response latency was observed in the knockout mice. These studies were
conducted with equal numbers of male and female mice and no consistent effects of gender were observed (data not shown). There were no significant differences in baseline core body temperatures or hot-plate reaction times between the three genotypes when duplicating the time course out to 90 min in untreated animals (data not shown).

**Motor Performance (Rotarod) Studies.** The mice of the three genotypes showed no differences in their ability to remain on an accelerating rotarod (Fig. 7A). Treatment of the mice with NT-2 at 1 mg/kg i.p. caused roughly a 50% reduction in the pretest performance of the /H11001/H11002 mice at 45 and 90 min (Fig. 7B). No loss in performance, however, was observed in the NTR1 knockout mice after NT-2.

**Colonic Propulsion Studies.** NT-2 was tested in the three genotypes for effects on colonic motility, as measured by the rate at which a rectally placed glass bead was expelled. In mice of each gender and genotype, all of the vehicle-treated mice expelled the bead within 20 min (data not shown). Treatment with NT-2 (1 mg/kg i.p.) inhibited the expulsion of the glass bead in 95% of the /H11001 mice after 20 min, a reflection of reduced colonic propulsion (Fig. 8A). This inhibition was partially and gradually reversed over a 3-h time frame. Similar to vehicle-treated wild-type mice, treatment of NTR1 knockout mice with NT-2 did not result in any disturbance in colonic propulsion at any of the time points measured, with all mice expelling the bead within 20 min. The magnitude of inhibitory effect in /H11001 mice by NT-2 was intermediate, suggestive of a gene-dose effect (Fig. 8A). In contrast, the inhibitory effects of clonidine on colonic propulsion were similar for all three genotypes (Fig. 8B).

**Discussion**

Upon central administration, NT and NT agonist analogs have been shown over the years to produce a constellation of effects in rodents, including hypothermia, analgesia, sedation, and antipsychotic-like activity (Bisette et al., 1976; Nemeroff et al., 1977; Clineschmidt et al., 1979; Nemeroff, 1980; Kinkead et al., 1999; Cusack et al., 2000). Most, if not all, of these CNS effects of NT can be mimicked by systemic dosing of metabolically stable analogs of NT, most notably NT-2 (Machida et al., 1993; Sarhan et al., 1997) and NT69L (Cusack et al., 2000; Tyler-McMahon et al., 2000). NT is also known for its pharmacological effects on certain peripheral functions, including effects on intestinal motility and secretory activity (Kitabgi and Freychet, 1978), as well as certain ef-
Effects on endocrine and neuroendocrine function (Brown and Miller, 1982).

Several receptor subtypes (NTR1, NTR2, NTR3) have now been cloned from human and rodent species that are candidates for mediating the effects of NT (Tanaka et al., 1990; Vita et al., 1993; Chalon et al., 1996; Mazella et al., 1996; Botto et al., 1998; Vincent et al., 1999; Navarro et al., 2001). Attempts at defining the receptor subtype mediating two prominent CNS effects of NT, hypothermia and antinociception, have not provided a consistent picture. Although systemic dosing of SR48692 blocked centrally mediated NT-induced turning behavior, it failed to alter NT-induced hypothermia and analgesia (Gully et al., 1993; Dubuc et al., 1994), suggesting non-NTR1 (i.e., NTR2) involvement in these responses to NT. A role for NTR2 in NT-induced analgesia (but not hypothermia) was further suggested by Dubuc et al. (1999b) by using an NTR2 antisense knock-down strategy. On the other hand, use of peptide nucleic acids to downregulate NTR1 expression provided evidence for involvement of NTR1 in the antinociception and hypothermia (Tyler et al., 1998a). Other studies showed that the highly NTR2-selective levocabastine had no effect on baseline core body temperature or NT-induced hypothermia or baseline analgesia (Tyler et al., 1998b; Dubuc et al., 1999a), but either partially inhibited NT-induced analgesia at low (but not high) doses (Tyler et al., 1998b) or completely blocked it (Dubuc et al., 1999a). Peripherally, SR48692 blocks many of the motility effects of NT on intestinal tissue in vitro (Labbe-Jullie et al., 1994; Croci et al., 1999), suggesting mediation by NTR1. Complicating the interpretation somewhat, however, is the fact that SR48692 is not highly NTR1 selective (versus NTR2) (Gully et al., 1993; Chalon et al., 1996; Botto et al., 1998; Vita et al., 1999, 1998) and in fact has significant affinity for NTR2 where it can act as an agonist (Botto et al., 1997; Vita et al., 1998; Yamada et al., 1998).

In the present article, we describe the generation of NTR1 knockout mice to study more clearly the functions of this receptor subtype. The gene deletion was accomplished by homologous recombination and was confirmed by RT-PCR, in situ hybridization, and radioligand binding. Although NTR1 expression was eliminated, the level of NTR2 expression appeared unchanged by Northern blot analysis and binding studies. These mice showed no obvious abnormalities by gross inspection, histologically or in overt behavior. Our

Fig. 7. Effect of NT-2 on rotarod performance in NTR1 mutant mice. Mice of the three genotypes (n = 12/group, six male, six female) were tested for motor coordination on an accelerating (4–40 rpm over 5 min) rotarod after either no treatment (A, control) or after NT-2 (B, NT-2) at 1 mg/kg i.p. Data are represented as mean percentage of pretest baseline. **, P < 0.01 compared with wild-type at the individual time points.

Fig. 8. Effect of NT-2 or clonidine on colonic propulsion in NTR1 mutant mice. Mice of the three genotypes (n = 20/group, 10 male, 10 female) were treated with either NT-2 (1 mg/kg i.p.) (A) or clonidine (100 μg/kg i.p.) (B), and the time required for glass bead expulsion was recorded as described under Materials and Methods and expressed as percentage of mice with bead expelled. Values are group means. *, P < 0.05; **, P < 0.01 compared with wild-type at the individual time points.
studies with the NTR1 knockout mice, however, clearly indicate an important involvement of NTR1 receptors in several prominent effects of NT receptor stimulation in the CNS (hypothermia, hot-plate analgesia, and motor coordination based on rotarod performance) and on gastrointestinal function (colonic propulsion).

Intraventricular administration of NT to wild-type C57BL6/129J F2 hybrid mice caused a marked drop on core body temperature and, in parallel, significant hot-plate analgesia, in agreement with other studies in rats and mice (Nemeroff et al., 1977; Clineschmidt et al., 1979; Dubuc et al., 1994). These responses to NT, however, were completely absent in the mutant mice in which the NTR1 gene had been deleted. Like i.c.v. NT, the peripheral administration of the metabolically stable, brain-penetrant NT analog NT-2 (Machida et al., 1993; Banks et al., 1995) to wild-type mice also caused hypothermia, hot-plate analgesia, and reduced rotarod performance in vivo, but again these effects were absent in the NTR1 knockout mice. These results substantiate the importance of NTR1 in these activities and confirm the utility of these brain-penetrant NT analogs in studying the CNS actions of NT (Pugsley et al., 1995; Sarhan et al., 1997; Tyler et al., 1999; Cusack et al., 2000; Tyler-McMahon et al., 2000).

The lack of effect of peripherally dosed SR48692 on NT-induced hypothermia or analgesia reported elsewhere (Dubuc et al., 1994; Pugsley et al., 1995; Gully et al., 1997) would seem to be at odds with the present findings implicating a role for NTR1 in these effects, but some possible explanations may be offered for the discrepancy: 1) NTR2 agonist-like activity of SR48692 may somehow interfere with its NTR1 antagonist effects on these endpoints, or 2) effective free concentrations of SR48692 in brain areas controlling the hypothermic/analgesic effects on NT may not be reached after systemic administration, in contrast to the striatum where systemic SR48692 exerted inhibitory effects on turning behavior stimulated by intrastriatally dosed NT (Gully et al., 1993). In addition, multiphasic interactions have been noted recently for pain responses to various doses of NT and SR48692 (Smith et al., 1997), which may reflect the complex ways in which these compounds can interact in vivo.

Our present results also show the role of NTR1 in gastrointestinal function as well as the utility of NT-2 in studying peripheral mechanisms. NT-2 administration to wild-type mice reduced colonic propulsion, similar to the effects of SR48692 (Smith et al., 1997; Vita et al., 1998; Yamada et al., 1998). The involvement of NTR1 in gastrointestinal function is further exemplified by our in vitro isolated tissue studies demonstrating loss of contractile and/or relaxant effects in gastric, ileal, and colonic tissue taken from −/− mice.

The fact that several diverse effects of NT are eliminated in the absence of NTR1 expression suggests that they may all be mediated directly and independently by NTR1 receptors. Our results, however, do not rule out that certain of the responses to NT may be dependent on, or downstream of others. For example, it is not possible from our studies to determine whether the effect of NT on hot-plate reaction time is an independent effect, or one arising from the marked hypothermia. Other investigators, however, have presented evidence that the hypothermic and antinociceptive effects of NT are dissociable (Clineschmidt et al., 1979; Tyler et al., 1998c; Dubuc et al., 1999a,b).

In summary, these initial results with NTR1 knockout mice implicate a major role of NTR1 in mediating several of the well known central and peripheral effects of NT, including body temperature control, hot-plate analgesia, rotarod performance, and gastrointestinal motility effects. Additional study of these knockout mice will help define further the in vivo activities of NTR1, as well as possibly provide insights into the unknown physiological role(s) of NTR2 (and NTR3).

Acknowledgments
We thank Dr. XiaoI Hou (Biometrics Research, Merck Research Laboratories) for expert assistance with the statistical analysis.

References


Address correspondence to: Dr. Douglas J. Pettibone, Department of Neuroscience, Building W46-300, Merck Research Laboratories, West Point, PA 19486. E-mail: doug.pettibone@merck.com