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The Orphan Transporter Rxt1/NTT4 (SLC6A17) Functions as a Synaptic Vesicle Amino Acid Transporter Selective for Proline, Glycine, Leucine, and Alanine

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ABSTRACT

Rxt1/NTT4 (SLC6A17) belongs to a gene family of "orphan transporters" whose substrates and consequently functions remain unidentified. Although Rxt1/NTT4 was previously thought to function as a sodium-dependent plasma membrane transporter, recent studies localized the protein to synaptic vesicles of glutamatergic and GABAergic neurons. Here, we provide evidence indicating that Rxt1/NTT4 functions as a vesicular transporter selective for proline, glycine, leucine, and alanine. Using Western blot, immunoprecipitation, immunocytochemistry, and polymerase chain reaction approaches, we demonstrate that PC12 cells express the Rxt1/NTT4 gene and protein. Small interfering RNA (siRNA)-mediated knockdown of Rxt1/NTT4 in PC12 cells resulted in selective reductions in uptake levels for proline, glycine, leucine, and alanine. Likewise, gas chromatography analysis of amino acid content in an enriched synaptic vesicle fraction from wild-type and siRNA-Rxt1/NTT4 PC12 cells revealed that proline, glycine, leucine, and alanine levels were decreased in siRNA-treated cells compared with wild-type cells. Furthermore, Rxt1/NTT4-transfected Chinese hamster ovary (CHO) cells exhibited significant uptake increases of these amino acids compared with mock-transfected CHO cells. Finally, proline uptake in both PC12 cells and Rxt1/NTT4-transfected CHO cells was dependent on the electrochemical gradient maintained by the vacuolar-type H^+-ATPase. These data indicate that the orphan Rxt1/NTT4 protein functions as a vesicular transporter for proline, glycine, leucine, and alanine, further suggesting its important role in synaptic transmission.

Neurotransmitter transporters play a crucial role in the regulation of neuronal transmission by removing transmitters from the synaptic cleft and loading synaptic vesicles for subsequent release. Based on their subcellular localization, neurotransmitter transporters can be classified as either plasma membrane or vesicular transporters. Plasma membrane transporters mediate high-affinity uptake of released transmitters into pre- and/or postsynaptic sites and glia cells (for review, see Torres and Amara, 2007). Molecular cloning techniques have further divided these plasma membrane transporters into two major groups based on amino acid homology. The first group is made up of transporters for dopamine (DA), norepinephrine, serotonin, GABA, and glycine, all of which contain 12 transmembrane putative spanning domains and transport their substrates in a sodium- and chloride-dependent manner (Nelson, 1998). The second group includes glutamate transporters, for which five genes

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ABBREVIATIONS: FITC, fluorescein isothiocyanate; HEK, human embryonic kidney; CHO, Chinese hamster ovary; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; siRNA, small interfering RNA; PNS, postnuclear supernatant; NaGluc, sodium gluconate; RT, reverse transcription; DA, dopamine; GFP, green fluorescent protein; VMAT, vesicular monoamine transporter; VAChT, vesicular acetylcholine transporter; VGLUT, vesicular glutamate transporter; VIAAT, vesicular inhibitory amino acid transporter; V-ATPase, vacuolar-type H^+-ATPase; NMDA, *N*-methyl-D-aspartic acid; GlyT, glycine transporter; WT, wild type.

have been identified (Seal and Amara, 1999). In contrast, vesicular transporters package neurotransmitters into synaptic vesicles through a mechanism involving a proton electrochemical gradient (Chaudhry et al., 2008a,b). To date, molecular cloning approaches have identified vesicular transporters for monoamines (Erickson et al., 1992; Liu et al., 1992), acetylcholine (Roghani et al., 1994), GABA (McIntire et al., 1997; Sagné et al., 1997), and glutamate (Ni et al., 1994; Aihara et al., 2000; Bellocchio et al., 2000; Takamori et al., 2000, 2001, 2002; Bai et al., 2001; Fremeau et al., 2001, 2002; Herzog et al., 2001; Gras et al., 2002; Schäfer et al., 2002).

In addition to these transporters with established substrates, several groups have cloned a family of putative transporter proteins whose substrates and consequently functions remain unidentified (Uhl et al., 1992). These proteins, referred to as "orphan transporters," include Rxt1/NTT4 (Liu et al., 1993; el Mestikawy et al., 1994), XT2/ROSIT (Wasserman et al., 1994), XT3/rB21a (Smith et al., 1995), NTT5 (Farmer et al., 2000), and v7-3/NTT7 (UhI et al., 1992; Sakata et al., 1999; Farmer et al., 2000). Although these orphan transporters share approximately 30 to 65% homology among themselves and approximately 30 to 45% homology to the sodium- and chloride-dependent plasma membrane transporters, their tissue distributions and subcellular localizations have been found to be very diverse. XT2/ROSIT, XT3/rB21a, and NTT5 are found predominantly in peripheral tissues (Wasserman et al., 1994; Smith et al., 1995; Farmer et al., 2000), whereas Rxt1/NTT4 and v7-3/NTT7 are expressed predominantly in brain (UhI et al., 1992; Liu et al., 1993; el Mestikawy et al., 1994; Masson et al., 1996; Farmer et al., 2000). Indeed, Rxt1/NTT4 is present primarily in glutamatergic and GABAergic neurons (el Mestikawy et al., 1994, 1997; Masson et al., 1995). Furthermore, biochemical and immunocytochemical studies revealed an unexpected synaptic vesicle localization for Rxt1/NTT4 in glutamatergic neurons (Fischer et al., 1999; Masson et al., 1999). Based on these observations, it has been suggested that Rxt1/NTT4 might function as a vesicular transporter, or alternatively, reside in intracellular vesicles as a reserve pool from which they need to be translocated to the plasma membrane to become functional. In this study, we provide evidence supporting the contention that the orphan Rxt1/NTT4 transporter exists and functions as a vesicular amino acid transporter with selectivity for proline, glycine, leucine, and alanine. The physiological significance of our findings is discussed.

Materials and Methods

Materials. The polyclonal antibody against Rxt1/NTT4 was generated by immunizing rabbits with a fusion protein derived from the carboxyl-terminal domain of the rat Rxt1/NTT4 (amino acids 304–322) and affinity purified as described in Masson et al. (1995). The monoclonal anti-synaptophysin antibody was obtained from BD Biosciences (San Jose, CA), whereas antibodies against calnexin and the transferrin receptor were from Millipore Corporation (Billerica, MA) and Zymed Laboratories (South San Francisco, CA), respectively. Peroxidase- and FITC-conjugated secondary antibodies were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK), whereas Texas Red-conjugated antibody was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Sprague-Dawley rats were supplied by Hilltop Laboratory Animals, Inc. (Scottsdale, PA), and C57BLC mice were supplied by The Jackson Laboratory (Bar Harbor, ME). Cell culture media were from Invitrogen (Carlsbad, CA), and protease inhibitors were from Pierce Chemical (Rockford, IL). Radiolabeled substrates were from Perkin-Elmer Life and Analytical Sciences (Waltham, MA). All other materials were from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Cell Culture. Human embryonic kidney (HEK) 293, the human neuroblastoma cell line SKNSH, PC12, and Chinese hamster ovary (CHO) cells were purchased from American Type Culture Collection (Manassas, VA). HEK293 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine, and 50 μg/ml each penicillin and streptomycin at 37°C in a humidified, 5% CO_2 incubator. SKNSH and PC12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% FBS, 5% horse serum, 1 mM glutamine, and 50 μg/μl each of penicillin and streptomycin at a 37°C in a humidified, 10% CO_2 incubator. CHO cells were cultured in F-12 + Glutamax, 5% FBS, and 50 μg/μl each penicillin and streptomycin at a 37°C in a humidified, 5% CO_2 incubator. If necessary, cells were transfected with 5 μg of DNA using Transfectin (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommendations. Transfected cells were allowed to grow for an additional 48 h before further studies.

Preparation of Brain and Cell Lysates. Cells, striata, or whole brain from rats or mice were homogenized in radioimmunoprecipitation assay buffer (100 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS) containing protease inhibitors and incubated with rotation for 1 h at 4°C. Brain lysates were centrifuged at 16,000*g* for 10 min and at 20,000*g* for 60 min at 4°C, whereas cell lysates were centrifuged at 13,000*g* for 10 min at 4°C. The final supernatants were then collected, and protein concentrations were measured using the DC Protein Assay (Bio-Rad) and used in subsequent experiments.

Immunoprecipitations. PC12 cell lysates were incubated with either preimmune serum or anti-Rxt1/NTT4 (1:200) antibody overnight at 4°C. Then, 40 μl of protein A-Sepharose (GE Healthcare) was added to all samples. Samples were incubated again for 1 h at 4°C and centrifuged at 13,000*g* for 1 min. The resulting pellets were washed twice with radioimmunoprecipitation assay buffer and twice with PBS. The immunoprecipitated proteins were eluted using Laemmli sample buffer (Bio-Rad) containing 10% β-mercaptoethanol. Then, samples were heated at 37°C for 30 min and resolved by polyacrylamide gel electrophoresis and Western blot.

Western Blot Analysis. Samples were separated by polyacrylamide gel electrophoresis on 10% Tris-HCl polyacrylamide gels and

TABLE 1
Primers used to amplify Rxt1/NTT4 in RT-PCR

Position	Primer Sequence	Expected Size
		bp
475–2658		2184
Forward	5′-ATGCCGAAGAACAGCAAGGTG-3′	
Reverse	5′-TCACAGCTCTGACTCAGGGGT-3′	
475–2648		2174
Forward	5′-ATGCCGAAGAACAGCAAGGTG-3′	
Reverse	5′-GACTCAGGGGTGCTGGCCAAG-3′	
475–2112		2174
Forward	5′-ATGCCGAAGAACAGCAAGGTG-3′	
Reverse	5′-GACTCAGGGGTGCTGGCCAAG-3′	
475–2112		1638
Forward	5′-ATGCCGAAGAACAGCAAGGTG-3′	
Reverse	5′-AATCCAGGCCACAGCGATGTT-3′	
718–2112		1395
Forward	5′-AACATCTGGAGGTTCCCCTAC-3′	
Reverse	5′-AATCCAGGCCACAGCGATGTT-3′	
2401–2658		258
Forward	5′-TTGCTCTCCGACGGGTCCAAC-3′	
Reverse	5′-TCACAGCTCTGACTCAGGGGT-3′	
475–678		204
Forward	5′-ATGCCGAAGAACAGCAAGGTG-3′	
Reverse	5′-CTGCAGCTTGCTATTCCAGGC-3′	

bp, base pairs.

transferred to nitrocellulose membranes using the Bio-Rad system. Whole brain or striata lysate (50 μg) was used as a positive control in all experiments. Nitrocellulose membranes were first blocked for 1 h in Tris-buffered saline buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween 20) containing 5% dry milk, followed by incubation with the primary antibody for 1 h in blocking buffer. After washing the membrane three times for 10 min each in Tris-buffered saline buffer, a horseradish peroxidase-conjugated secondary antibody was added in blocking buffer for 1 h. The membrane was washed a final three times, and West Pico chemiluminescence (Pierce Chemical) was used to visualize protein bands.

Immunocytochemistry. Wild-type PC12 cells were grown on poly-D-lysine-coated coverslips overnight and then fixed using 4% paraformaldehyde for 10 min, followed by washing with PBS. After permeabilization with 0.05% Triton X-100 for 10 min, the samples were incubated with blocking solution (5% goat serum and 1% bovine serum albumin) for 30 min. Preimmune or anti-Rxt1/NTT4 was incubated at a 1:200 dilution in blocking solution for 1 h, followed by three washes in PBS, and an additional 1-h incubation with FITC-conjugated secondary antibody at a 1:1000 dilution in blocking solution. For colocalization experiments, samples were also incubated with synaptophysin, calnexin, or transferrin receptor antibodies all at 1:200 dilution, followed by Texas Red-conjugated secondary antibody at a 1:500 dilution for 1 h. Immunofluorescent experiments were also performed on CHO cells transfected with Rxt1/NTT4 tagged with GFP (GFP-Rxt1/NTT4). In all cases, cells were mounted with Fluoro mounting medium (MP Biomedicals, Irvine, CA) and visualized using confocal microscopy (TCS SL; Leica Microsystems, Inc., Deerfield, IL).

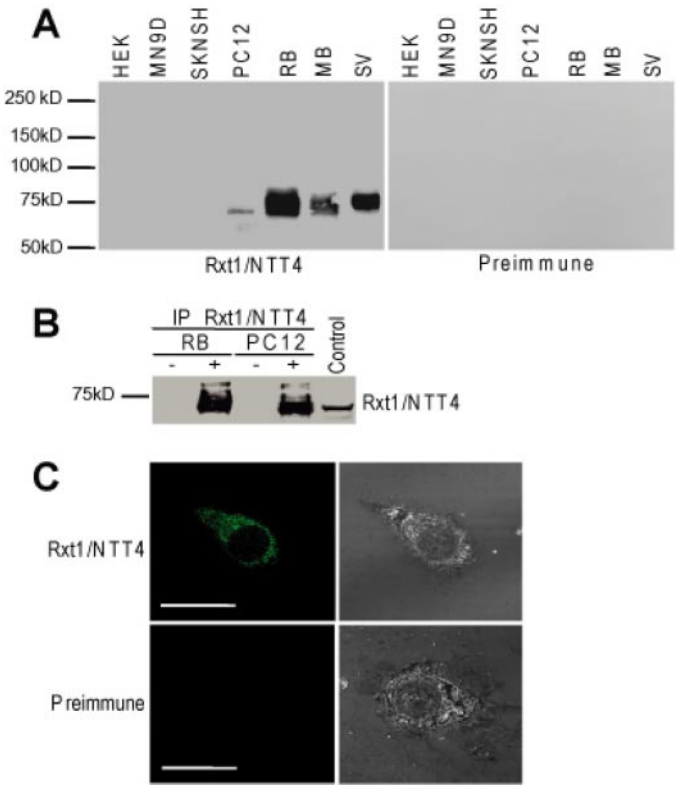

Fig. 1. Rxt1/NTT4 is expressed in PC12 cells. A, Western blot analysis of Rxt1/NTT4 using four different cell lines. Positive controls from an enriched synaptic vesicle preparation (SV) and whole brain lysates from rat (RB) and mouse (MB) are also shown. Blots were also probed with preimmune serum as a specificity control. B, immunoprecipitation of Rxt1/NTT4 from PC12 cells. Immunoprecipitations using rat brain (RB) lysate were also used as a positive control. Samples were incubated with preimmune serum (−) or Rxt1/NTT4 antibody (+). C, immunocytochemical analysis of Rxt1/NTT4 in PC12 cells. Fixed cells were immunostained with the anti Rxt1/NTT4 (top; scale bar, 20 μm) or preimmune serum (bottom; scale bar, 18 μm) and labeled with a FITC-conjugated antibody. Contrast phase controls also shown (right).

RNA Isolation, cDNA Synthesis, and Cloning. Total RNA from rat brain, PC12, or CHO cells was isolated using TRIzol reagent (Invitrogen) as recommended by the manufacturer. Reverse transcription was performed using SuperScript III (Invitrogen). The cDNA was purified using the QIAquick polymerase chain reaction (PCR) purification kit (QIAGEN, Valencia, CA), and the full coding sequence for Rxt1/NTT4 was amplified using iTaq DNA polymerase (Bio-Rad) and PCR. The primers used for the PCR and nested PCR are listed in Table 1. PCR products were purified by agarose gel electrophoresis, isolated using the QIAquick gel extraction kit (QIAGEN), subcloned using the pcDNA3.1/V5-His TOPO vector (Invitrogen), and sequenced by automated DNA sequencing.

Generation of siRNA Stable Clones. The pSilencer 4.1-CMV Puro vector was obtained from Ambion (Austin, TX) and used to produce siRNAs against Rxt1/NTT4. According to the manufacturer's instructions, 21 pairs of annealed DNA oligonucleotides were inserted into this vector between the BamHI and HindIII restriction sites to construct the siRNAs. The Rxt1/NTT4 siRNA sequence was 5′-AAACAGGACAATAACTGCCAC-3′ corresponding to nucleotides 1444 to 1464 of rat Rxt1/NTT4. The sequence 5′-AAACTACCGTTGTTATAGGTG-3′ was used as a scramble control. Inserted sequences were confirmed by DNA sequencing. To isolate siRNA-containing clones, PC12 cells were transfected with pSilencer 4.1-CMV-Rxt1/NTT4 siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, 200 ng/ml puromycin was maintained in the medium for selection. Western blot analysis for Rxt1/NTT4 was performed as described under Western blot analysis.

Postnuclear Supernatant Preparation and Vesicle Isolation. Media were removed from confluent 10-cm plates of either PC12 or CHO cells. After rinsing the plate with PBS, cells were

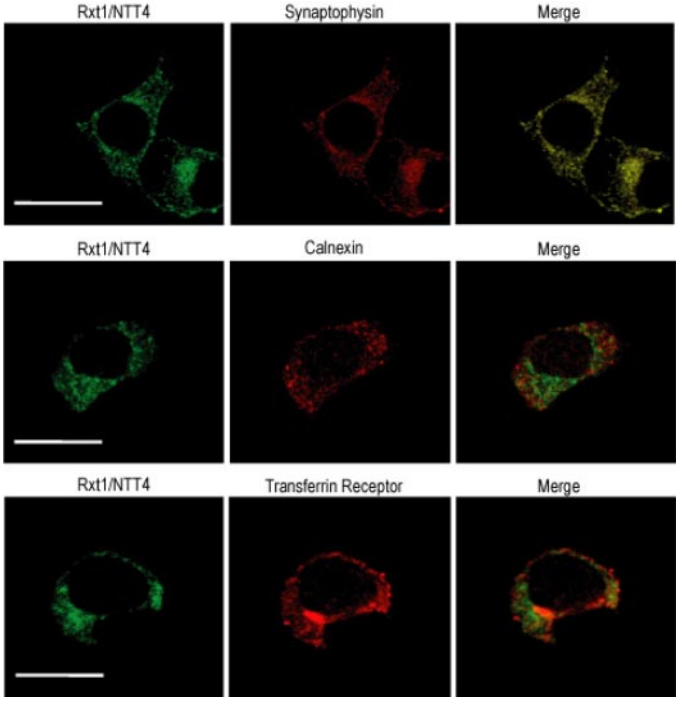

Fig. 2. Rxt1/NTT4 colocalizes with synaptophysin in PC12 cells. Fixed PC12 cells were immunostained with Rxt1/NTT4 (green; left), synaptophysin, calnexin, or transferrin receptor antibodies (red; middle). The merged image shows an almost complete colocalization of Rxt1/NTT4 with synaptophysin (right, top; scale bar, 20 μm). In contrast, the endoplasmic reticulum marker calnexin (right, middle; scale bar, 18 μm) and the endosomal marker transferrin receptor (right, bottom; scale bar, 8 μm) showed little overlap with Rxt1/NTT4.

scraped from dishes and pelleted at 305*g* for 3 min. The resulting pellet was resuspended in buffer A (320 mM sucrose, 0.1 $MgCl_2$, 0.5 EGTA, and 10 HEPES, pH 7.4) containing protease inhibitors. After homogenization using 25 strokes in a Wheaton homogenizer (Wheaton Science Products, Millville, NJ) followed by 25 passages through a 25-gauge needle, the sample was centrifuged at 1000*g* for 5 min at 4°C, resulting in the postnuclear supernatant (PNS) preparation. Further isolation of synaptic vesicles was obtained by additional centrifugations at 27,000*g* for 35 min and 180,000*g* for 2 h at 4°C. The resulting pellet contained an enriched synaptic vesicle fraction and was called P3.

Determination of Vesicular Amino Acids by Capillary Gas Chromatography. P3 fractions from PC12 cells (wild type, siRNA-Rxt1/NTT4, and siRNA-scramble) were resuspended and sonicated in 300 μl of water. Aliquots of lysed cells were used for amino acid analysis according to the procedure established by the EZ:faast free amino acid kit (Phenomenex, Torrance, CA). In brief, 100 μl of Norvaline solution (20 nmol) was added to each sample as an internal standard. The mixture was slowly passed through a sorbent tip, and the filtrate was discarded. The sorbent tip was washed with 200 μl of 2-propanol/H_2O. The amino acids were then recovered by eluting the sorbent tip with 200 μl of a sodium hydroxide and *n*-propanol mixture [3:2 (v/v)]. Finally, the recovered amino acids were derivatized by adding 50 μl of chloroform, 100 μl of isooctane, and 100 μl of 1 N HCl solvents consecutively with vigorous vortexing in between the solvent addition. Then, 100 μl of the upper layer was used for amino acid determination using capillary gas chromatography. The amino acid derivatives were separated on a 10-m Zebron ZB-AAA column (0.25 mm i.d.) with helium as the carrier gas (1.5 ml/min) using a Hewlett-Packard capillary gas chromatograph model 5890 series II, equipped with a hydrogen flame ionization detector. The sample was injected under a split ratio of 15:1. Oven temperature was initially set at 110°C and then programmed to 280°C at 32°C/min. The oven temperature remained at 280°C for another 5 min before the end of the run. Total run time was approximately 10.3 min. Peaks were identified by comparing the retention times of the samples with those of standard mixtures and calculated by ChemStation (rev. A.09.03; Agilent Technologies, Santa Clara, CA) using an internal standard mode. These results were normalized using protein concentration.

Vesicular Uptake. PNS (200 μg) in 50 μl was added to 200 μl of uptake buffer (20 mM HEPES, 100 mM NaCl, mM 2.5 $MgSO_4$, and 2 mM ATP, pH 7.4) warmed to 29°C, followed by the addition of radiolabeled substrate (50 nM). After incubation for 6 min, the samples were filtered through a 0.2-μm SUPOR membrane filter (Pall Corporation, East Hills, NY). The reaction was stopped by washing twice with 1.5 ml of ice-cold uptake buffer. The filter was then added to 4 ml of Biosafe II scintillation liquid (Research Products International, Mt. Prospect, IL), and radioactivity was counted in an LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). In some cases, the uptake buffer was altered to contain 100 mM LiCl or 100 mM sodium gluconate (NaGluc) instead of NaCl; depleted of ATP; or supplemented with 10 μM bafilomycin A1, 20 μM valinomycin, 5 μM nigericin, or 1 mM ouabain. To determine kinetic constants for [^{3}H]Pro and [^{3}H]Gly transport, we used 200 μg of PNS from Rxt1/NTT4-transfected CHO cells using normal uptake conditions. The substrate [^{3}H]Pro or [^{3}H]Gly (at 50 nM) and increasing concentrations of unlabeled Pro or Gly ranging in concentration from 1 μM to 3 mM were used. Nonspecific uptake was determined in the presence of 50 mM Pro or Gly.

Data Analysis. Results are presented as mean ± S.E. Significant differences between means were determined by Student's *t* test, with $p < 0.05$ considered statistically significant.

Results

Rxt1/NTT4 Is Expressed in PC12 Cells. Although previous in vivo electron microscopy studies have indicated that Rxt1/NTT4 is localized to synaptic vesicles of glutamatergic and/or GABAergic neurons (Fischer et al., 1999; Masson et al., 1999), we were unaware of any cell lines endogenously expressing Rxt1/NTT4. Thus, in an effort to find a suitable cell system to use for the identification of the substrate(s) for Rxt1/NTT4, we screened several cell lines for endogenous expression of this protein. Tested cell lines included HEK293, SKNSH, MN9D, and PC12 cells, whereas whole brain lysates and synaptic vesicle preparations from mouse and rat were used as positive controls. Western blot analysis using an affinity-purified polyclonal antibody against Rxt1/NTT4 revealed the presence of an approximately 75-kDa band in both PC12 cells and in the positive controls (Fig. 1A). No bands were detected in HEK293, SKNSH, or MN9D cells. As a control for specificity, blots were also incubated with preimmune serum, and no bands were detected in any of the cell lines or positive controls (Fig. 1A). As an additional approach, we performed immunoprecipitation on PC12 cells using either the Rxt1/NTT4 antibody to detect the transporter. Consistent with our previous data, the transporter successfully coprecipitated with the Rxt1/NTT4 antibody but not with the preimmune serum in both PC12 cells and rat brain lysates (Fig. 1B). Next, we examined the subcellular localization of Rxt1/NTT4 in PC12 cells using confocal microscopy. PC12 cells exhibited extensive Rxt1/NTT4 staining throughout the cell only in those samples incubated with the antibody against Rxt1/NTT4 but not in those cells incubated with the preimmune serum (Fig. 1C). Furthermore, to test whether Rxt1/NTT4 was present in synaptic vesicles, we also conducted colocalization experiments using synaptophysin as a

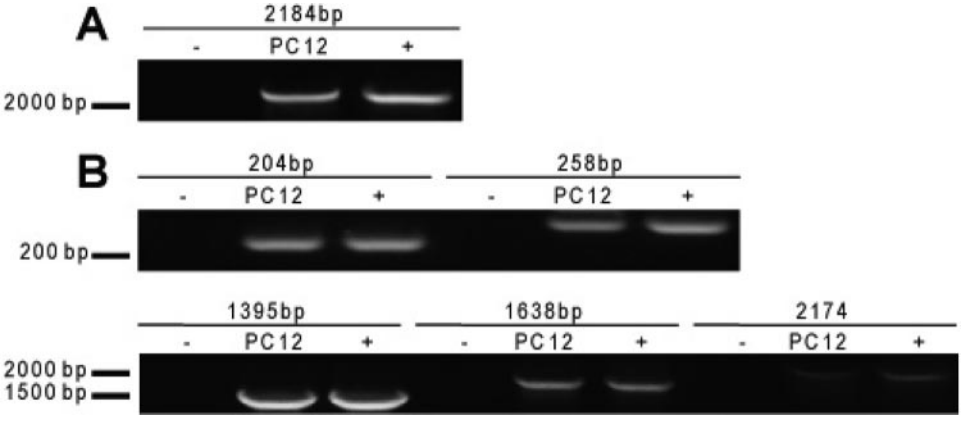

Fig. 3. PC12 cells express the Rxt1/NTT4 gene. A, RT-PCR analysis of Rxt1/NTT4 mRNA from PC12 cells reverse transcribed into cDNA. Rxt1/NTT4 cDNA from rat brain was used as a positive control (+), whereas the negative control contained no cDNA (−). B, to confirm the amplification of the desired sequence, nested PCR followed first-round amplification. The isolated PCR fragment was initially cloned using pcDNA3.1/V5-His-TOPO cloning kit, and its sequence was confirmed by DNA sequencing.

synaptic vesicle marker. Merged images show an almost complete overlap of synaptophysin and Rxt1/NTT4 (Fig. 2, top). In contrast, little colocalization was found between Rxt1/NTT4 and the ER marker calnexin (Fig. 2, middle) or the endosomal marker transferrin receptor (Fig. 2, bottom). Our findings are consistent with the synaptic vesicle localization of Rxt1/NTT4 in neurons as reported by Masson et al. (1999).

Because approaches using antibodies cannot confirm the identity of a given protein, we sought to isolate the Rxt1/NTT4 gene from PC12 cells. To do this, we performed RT-PCR using flanking primers against the transporter sequence. As shown in Fig. 3A, a band of approximately 2200 base pairs corresponding to the size of the full-length Rxt1/NTT4 was detected in PC12 cell samples. This band was purified and used as a template for nested PCR. Using five sets of internal primers (Table 1), we were able to visualize bands of the expected sizes (Fig. 3B). Finally, the identity of Rxt1/NTT4 was confirmed by DNA sequencing (data not shown). Taken together, these data demonstrate that Rxt1/NTT4 is expressed in PC12 cells, which represent a suitable model system to be used in the identification of the transporter's substrate(s).

Rxt1/NTT4-Mediated Vesicular Uptake in PC12 Cells. Previous studies failed to identify the substrate(s) for Rxt1/NTT4 because it was assumed that the transporter was targeted to the plasma membrane. However, more recent findings show that this transporter is actually targeted to synaptic vesicles, suggesting the possibility that Rxt1/NTT4 functions as a vesicular transporter. To test this hypothesis, we developed a strategy in which vesicular content and uptake were measured in wild-type PC12 cells and compared with cells in which the expression of Rxt1/NTT4 was reduced by siRNA. In our hands, overexpressing an siRNA construct against nucleotides 1444 to 1464 in Rxt1/NTT4 results in reduction of protein levels by approximately 50% compared with wild-type cells or cells overexpressing a scramble-siRNA sequence (Fig. 4, A and B). Under the same conditions, the synaptic vesicle proteins SV2, synaptophysin, and synaptobrevin were not affected (Fig. 4A). Immunofluorescent experiments further confirmed this effect of siRNA on Rxt1/NTT4 expression in PC12 cells (Fig. 4C). Next, we tested multiple substrates in an effort to find an uptake activity that would be decreased in siRNA cells compared with wild-type or scramble-siRNA cells. The tested substances included norepinephrine, epinephrine, Asp, Lys, Tyr, Met, dopamine, serotonin, Trp, Cys, Arg, Glu, GABA, Ser, Ala, Pro, Gly, and Leu. All were used at a concentration of 50 nM. Under these experimental conditions, the transport of Pro, Gly, and Leu were all decreased by approximately 70%, and Ala transport was decreased 50% in Rxt1/NTT4 knockdown cells compared with wild-type or scramble-siRNA cells (Fig. 5A). In contrast, vesicular transport for all other tested substances remained unchanged in all three cell lines (Fig. 5A). To complete testing of the remaining amino acids as potential substrates for Rxt1/NTT4, we used unlabeled substrates as potential competitors for [^{3}H]Pro uptake. Using this rationale, we used 50 mM unlabeled GABA, His, Glu, Gln, Phe, Thr, Val, Ile, Ala, Leu, Gly, and Pro. The addition of unlabeled Pro resulted in a 35% decrease of [^{3}H]Pro uptake (Fig. 5B). In agreement with our previous findings, Leu, Gly, and Ala also significantly inhibited [^{3}H]Pro uptake. In contrast, we did not observed significant changes in [^{3}H]Pro uptake in the presence of any of the remaining substances (Fig. 5B). Although it is intriguing that [^{3}H]Pro uptake was reduced only 35% when competing with 50 mM unlabeled Pro, similar reduction levels of [^{3}H]Pro uptake were observed when unlabeled Pro was used at 0.5 or 5 mM (Fig. 5C). This indicates that the [^{3}H]Pro uptake that remains after competition is most likely due to background unrelated to Rxt1/NTT4 function. Taken together, these data suggest that Rxt1/NTT4 can transport Pro, Gly, Leu, and Ala in PC12 cells.

Rxt1/NTT4-Mediated Vesicular Content in PC12 Cells. Because our first experiments were performed using exogenous substrates, we sought to determine whether in fact these substrates were present in PC12 vesicles and whether their content was reduced in cells expressing siRNA against Rxt1/NTT4. Thus, we obtained an enriched synaptic vesicle fraction (called P3) from PC12 cells. Our data confirmed that Rxt1/NTT4 and other synaptic vesicle proteins including SV2 and synaptophysin were enriched in our P3 fraction compared with the initial homogenate (Fig. 6A). Next, we analyzed the amino acid content of the P3 fraction by gas chromatography in wild-type, scramble-siRNA, and siRNA-Rxt1/NTT4 PC12 cells. Consistent with the uptake data, the vesicular content of Pro, Gly, Leu, and Ala were significantly reduced in siRNA cells compared with wild-type or scramble cells (Fig. 6, B and C). No significant differences in vesicular content of Val, Ile, Thr, Met, Phe, Trp, His, Tyr, or Lys were detected (Fig. 6C). These findings are consistent with the notion that Rxt1/NTT4 functions as a vesicular transporter selective for Pro, Gly, Leu, and Ala.

Rxt1/NTT4 Confers Pro, Gly, Leu, and Ala Uptake in CHO Cells. To provide definitive proof that Pro, Gly, Leu, and Ala are substrates of Rxt1/NTT4, we next determined whether in fact the coding sequence of Rxt1/NTT4 is sufficient to transport these amino acids. CHO cells, which do not express Rxt1/NTT4 as demonstrated by RT-PCR (Fig. 7A)

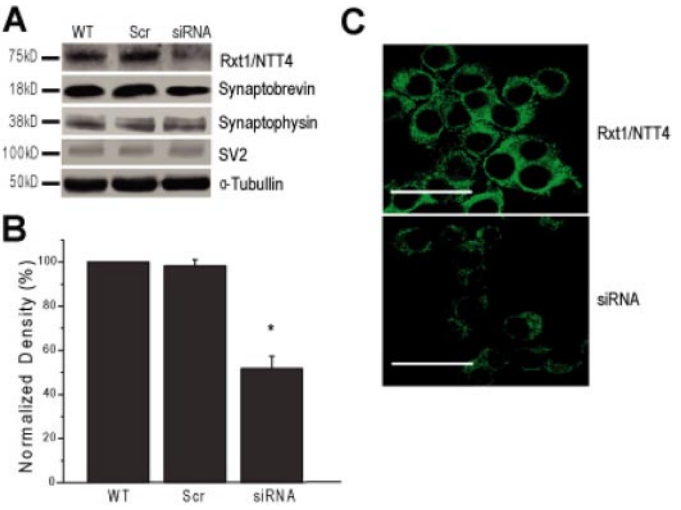

Fig. 4. Rxt1/NTT4 expression is decreased by 50% when knocked down by siRNA in PC12 cells. A, representative immunoblots of wild-type (WT), scramble-siRNA (Scr), and Rxt1/NTT4-siRNA PC12 cells. Rxt1/NTT4 expression is reduced in the siRNA-transfected cells, whereas the synaptic vesicle markers synaptobrevin, synaptophysin, and SV2 are not altered. B, densitometry analysis of Rxt1/NTT4 expression normalized to arbitrary densitometry units of α-tubulin ($n = 3$; *, $p < 0.05$). C, immunofluorescence analysis of Rxt1/NTT4 in wild-type PC12 cells (top; scale bar, 20 μm) and Rxt1/NTT4 siRNA-treated PC12 cells (bottom; scale bar, 24 μm). Fixed cells were immunostained with the anti-Rxt1/NTT4 and labeled with a FITC-conjugated secondary antibody.

and immunoblotting (Fig. 7B), were transfected with a GFP-tagged Rxt1/NTT4, allowing detection by Western blot and confocal microscopy (Fig. 7, B and C). Next, we performed uptake experiments using a postnuclear preparation (PNS) from mock and GFP-Rxt1/NTT4-transfected CHO cells. Mock-transfected cell uptake was considered to be 100% in each individual condition and increases were expressed relative to this percentage. Uptake levels were significantly increased for Pro (10,492%), Gly (611%), Leu (160%), and Ala (206%) in cells transfected with GFP-Rxt1/NTT4 compared with mock cells (Fig. 7D). Similar levels of uptake were obtained in cells transfected with an untagged Rxt1/NTT4 construct (data not shown). These percentages are dramatically high, especially for Pro and Gly, because the uptake of these substrates is almost undetectable in nontransfected cells. No significant differences in uptake levels were observed for GABA or DA. These data demonstrate that the coding sequence of Rxt1/NTT4 is sufficient to confer Pro, Gly, Leu, and Ala uptake in CHO cells. Pro uptake was saturated, with an estimated K_m value of 0.86 ± 0.17 mM and a V_{max} value of 172 ± 12.9 pmol/min/mg protein, whereas K_m and V_{max} values for Gly were 1.72 ± 0.3 mM and 199 ± 16.6 pmol/min/mg protein, respectively (Fig. 8). The K_m values obtained for Pro and Gly in the low millimolar range are consistent with what has been described for other vesicular transporters (Table 2). In fact, with the exception of VMAT2, all the vesicular transporters, including VAChT, VGLUTs, and VIAAT, have K_m values in the low millimolar range. This is in contrast to K_m values of plasma membrane transporter, which are usually in the low micromolar range. This supports the idea that plasma membrane transporters are high-affinity carriers that primarily accumulate substrates in the terminal at concentrations high enough to allow the vesicular transporter with lower affinity to sequester these substrates.

Rxt1/NTT4 Uptake Mechanism. Because Rxt1/NTT4 shares approximately 30 to 45% homology with the sodium chloride-dependent plasma membrane transporters, we wanted to explore the ion dependence of this transporter's novel function. Thus, we measured Pro uptake in PNS from wild-type PC12 cells and CHO cells transfected with Rxt1/NTT4 because our previous data revealed that Rxt1/NTT4 expression correlated the highest level with Pro uptake. The use of both wild-type PC12 and transfected CHO cells enabled this study to be conducted using native and recombi-

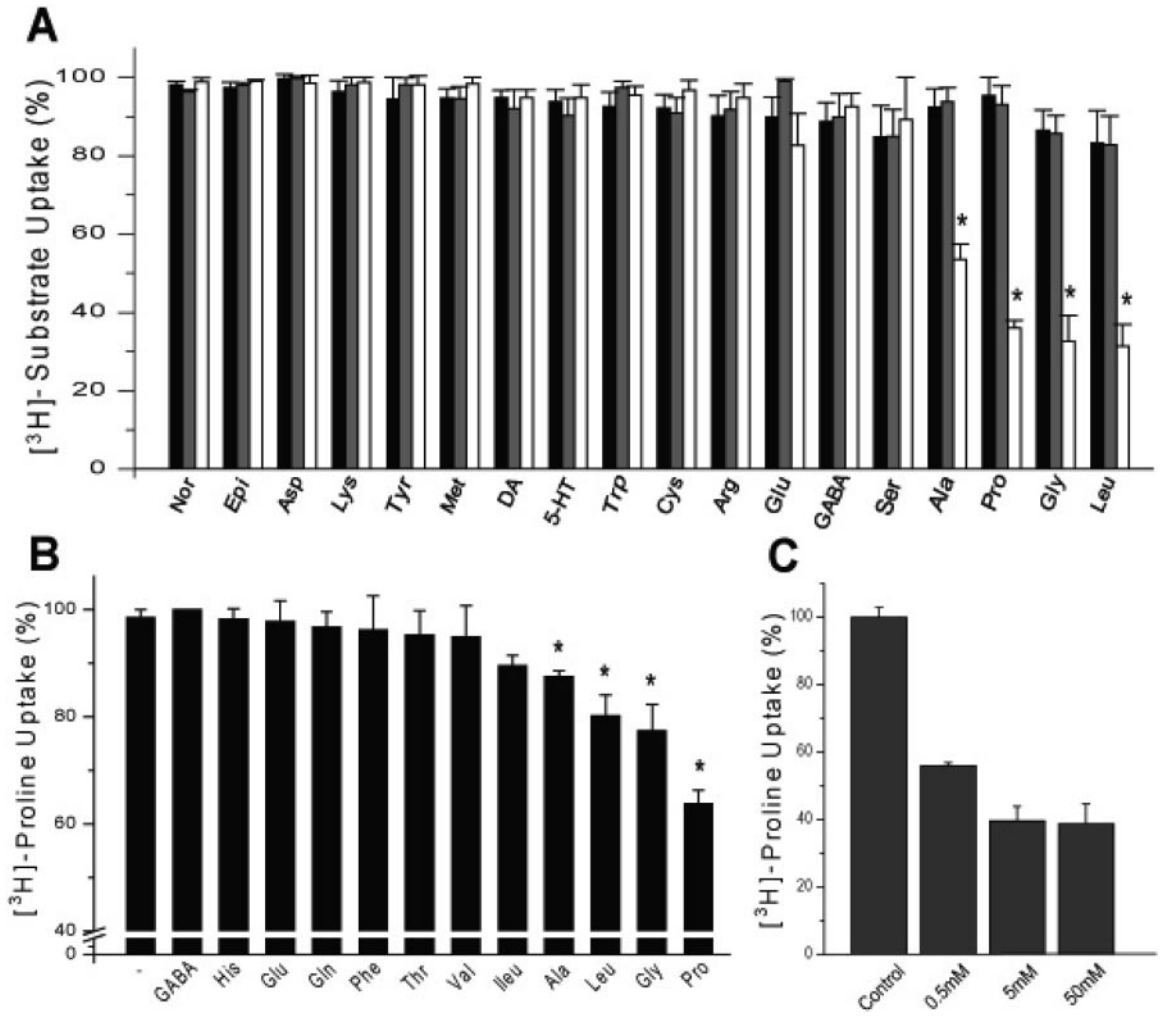

Fig. 5. Rxt1/NTT4 can transport Pro, Gly, Leu, and Ala. A, amino acid uptake (50 nM) in 200 μg of a PNS preparation from wild-type (black), scramble-siRNA-transfected (gray), and Rxt1/NTT4-siRNA-transfected PC12 cells (white). Uptake was determined for a period of 6 min. Nor, norepinephrine; Epi, epinephrine; DA, dopamine; 5-HT, 5-hydroxytryptamine (serotonin). B, [^{3}H]Pro uptake (50 nM) in PNS from wild-type PC12 cells was performed in the presence or absence of 50 mM unlabeled amino acids ($n \geq 9$; *, $p < 0.05$). C, [^{3}H]Pro uptake (50 nM) in the presence of unlabeled Pro at 0.5, 5, or 50 mM.

nant Rxt1/NTT4, respectively. Specifically, NaCl in the uptake buffer was substituted for either NaGluc or LiCl. Our results showed that Pro uptake was unaffected by the use of NaGluc in both PC12- and CHO-transfected cells, indicating that Rxt1/NTT4 function is not chloride-dependent (Fig. 9A). Likewise, both cell lines exhibited approximately 70% of the control uptake when buffer containing LiCl was used, suggesting a partial dependence on sodium (Fig. 9A). Thus, it seems that the function of Rxt1/NTT4 is not consistent with the signature plasma membrane carrier characteristic of sodium and chloride-dependent transport (for review, see Nelson, 1998). Therefore, we next explored whether the function of Rxt1/NTT4 is affected by the vacuolar-type H^+-ATPase (V-ATPase) that is typically coupled to vesicular transporters, including VMAT2, VGLUT, VIAAT, and VAChT (Naito and Ueda, 1985; Moriyama and Futai, 1990; Hell et al., 1991; Eiden et al., 2004). In these experiments, uptake buffer with and without ATP or the specific inhibitor of V-ATPase bafilomycin A1 was used to measure Pro uptake in both wild-type PC12 and Rxt1/NTT4-transfected CHO cells. In the presence of bafilomycin A1 or in the absence of ATP, Pro uptake was almost abolished in both wild-type PC12 cells and Rxt1/NTT4-transfected CHO cells (Fig. 9B). The electrochemical gradient of protons maintained by V-ATPase consists of two components, a proton gradient and the membrane potential (Moriyama et al., 1992). To further dissect the contribution of

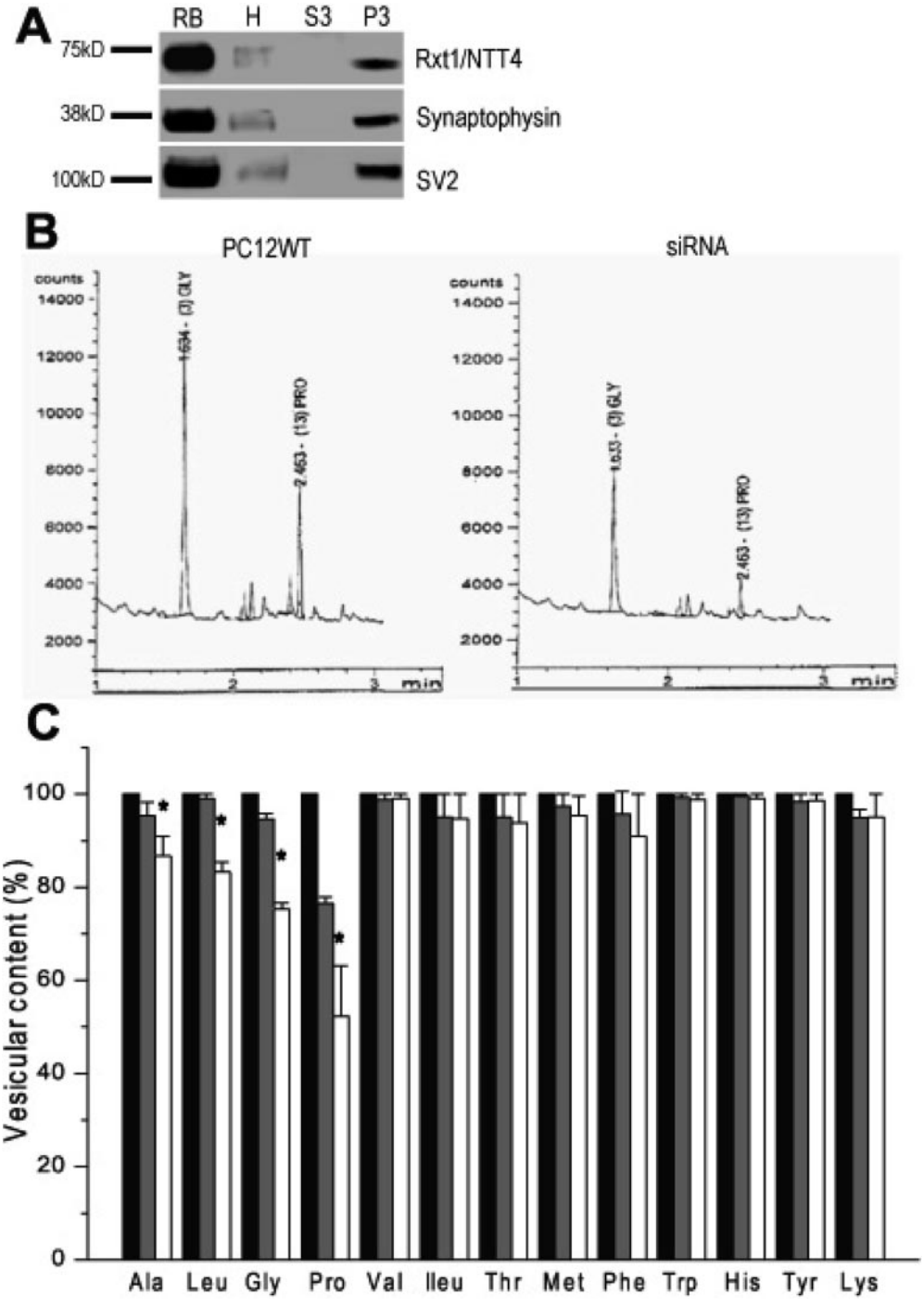

Fig. 6. Rxt1/NTT4 mediates vesicular content of Pro, Gly, Leu, and Ala in PC12 cells. A, Rxt1/NTT4 and the synaptic vesicle marker proteins synaptophysin and SV2 are enriched in the P3 fraction compared with the original homogenate (H). B, representative gas chromatograph analyses of P3 lysates from wild-type PC12 cells (left) and Rxt1/NTT4-siRNA-transfected PC12 samples (right). C, amino acid vesicular content levels were normalized to protein levels. Changes are represented as a percentage of amino acid vesicular content for scramble-siRNA transfected PC12 cells (gray) and Rxt1/NTT4-siRNA-transfected PC12 cells (white) compared with wild-type PC12 cells (black) ($n = 3$; $*$, $p < 0.05$). Ileu, isoleucine.

these two components in Pro uptake, we used the ionophores nigericin and valinomycin. Pro uptake was reduced by approximately 75% in the presence of nigericin, which selectively dissipates ΔpH. In contrast, valinomycin, an ionophore more selective to dissipate membrane potential, reduced Pro uptake by only 50% (Fig. 9B). In contrast, ouabain, an agent known to inhibit plasma membrane Na^+,K^+-ATPase, did not affect Pro uptake. Collectively, these results suggest that Rxt1/NTT4-mediated uptake of Pro is coupled to an ATP-dependent electrochemical gradient that is influenced mostly by the proton gradient and is less affected by the membrane potential, further demonstrating that Rxt1/NTT4 acts as a vesicular transporter.

Discussion

In this report, we have used multiple approaches to identify the orphan transporter Rxt1/NTT4 as a vesicular transporter selective for Pro, Gly, Leu, and Ala. Our results demonstrate that PC12 cells endogenously express Rxt1/NTT4 and, thus represent a suitable cell model to identify the substrate of this transporter. Three lines of evidence are consistent with the contention that Rxt1/NTT4 exists and functions as a transporter for Pro, Gly, Leu, and Ala. First, uptake for Pro, Gly, Leu, and Ala was demonstrated in a PNS preparation from PC12 cells and was decreased in Rxt1/NTT4 knockdown cells. Second, an enriched fraction of vesicles from PC12 cells express Rxt1/NTT4 and contain Pro, Gly, Leu, and Ala as measured by gas chromatography. Furthermore, the vesicular content of these amino acids was selectively and significantly decreased in cells where Rxt1/NTT4 expression was down-regulated by siRNA. Finally, overexpression of Rxt1/NTT4 in CHO cells allowed Pro and Gly transport and increased Leu and Ala uptake in a PNS preparation. Thus, these findings demonstrate that the pres-

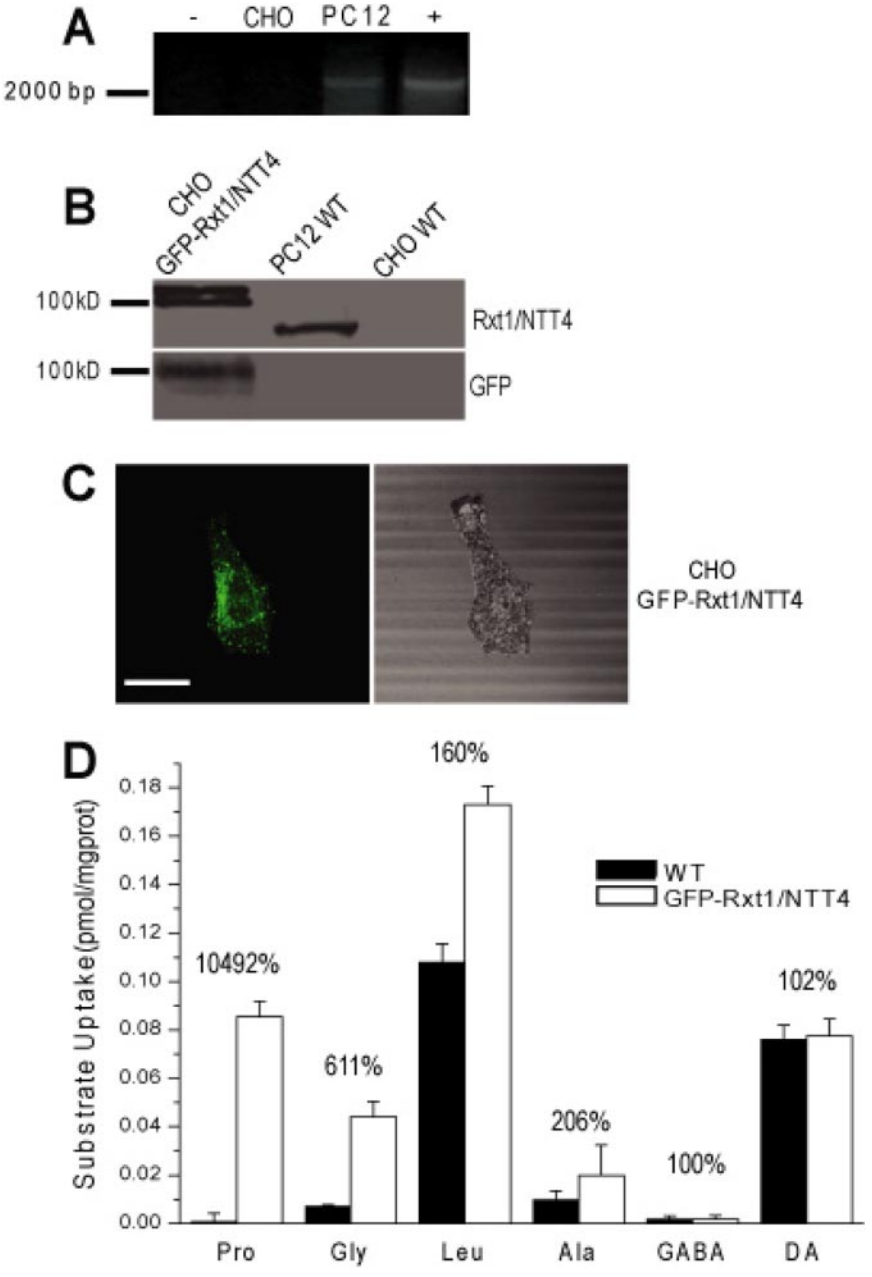

Fig. 7. Rxt1/NTT4 confers Pro, Gly, Leu, and Ala uptake in CHO cells. A, RT-PCR analysis of Rxt1/NTT4 mRNA from CHO cells reverse transcribed into cDNA. Rxt1/NTT4 cDNA from rat brain (+) and PC12 cells were used as a positive controls, whereas the negative control contained no cDNA (−). B, Western blot analysis in GFP-Rxt1/NTT4-transfected CHO cells, PC12 WT cells, and CHO WT cells using both the Rxt1/NTT4 (top) and GFP (bottom) antibodies. C, confocal microscopy images showing CHO cells transfected with a GFP-tagged Rxt1/NTT4 cDNA (scale bar, 15 μm). D, amino acid uptake using 200 μg of PNS from mock (black) and GFP-Rxt1/NTT4-transfected (white) CHO cells. Uptake levels were significantly increased for Pro (10,492%), Gly (611%), Leu (160%), and Ala (206%) in cells transfected with GFP-Rxt1/NTT4 compared with mock cells. No significant differences were observed for GABA (100%) and DA (102%) uptake between the mock-transfected and GFP-Rxt1/NTT4-transfected CHO cells.

ence of Rxt1/NTT4 is sufficient to confer transporter function for these amino acids.

The relatively moderate degree of sequence homology between Rxt1/NTT4 and members of the sodium- and chloride-dependent plasma membrane transporters led to the assumption that Rxt1/NTT4 was also a plasma membrane carrier (Liu et al., 1993; el Mestikawy et al., 1994). However, electron microscopy studies revealed an unexpected vesicular localization for Rxt1/NTT4 in both brain slices and neurons in culture (Fischer et al., 1999; Masson et al., 1999). Based on these findings, two potential scenarios for the role of Rxt1/NTT4 were proposed: either Rxt1/NTT4 functions as a bona fide vesicular transporter; or; alternatively, Rxt1/NTT4 proteins reside as a reserve pool in intracellular vesicles from which they need to be translocated to the plasma membrane to become functional. Our results strongly support the contention that Rxt1/NTT4 resides and functions as a vesicular transporter selective for Pro, Gly, Leu, and Ala. Our data showed that the transporter activity was not dependent on sodium or chloride, a signature characteristic of plasma membrane carriers from this family (for review, see Nelson, 1998). In addition, as seen with other vesicular transporter systems, the Rxt1/NTT4-mediated uptake was ATP-dependent and sensitive to agents that disrupt the vesicular proton gradient (bafilomycin A1 and nigericin) and the membrane potential (valinomycin). A similar phenomenon was reported for the vesicular glutamate transporter (Bellocchio et al., 2000), which was originally described as a brain-specific plasma membrane Na^+-dependent inorganic phosphate transporter (Ni et al., 1994).

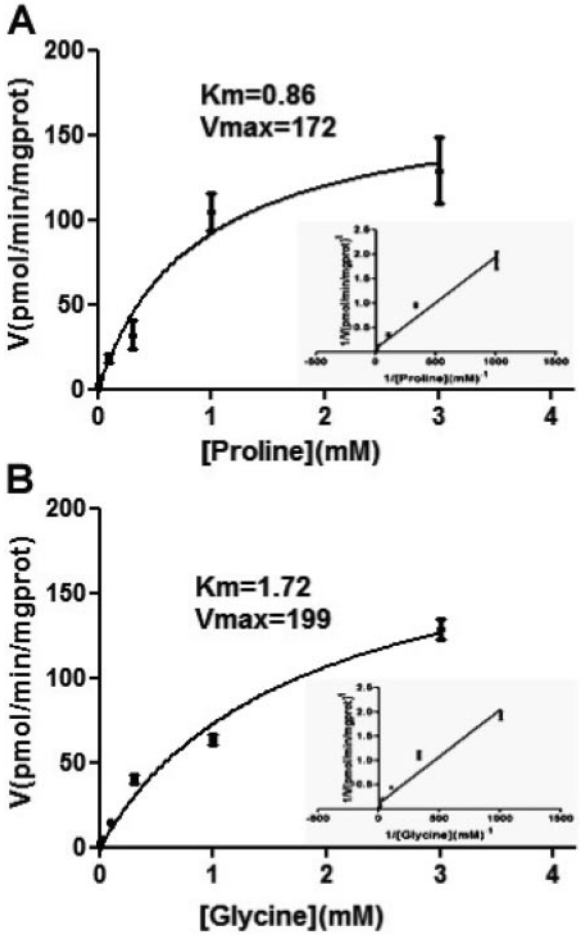

Fig. 8. Kinetic analysis of proline and glycine uptake through Rxt1/NTT4 in CHO cells. A, PNS from CHO cells transfected with Rxt1/NTT4 were resuspended in buffer containing 50 nM [^{3}H]Pro and various concentrations of unlabeled Pro. Uptake was performed for 6 min at 37°C. Lineweaver-Burk analysis (inset) showed a K_m value of 0.86 ± 0.17 mM and a V_{max} value of 172 ± 12.9 pmol/min/mg protein. The values represent the means of triplicate experiments. B, similar experiments were conducted using 50 nM [^{3}H]Gly and increasing concentrations of unlabeled Gly. A K_m value of 1.72 ± 0.3 mM and a V_{max} value of 199 ± 16.6 pmol/min/mg protein were obtained.

Our findings demonstrating that Pro, Gly, Leu, and Ala are substrates of Rxt1/NTT4 and stored in synaptic vesicles leads to questions regarding the physiological role of a vesicular transporter selective for these amino acids. Indeed, several lines of evidence already suggest that Pro might function as a neurotransmitter. Pro is biosynthesized in the brain through the action of the enzyme pyrroline 5-carboxylate reductase (Yoneda and Roberts, 1982). Furthermore, Pro produces electrophysiological actions in the spinal cord and brain (Felix and Künzle, 1976). Previous studies have shown that potassium stimulation of neurons in brain and spinal cord slices leads to release of radiolabeled Pro (Mulder and Snyder, 1974). In addition, a plasma membrane Pro transporter has been identified and is widely distributed in rat brain (Fremeau et al., 1992). Finally, immunohistochemistry analysis with a selective Pro antibody demonstrated that Pro is localized within terminals in several groups of neurons (Takemoto and Semba, 2004). Thus, our data showing that Rxt1/NTT4 mediates vesicular uptake of Pro will stimulate further studies exploring the possibility that Pro has a role in synaptic transmission.

Regarding Gly, its actions as an inhibitory neurotransmitter as well as a necessary coagonist for activation of the ionotropic glutamate *N*-methyl-D-aspartate (NMDA) receptor are well established (Johnson and Ascher, 1987; Kemp and Leeson, 1993). Heteromeric NMDA receptors are ubiquitously distributed throughout the brain, participating in a variety of functions, including neuronal development, synaptic plasticity, learning, and memory (Scheetz and Constantine-Paton, 1994; Kato et al., 1999; Albensi, 2007; Lau and Zukin, 2007). NMDA receptor activation requires not only glutamate but also glycine (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Kemp and Leeson, 1993). Indeed,

TABLE 2
Apparent affinity of vesicular neurotransmitter transporters

Substrate	Transporter	K_m	References
Glutamate	VGLUT1	~1 mM	Bellocchio et al., 2000
	VGLUT2	~1.1–4.7 mM	Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001
	VGLUT3	~0.5–1.5 mM	Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002
GABA	VGAT/VIAAT	~1 mM	McIntire et al., 1997
Acetylcholine	VAChT	~1 mM	Varoqui et al., 1996; Kim et al., 1999
Serotonin	VMAT1	1.56 μM	Erickson et al., 1992
Dopamine	VMAT2	0.32 μM	Liu et al., 1992

Rxt1/NTT4 is expressed in glutamatergic neurons in which glycine could be coreleased with glutamate (Johnson and Ascher, 1987; Kemp and Leeson, 1993). Therefore, changes in extracellular Gly concentrations may lead to alterations in NMDA receptor function (Martina et al., 2004). Such changes in receptor function have been implicated in neurodegeneration and neuropsychiatric disorders, including schizophrenia (Krystal et al., 1998; Newcomer et al., 1999). Thus, it is interesting that although glycine transporters (GlyTs) are localized to inhibitory synaptic terminals, GlyT1 is broadly expressed in regions of the brain not known to have glycinergic inhibition (cortex, hippocampus, and thalamus), suggesting that this transporter influences excitatory synaptic signaling by regulating glycine concentrations. In fact, knockout of GlyT1 in mice causes neonatal death (Gomeza et al., 2003a), and mice deficient of GlyT2 died within 2 weeks of birth (Gomeza et al., 2003b). These findings illustrate the crucial role of glycine in brain function. In addition, administration of either the GlyT1 inhibitor *N*[3-(4′-aurophenyl)-3-(4′-phenylphenoxy)propyl] sarcosine or the GlyB site agonist D-serine potentiated NMDA-mediated responses in the prefrontal cortex (Chen et al., 2003). These results suggest that GlyB binding sites on NMDA receptors are likely to be unsaturated. In this context, Gly release from glutamatergic neurons might represent another regulatory component in the activation of NMDA receptors. A novel NMDA receptor subunit, NR3B, has been cloned from rat brain and shown to form a novel excitatory Gly receptor (Ciabarra et al., 1995; Sucher et al., 1995). Analysis of the NR3B subunit tissue distribution revealed that this protein is primarily expressed in motor neurons of the spinal cord and brainstem (Nishi et al., 2001; Matsuda et al., 2002). These new findings provide a novel mechanism to study the actions of Gly. In this context, it will be interesting to examine whether Rxt1/NTT4-mediated Gly release plays a role in the activation of this novel excitatory Gly receptor. Finally, we are not aware of any studies suggesting that Leu or Ala functions as a neurotransmitter. Further studies will be required to distinguish whether Leu and Ala exist as true neurotransmitters or alternatively uptake of these amino acids might represent a pharmacological property of this transporter.

Two groups have identified the substrate for another orphan transporter, v7-3/NTT7, as a plasma membrane neutral amino acid transporter for Pro, Leu, Met, and Ala (Bröer et al., 2006). Furthermore, in situ hybridization studies have revealed that v7-3/NTT4 and Rxt1/NTT4 have extensive overlapping expression patterns, especially in the olfactory bulb, cerebral cortex, hippocampus, and cerebellum (Inoue et al., 1996; Luque et al., 1996; Masson et al., 1996). Therefore, it is tempting to speculate that v7-3/NTT7 and Rxt1/NTT4 might function in concert to transport neutral amino acids through the plasma membrane and load them into synaptic

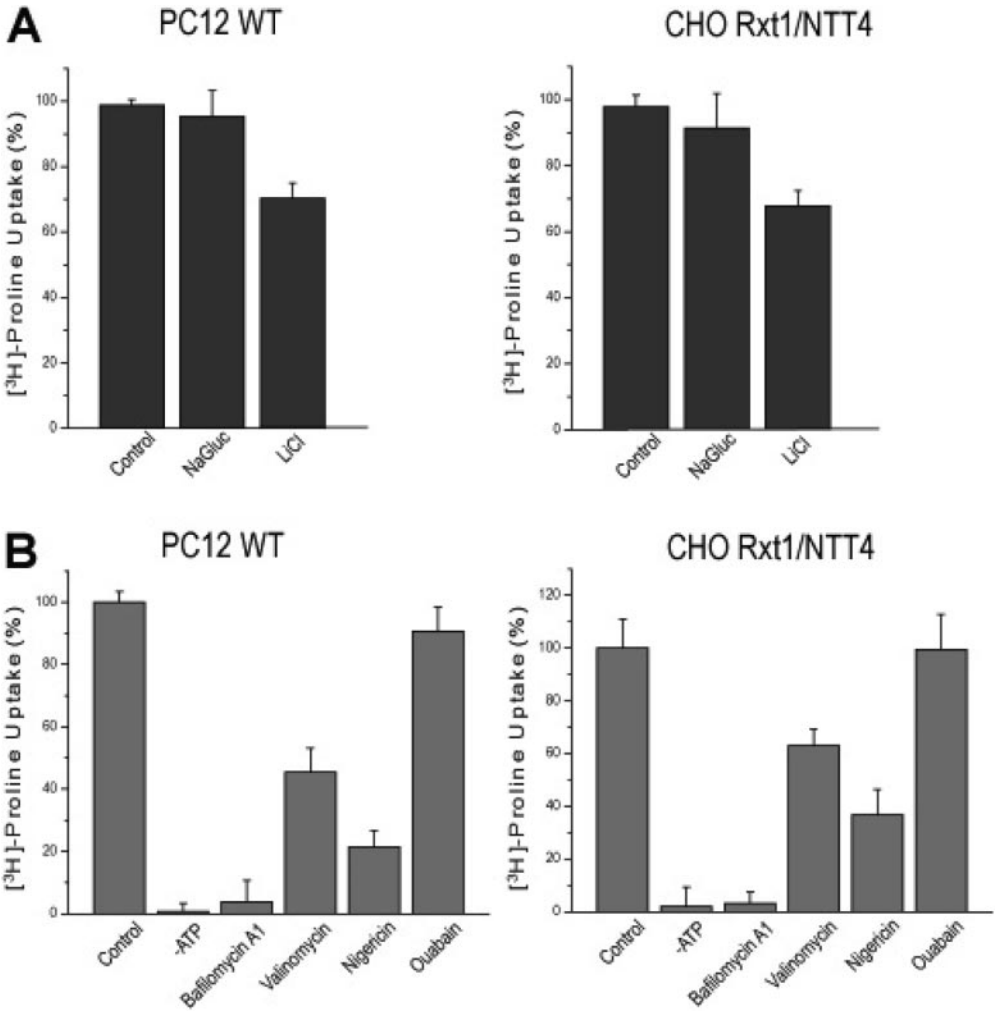

Fig. 9. Proline uptake in both PC12 cells and Rxt1/NTT4-transfected CHO cells was dependent on the electrochemical gradient maintained by the vacuolar-type H^+-ATPase. A, Pro uptake in 200 μg of PNS from wild-type PC12 cells (left) and Rxt1/NTT4-transfected CHO cells (right) were measured using control uptake buffer, buffer in which LiCl was substituted for NaCl, or buffer in which NaGluc was substituted for NaCl. B, Pro uptake in 200 μg of PNS from wild-type PC12 cells (left) and Rxt1/NTT4-transfected CHO cells (right) in which ATP was omitted (−ATP) or in the presence of 10 μM bafilomycin A1, 20 μM valinomycin, 5 μM nigericin, or 1 mM ouabain.

vesicles. Further studies will be necessary to test this hypothesis and uncover the physiological role of these novel transporter systems.

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