

and tensin homolog induced kinase-1 (PINK1) (Valente et al., 2004), and leucine-rich repeat kinase-2 (LRRK2) (Paisan-Ruiz et al., 2004; Zimprich et al., 2004).  $\alpha$ -Synuclein, UCH-L1 and LRRK2 are linked to the autosomal dominant form of PD, whereas parkin, DJ-1 and PINK1 are linked to the recessive form.

In 1998, UCH-L1 carrying an Ile to Met mutation at amino acid position 93 (I93M) was identified in one German family affected by autosomal dominant familial PD. UCH-L1, also known as PGP9.5, is an abundant protein in neuronal cells, comprising up to about 1–2% of total protein in the brain. Its function as de-ubiquitylating enzyme (Larsen et al., 1998; Wilkinson et al., 1989), ubiquitylating enzyme (Liu et al., 2002), de-neddylating enzyme (Hemelaar et al., 2004), and mono-ubiquitin stabilizer (Osaka et al., 2003) has been reported. *In vitro* analysis using recombinant human UCH-L1 indicated that I93M mutation results in the reduction of hydrolase activity of about 50% (Nishikawa et al., 2003). *Uchl1* gene deletion in mice, however, was reported to cause gracile axonal dystrophy (*gad*), a recessive neurodegenerative disease with distinct phenotype and pathological features from PD (Saigoh et al., 1999). Moreover, extensive analysis failed to find other PD patients with mutations in the *UCHL1* gene (Lincoln et al., 1999; Maraganore et al., 1999) and there was an incomplete penetrance in reported German family (Leroy et al., 1998). Thus, the correlation of I93M mutation and pathogenesis of PD was questioned.

To elucidate the pathological role of UCH-L1<sup>I93M</sup> expression in the pathogenesis of PD, *in vivo*, we generated transgenic mice expressing human UCH-L1<sup>I93M</sup>.

## 2. Experimental procedures

### 2.1. Generation of hUCHL1<sup>WT</sup> and hUCHL1<sup>I93M</sup> transgenic mice

We generated transgenes by cloning either the wild-type or I93M mutant human UCH-L1 cDNAs under the control of the human platelet-derived growth factor B chain (*PDGF-B*) promoter (Fig. 1A) (Sasahara et al., 1991). Sequences encoding *UCHL1* were amplified from a human brain cDNA library (Stratagene, La Jolla, CA) by PCR and subcloned into the *XhoI* and *NotI* sites of pCI-neo (Promega, Madison, WI). The I93M substitution was obtained using QuikChange (Stratagene). The 5' flanking region of the human *PDGF-B* chain gene was isolated from the human genomic DNA and inserted into the *BglIII* and *XhoI* site of pCI-neo which results in the replacement of promoter from CMV to *PDGF-B*. The plasmid was linearized by digestion with *HindIII* and *AatII*. A 2  $\mu$ g/ml solution of the linearized plasmid of each transgene was then micro-injected into the pronuclei of newly fertilized C57BL/6J mouse eggs. Offspring were screened for the presence of the transgene by PCR of tail DNA using specific primers (forward: PD-UCH-2, 5'-GCACTCTCCCTTCTCCTTTATA-3'; reverse: PD-UCH-5, 5'-CCTGTATGGCCTCATTCTTTTC-3'). Expression of hUCH-L1<sup>I93M</sup> in a low-expressing mouse line only occurred in male mice. Thus, all experiments were done using male heterozygous transgenic mice. Animal care and handling were in accordance with institutional regulations for animal care and were approved by the Animal Investigation Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan which conforms the National Institute of Health guide for the care and use of Laboratory animals.

### 2.2. Quantitative RT-PCR analysis

Primers specific for mouse *Uchl1* (forward: mL1-7, 5'-CCTTGGTTTGCA-GCTTAGCA-3'; reverse: mL1-8, 5'-GGGCTGTAGAACGCAAGAAGA-3')

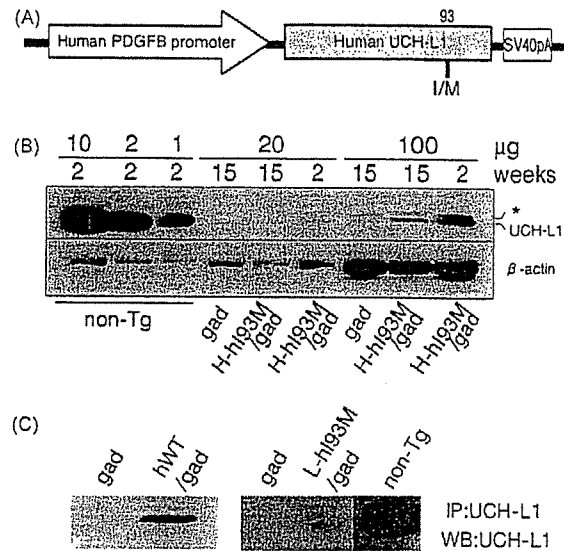


Fig. 1. Generation of transgenic mice expressing hUCH-L1<sup>WT</sup> and hUCH-L1<sup>I93M</sup>. (A) UCH-L1<sup>I93M</sup> was constructed under control of the *PDGF-B* promoter, as depicted. (B) Immunoblotting analysis of endogenous mouse UCH-L1 and transgenic human UCH-L1 expression in mouse midbrain. To detect exogenous human UCH-L1 levels specifically, we generated transgenic mice in the *gad* background (H-hI93M/*gad*), which corresponds to the null mutant of *Uchl1*. Notice that the faint band corresponding to UCH-L1 is detected at 2 weeks of age when 20  $\mu$ g protein/lane was loaded for the detergent-soluble fraction of midbrain origin in H-hI93M/*gad* mice. When the applied protein was increased to 100  $\mu$ g/lane, UCH-L1 was easily detected at 2 weeks in H-hI93M/*gad* mice, and UCH-L1 levels markedly decreased by age 15 weeks. Faint bands indicated by the asterisk may correspond to UCH-L3, which cross-reacted with the UCH-L1 antibody when a large amount of protein was loaded per lane. (C) Immunoprecipitation analysis of exogenous human UCH-L1 in hWT/*gad* (left) and L-hI93M/*gad* (right) brains. Brain lysates from hWT/*gad* (left) or L-hI93M/*gad* (right) were both immunoprecipitated and detected using anti-UCH-L1 antibody. The band corresponding to the UCH-L1 can be found in both hWT/*gad* and L-hI93M/*gad* lysates but not in *gad* lysates indicating the exogenous human UCH-L1 expression.

and human *UCHL1* (forward: L1Tg-F2, 5'-TGGCAACTTCTCCTCCTGCA-3'; reverse: L1Tg-R2, 5'-ACAGCACTTTGTTTCAGCATC-3') were designed, and SYBR Green-based real-time quantitative RT-PCR was performed using the ABI PRISM 7700 (Applied Biosystems, Foster City, CA) using total RNA from mouse brain ( $n = 3$  for each line) (Aoki et al., 2002). GAPDH was used as an internal control.

### 2.3. Fractionation and immunoblotting and immunoprecipitation

For the immunoblotting of total UCH-L1, the soluble fraction in RIPA (20 mM Tris-HCl, pH 7.5; 0.1% SDS; 1.0% (w/v) Triton X-100; 1.0% sodium deoxycholate) with Complete EDTA-Free Protease Inhibitors (Roche, Basel, Switzerland) was extracted from H-hI93M/*gad* ([high-expressing] UCH-L1<sup>I93M</sup>), *Uchl1*<sup>gad/gad</sup>, *gad* and non-Tg mouse midbrains. The extracted samples were loaded as indicated in Fig. 1.

For subfractionation, the cortex and hippocampus were removed from the midbrains of a H-hI93M mouse or a non-Tg littermate and bottom half under the aqueduct were used as the substantia nigra fraction. The fractionation method was modified from that of Kahle et al. (2001). Each sample was homogenized with 9 volumes of 5% SDS/TBS lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% SDS) with Complete EDTA-Free Protease Inhibitors using a 23G syringe. After three times of 10 s sonication, samples were ultra-centrifuged in 130,000  $\times$  g for 1 h, and the supernatant were pooled as 5% SDS fraction. The pellets were washed with 5% SDS/TBS solution once and further homogenized in 8 M urea/5% SDS/TBS lysis buffer

(8 M urea, 5% SDS, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl) with 23 G syringe. The resulting supernatant was used as 8 M urea/5% SDS fraction. The protein concentration was assessed by a DC-protein assay kit (Bio-Rad). 1.25 µg of 5% SDS fraction and 0.5 µg of 8 M urea/5% SDS fraction were subjected to SDS-PAGE using 15% gels (Perfect NT Gel; DRC, Tokyo, Japan). Anti-UCH-L1 (1:5000, RA95101; Ultracelone, Isle of Wight, UK) and anti-β-actin (1:5000, clone AC15; Sigma, St. Louis, MO) were used to detect each protein. Signals were detected using a chemiluminescent SuperSignal West Dura Extended Duration Substrate kit or West Femto Maximum Sensitivity Substrate kit (Pierce, Rochford, IL) and analyzed with a ChemImager (Alpha Innotech, San Leandro, CA). For the internal control of 8 M urea/5% SDS fraction, 1 µg protein were dot blotted to PVDF membrane and stained with Ponceau S staining (Rane et al., 2004). Statistical analyses were conducted using the two-tailed Student's *t*-test with total of four samples for each group.

For the immunoprecipitation, half of the brain (for hWT/gad) or mid-brain region (for L-hI93M/gad) were homogenized in 2 ml ice-cold modified RIPA buffer (50 mM Tris–HCl, pH 7.4; 1% (w/v) Nonidet P40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA) with Complete EDTA-Free Protease Inhibitors and centrifuged at 16,000 × *g* at 4 °C for 20 min. The protein concentration of the resulting supernatants was determined with the Protein Assay Kit (Bio-Rad, Hercules, CA). Immunoprecipitation was performed with a Seize X Mammalian Immunoprecipitation kit (Pierce, Rockford, IL) with some modifications. Briefly, 300 µg of protein was added to a 50 µl slurry of immobilized protein G cross-linked with rabbit polyclonal anti-human UCH-L1 (AB1716; Chemicon, Temecula, CA) or normal rabbit IgG and rotated at 4 °C overnight. The samples were then washed three times with 500 µl of 0.1B buffer (20 mM Tris–HCl, pH 8.0; 0.1 M KCl; 5 mM MgCl<sub>2</sub>; 10% (w/v) glycerol; 0.1% (w/v) Tween 20; 10 mM β-mercaptoethanol). Elution of samples was performed by adding 20 µl of 5 × SDS-PAGE sample buffer, and samples were boiled at 100 °C for 5 min.

#### 2.4. Immunohistochemistry, immunofluorescence and electron microscopy

Brain and peripheral (sciatic) nerve sections from 2-, 7- and 20-week-old mice were analyzed (*n* = 3 for each line) by immunocytochemistry as previously described (Wang et al., 2004; Watanabe et al., 1977) using antibodies to UCH-L1 (1:4000; RA95101, Ultracelone), TH (1:1000; Chemicon) and ubiquitin (1:1000; Sigma–Aldrich, St. Louis, MO). Antibody binding was detected with 3,3'-diaminobenzidine tetrachloride (DAB) or 3-amino-9-ethylcarbazole (AEC) as a peroxidase substrate or Alexa-488- or Alexa-568-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). Sections were then counterstained with hematoxylin. Ultrastructural electron microscopic studies of the substantia nigra were performed as described (Watanabe et al., 1977) using midbrain sections.

#### 2.5. MPTP treatment

For MPTP treatment, the mice received four injections of 30 mg/kg MPTP–HCl intraperitoneally (Research Biochemicals, Natick, MA) in saline at 24-h intervals (Mochizuki et al., 2001).

#### 2.6. Tyrosine hydroxylase-positive cell counting and biochemical analysis

Samples for both histochemistry and biochemical analysis were obtained from the same mouse. Each animal was deeply anesthetized with pentobarbital and perfused transcardially with 10 ml of ice-cold phosphate-buffered saline, and the brain was removed and divided into forebrain and midbrain–hindbrain regions.

For the tyrosine hydroxylase (TH)-positive cell counting, midbrain–hindbrain was fixed with chilled 4% formaldehyde solution (pH 7.4). The procedure of TH-positive cell counting was described previously (Furuya et al., 2004) with minor modifications. Briefly, the substantia nigra was cut into serial sections (30 µm), and every third section was subjected to

immunostaining for TH using a polyclonal antibody to TH (a kind gift from I. Nagatsu, Fujita Health University, Aichi, Japan). The Vectorstain Elite ABC kit (Vector Labs, Burlingame, CA) was used for subsequent antibody detection with DAB as a peroxidase substrate. The number of viable TH-positive neurons was assessed by manual counting by a blind observer using coded slides (Furuya et al., 2004). The number of total neuronal cells outside the substantia nigra was counted after Bodian staining in the cerebral cortex (1 mm<sup>2</sup>, seven regions per section), cerebellum (total of all lobules) and hippocampus (total number in CA1, CA2, CA3 and dentate gyrus). Statistical analysis were done by one-way ANOVA followed by post hoc test (Fisher's PLSD).

For the biochemical analysis, the striatum was quickly dissected from the forebrain, and the striatal tissue samples were weighed (~30 mg) and homogenized in 10 volumes (w/v) of ice-cold 0.05 M sodium acetate (pH 6.0). Homogenates were centrifuged (18,000 × *g*, 10 min at 4 °C), and the supernatant was frozen immediately on dry ice and stored frozen at –80 °C until use.

For the striatal dopamine measurement, supernatant (50 µl) from the striatal lysate was mixed with an equal volume of 0.2 M perchloric acid containing 0.2 mM EDTA and centrifuged (18,000 × *g*, 10 min at 4 °C), and the supernatant was applied to an HPLC system. Chromatographic separation was achieved using a C18 reversed-phase column (150 mm × 4.6 mm i.d., Model S-100; TOSOH, Tokyo, Japan). The mobile phase (50 mM citrate, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1 mM EDTA, 4.36 mM 1-heptanesulfonate, 2.35% acetonitrile, 5.72% MeOH, pH 2.5) was pumped through the chromatographic system at a rate of 1.0 ml/min. A Coulchem electrode array system (ESA Inc., MA) with eight coulometric electrodes was used to quantify the eluted catecholamines and their metabolites. Statistical analysis was done by one-way ANOVA followed by post hoc test (Fisher's PLSD).

TH activity was assayed following the method of Hooper (1997) with minor modifications (Hooper et al., 1997; Naoi et al., 1988). The incubation mixture contained 50 µl of diluted sample and included the following components in a total volume of 200 µl: 0.2 M sodium acetate (pH 6.0), 0.2 M glycerol, 20,000 U/ml catalase, 1.0 mM 6-MPH4, 4.0 U/ml dihydropteridine reductase, 1 mM NADPH and 200 µM L-tyrosine. Incubations were carried out at 37 °C for 10 min in a shaking water bath. Reactions were terminated by adding 600 µl of ice-cold 0.33 M perchloric acid, 17 mM EDTA including 50 pmol of α-methyl DOPA as the internal standard. The L-DOPA produced was extracted onto alumina, and the catechols were eluted with 0.16 M acetic acid followed by 0.02 M phosphoric acid. A sample incubated on ice instead of 37 °C was used as a blank. The amount of L-DOPA was quantified with the HPLC system, as mentioned above. Statistical analysis was done by one-way ANOVA.

#### 2.7. Silver staining

Sixty-micrometer brain sections from 12-week-old mice (*n* = 3 for each group) were stained using FD NeuroSilver kit (FD Neuro-Technologies, Catonsville, MD) according to the manufacturer's protocol to detect argyrophilic grain-positive degenerating neurons.

#### 2.8. Behavioral tests

H-hI93M mice and non-Tg littermates were used for all behavioral analyses. For the accelerated rota-rod test, 20–25-week-old mice were placed on the rod (Ohara, Japan) at a speed of 5 rpm, and the speed was accelerated to 50 rpm in 5 min. The length of time that each mouse was able to remain on the rod before falling was recorded. For the locomotor activity test, 11–13-week-old or 20–23-week-old mice were placed separately in a home cage 4 days before the beginning of analysis for habituation. Two to four mice were monitored at once for locomotor activity on the home cage monitor (Ohara, Japan) for 63 h beginning from 5:30 p.m. All mice were housed with a 12 h light/dark cycle, with the light cycle beginning at 8 a.m. The last 12 h of active night were used for the analysis. Mice were weighed after the analysis; there were no differences between the weights H-hI93M and non-Tg mice (data not shown). Statistical analyses were conducted using the two-tailed Student's *t*-test.

### 3. Results

#### 3.1. Generation of transgenic mice expressing human *UCHL1*<sup>193M</sup> in neurons of the substantia nigra

The human *PDGF-B* promoter was used to drive expression of the human *UCHL1* in Tg mice (Fig. 1A) (Sasahara et al., 1991). Germline transmission of *hUCHL1*<sup>193M</sup> was obtained in two independent Tg mouse lines (denoted L-hI93M and H-hI93M, where L and H denote low and high expression, respectively). Germline transmission of *hUCHL1*<sup>WT</sup> was obtained in one Tg mouse line (denoted hWT). The levels of transgenic mRNA and endogenous *Uchl1* mRNA were assessed by quantitative RT-PCR using primers designed to amplify specifically the *UCHL1* transgene and mouse *Uchl1*, respec-

tively. The estimated relative expression of *UCHL1* among the transgenic lines was H-hI93M > hWT > L-hI93M. The ratio of endogenous mouse *Uchl1* transcripts to transgenic human *UCHL1* transcripts was 111 in H-hI93M, 739 in hWT and 6015 in L-hI93M ( $n = 3$  for each line).

At the amino acid level, human and mouse UCH-L1 differ at only 11 discrete positions, and endogenous UCH-L1 is one of the most abundant protein in the brain. Therefore, we were not able to make distinction between the exogenous human UCH-L1 and endogenous mouse UCH-L1 in the brains of Tg mice (data not shown) using immunoblotting analysis with several antibodies against human UCH-L1 from different companies (Chemicon; UltraClone; Medac; Biogenesis). To ascertain the expression of transgene product, we used *gad* mice, which lack endogenous UCH-L1 (Saigoh et al., 1999). We mated mice

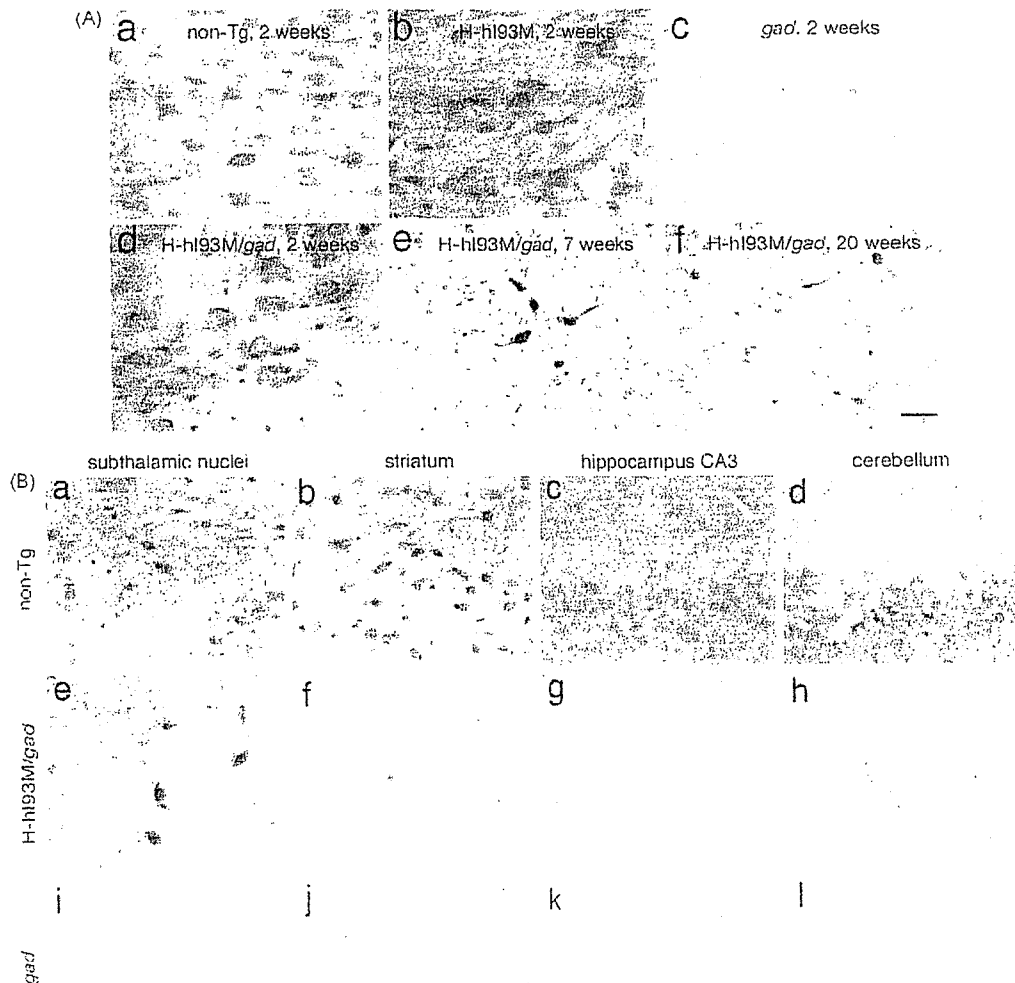


Fig. 2. Immunohistochemistry of UCH-L1 in coronal sections of the substantia nigra (A) and regions outside the substantia nigra (B) in H-hI93M, H-hI93M/*gad* and non-Tg mice. (A) Non-Tg mice (a), H-hI93M mice on a C57BL/6J background (b) and *gad* mice (c) at 2 weeks of age and H-hI93M/*gad* mice at 2 weeks (d), 7 weeks (e) and 20 weeks (f) of age. Neurons expressing UCH-L1 in the substantia nigra decreased in number and area, and densely stained neurons were observed in the aged substantia nigra. Scale bar: 30 μm. (B) UCH-L1 immunohistochemistry of coronal sections at the level of the subthalamic nuclei (a, e, i), striatum (b, f, j), hippocampus CA3 (c, g, k) and cerebellum (d, h, l). Upper row (a–d), non-Tg mice; middle row (e–h), H-hI93M/*gad* mice; lower row (i–l), *gad* mice. All mice were examined at 2 weeks of age. Scale bar: 30 μm.

from each transgenic line with mice homozygous for the *Uchl1*<sup>gad/gad</sup> allele (*gad* mice). Detergent-soluble (1% Triton X-100) fractions of mouse midbrain from H-hI93M/*gad* (*UCHL1*<sup>I93M/-</sup>, *Uchl1*<sup>gad/gad</sup>) at 2 and 15 weeks of age were subjected to SDS-PAGE and immunoblotted with anti-UCH-L1. We detected human UCH-L1 expression in H-hI93M/*gad* brains (Fig. 1B). Compared with endogenous mouse UCH-L1, which constitutes 1–2% of neuronal proteins, human UCH-L1 expression was substantially lower in H-hI93M/*gad* brains (~1% of endogenous UCH-L1 at 2 weeks of age; Fig. 1B). Interestingly, the level of transgenic human UCH-L1 was lower at 15 weeks than at 2 weeks of age (Fig. 1B). Although we could not detect human UCH-L1 in L-hI93M/*gad* and hWT/*gad* by standard immunoblotting methods, we were successful in detecting it by immunoprecipitation (Fig. 1C). These data suggest the expression of the human UCH-L1 in L-hI93M and hWT mice, which were much lower than in H-hI93M mice.

UCH-L1 is a cytosolic protein predominantly expressed in neuronal cells including dopaminergic neurons at substantia nigra with diffuse localization (data not shown). Thus, we next examined the immunohistochemical localization of the transgene products. In agreement with the data obtained by

Western blotting analysis, UCH-L1-immunoreactive cells were not observed in any brain region, including the substantia nigra, of the L-hI93M/*gad* and hWT/*gad* mice (data not shown). In H-hI93M/*gad* mice, however, human UCH-L1<sup>I93M</sup> was detected in the substantia nigra, the region that contains the central pathological lesions in PD, with relatively high intensities (Fig. 2A). Subthalamic nuclei, striatum, hippocampus CA3 and cerebellum also contained UCH-L1 immunoreactive cells in H-hI93M/*gad* mice (Fig. 2B). As with the previous report that CAT expression under control of the *PDGF-B* promoter in transgenic mice localizes to neuronal cell bodies (Sasahara et al., 1991), most UCH-L1-immunoreactive cells in H-hI93M/*gad* mice had a neuronal morphology (Fig. 2). Western blotting analysis of midbrain lysates showed a reduction of transgenic UCH-L1<sup>I93M</sup> at 15 weeks of age as compared with that at 2 weeks in H-hI93M/*gad* mice (Fig. 1B). Thus, we also performed immunohistochemical analysis of UCH-L1 on substantia nigra from 2-, 7- and 20-week-old H-hI93M/*gad* mice. We found many UCH-L1-positive neurons at 2 weeks. The number of positive cells had decreased by 7 weeks, however, at which time small-sized and densely stained neurons were observed, and UCH-L1-positive cells were barely

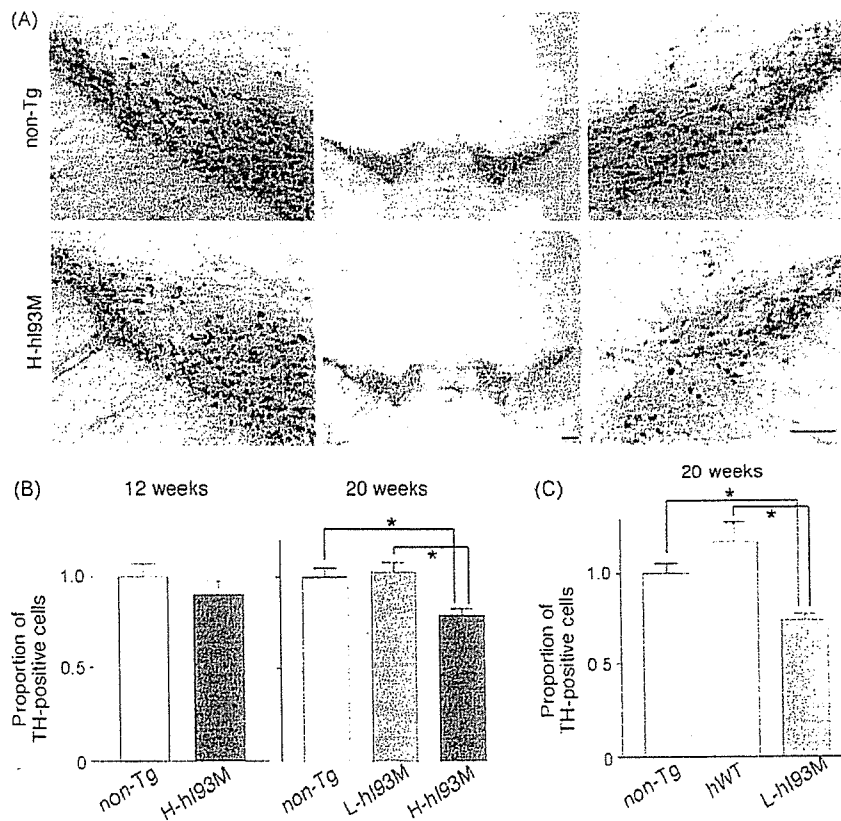


Fig. 3. TH-positive neurons of hI93M Tg mice were reduced as the animals aged. (A) Immunohistochemical staining of the substantia nigra with anti-TH in non-Tg (upper panels) and H-hI93M (lower panels) mice at 20 weeks of age. Scale bar: 1 mm. Left and right panels in the figure correspond to the left and right part of the middle panel, respectively. (B) Proportion of neurons stained with anti-TH in the substantia nigra from non-Tg and hI93M mice at 12 weeks (left panel) and 20 weeks (right panel) of age. Cell numbers were normalized to those for the non-Tg mice. Values are the mean  $\pm$  S.E.M.;  $n = 10$ . Significance was examined by a one-way ANOVA. \* $p < 0.01$ . (C) The number of TH-positive cells in the substantia nigra from 20-week-old non-Tg ( $n = 5$ ), hWT ( $n = 3$ ) and L-hI93M mice ( $n = 5$ ) after treatment with MPTP. The cell numbers were normalized to those for non-Tg mice. Values are the mean  $\pm$  S.E.M. Significance was examined by a one-way ANOVA. \* $p < 0.001$ .

detectable at 20 weeks of age (Fig. 2A). Together, our results indicate that hUCH-L1<sup>I93M</sup> is expressed in the neurons of the substantia nigra in H-hI93M mice, but the number of positive cells declines before 20 weeks of age. With the failure to detect hUCH-L1 protein in hWT/*gad* mice and L-hI93M/*gad* mice both in the Western blotting and the immunohistochemistry, we performed most of the analysis using H-hI93M mice with non-Tg mice as a control.

### 3.2. Loss of dopaminergic neurons in the substantia nigra of 20-week-old H-hI93M mice

We next determined whether the number of midbrain dopaminergic neurons was reduced in the substantia nigra of transgenic mice using TH immunohistochemistry. The number of TH-positive dopaminergic neurons in the substantia nigra at the same neuroanatomical level was compared and quantified for each transgenic mouse line. Surprisingly, we detected an

~30% reduction in TH-positive neurons in 20-week-old H-hI93M mice as compared with those in non-Tg control mice (Fig. 3A and B). This reduction was not seen in 12-week-old H-hI93M mice or 20-week-old L-hI93M mice. Together with the decrease in the level of UCH-L1<sup>I93M</sup> (Fig. 1B) and the reduction in UCH-L1-positive neurons in the substantia nigra of H-hI93M/*gad* mice, our data indicate that UCH-L1<sup>I93M</sup> expression in the dopaminergic neurons is sufficient to induce the degeneration of these neurons.

MPTP is a toxin used to induce an acute Parkinsonian syndrome that is indistinguishable from sporadic PD (Dauer and Przedborski, 2003). MPTP metabolite 1-methyl-4-pyridinium (MPP<sup>+</sup>), an inhibitor of complex I of the mitochondrial respiration chain, is taken up by the terminals of dopaminergic neurons via the dopamine transporter (DAT), thereby causing the selective death of nigral neurons (Dauer and Przedborski, 2003). Although neuronal loss was not observed in L-hI93M mice at 20 weeks of age, we speculated that dopaminergic

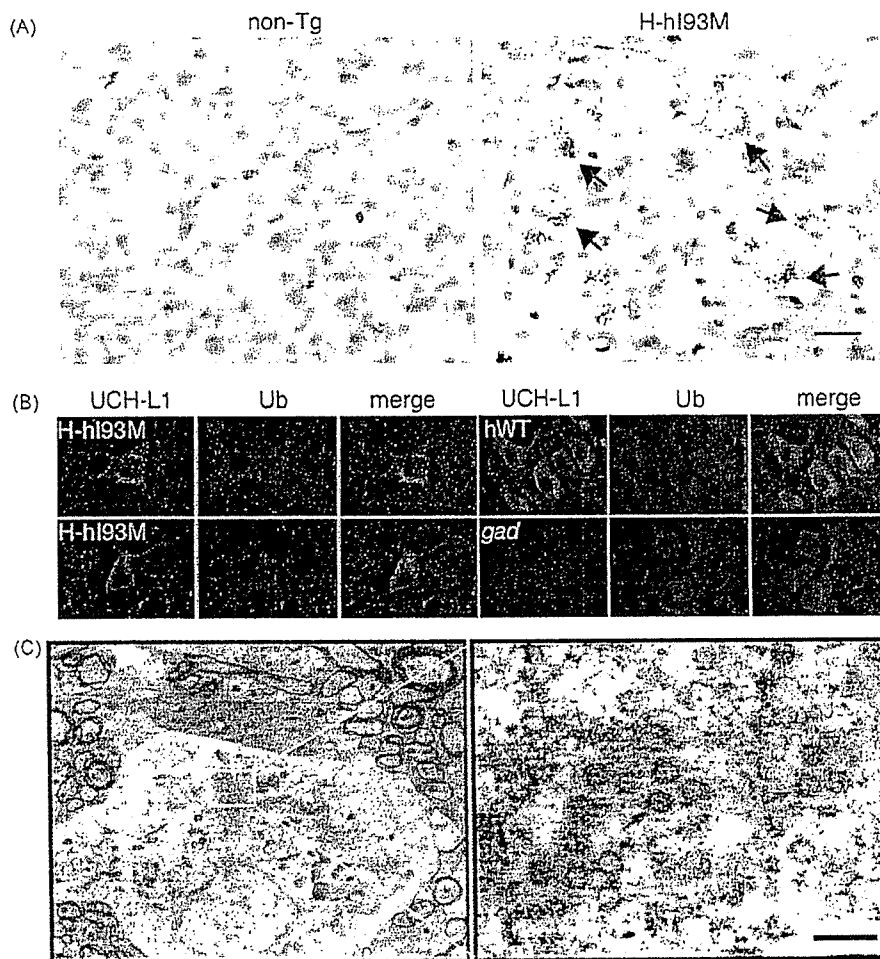


Fig. 4. Several neuropathological features reminiscent of PD are present in H-hI93M mice brains. (A) Silver staining of the substantia nigra at 12 weeks of age in non-Tg and H-hI93M mice. Note the presence of silver staining-positive argyrophilic grains in the cell bodies of some dopaminergic neurons in H-hI93M mice (arrows). This kind of abnormal structure was not seen in substantia nigra of non-Tg mice. Scale bar: 30  $\mu$ m. (B) Confocal images of dopaminergic neurons from hWT, H-hI93M and *gad* mice. H-hI93M mice showed the formation of ubiquitin-positive cytoplasmic inclusions (red) co-localized with UCH-L1 staining (green) in the remaining nigral neurons at 20 weeks of age. Compared with the diffuse, reduced staining of ubiquitin in *gad* mice, nigral neurons from hWT mice also showed a diffuse pattern of staining but with fine small granular cytoplasmic staining (red) co-localized with UCH-L1 (green). (C) Electron micrographs of a nigral neuron from a 20-week-old H-hI93M mouse at the level of the cell body (left panel), and dense-core vesicles (red arrows) at higher magnification (right panel). Scale bar: 1  $\mu$ m.

neurons of L-hI93M mice might be more susceptible to MPTP toxin compared to that of non-Tg mice or hWT mice. As expected, significantly fewer TH-positive neurons were observed in L-hI93M mice after MPTP treatment as compared with hWT or non-Tg control mice though hWT express higher *hUCHL1* compared to L-hI93M (Fig. 3C). The number of TH-positive neurons in MPTP-treated hWT mice was somewhat higher than that in non-Tg mice ( $p < 0.001$ ). Taken together with the fact that expression of human UCH-L1 in L-hI93M is lower than that in hWT, these results suggest that the UCH-L1<sup>I93M</sup> mutant, but not UCH-L1<sup>WT</sup>, is specifically toxic to dopaminergic neurons.

### 3.3. Presence of neuropathology in dopaminergic neurons from H-hI93M mice

To evaluate the degenerative process of dopaminergic neurons, silver staining was used to indicate argyrophilic degenerating neurons (Lo Bianco et al., 2004). In non-Tg mice, no silver staining was observed, whereas scattered neurons containing grains that were silver staining positive were present in the substantia nigra of H-hI93M mice (Fig. 4A). The presence of intracellular inclusions called Lewy bodies and Lewy neurites are neuropathological characteristics of PD and are silver staining positive (Sandmann-Keil et al., 1999; Uchihara et al., 2005). It is also known that UCH-L1 and ubiquitin, as well as  $\alpha$ -synuclein, are components of Lewy bodies (Lowe et al., 1990). Furthermore, UCH-L1 is tightly associated with mono-ubiquitin *in vivo* (Osaka et al., 2003). Thus, we expected that the silver staining-positive grains might have characteristic features of Lewy bodies. We therefore compared the immunohistochemical analysis of UCH-L1 and ubiquitin. Compared with reduced staining for ubiquitin in *gad* mice, strong and diffuse ubiquitin staining was observed in nigral neurons of hWT mice and non-Tg mice (data not shown), and this staining co-localized with UCH-L1, which is in agreement with our previous report (Osaka et al., 2003). In H-hI93M substantia nigra at 20 weeks of age, ubiquitin- and UCH-L1-positive cytoplasmic inclusions, a large aggregates with different morphology from small dots usually seen in hWT mice and non-Tg mice, were observed in a portion of the remaining nigral neurons (Fig. 4B). These inclusions were, however,  $\alpha$ -synuclein or hematoxylin–eosin (HE) negative (data not shown). We could not observe UCH-L1- and ubiquitin-positive inclusions in L-hI93M mice (data not shown).

Another cellular characteristic of PD neuropathology is dense-core vesicles of about 80–200 nm in perikarya, which are frequently observed along with Lewy bodies in PD patients (Watanabe et al., 1977). We observed electron dense-core vesicles in the cytoplasm of ~30% of nigral neurons in H-hI93M mice using electron microscopy (Fig. 4C). In non-Tg mice, such vesicles with a similar shape were not detected in cell bodies but rather were seen in synaptic terminals. Taken together, our data indicate that degenerating dopaminergic neurons in the substantia nigra of H-hI93M mice are devoid of Lewy bodies but show some neuropathological features such as silver staining-positive argyrophilic grains, aggregates with UCH-L1 and ubiquitin, and dense-core vesicles in the perikarya.

### 3.4. Increased amount of SDS-insoluble but urea/SDS-soluble UCH-L1 in the midbrain of H-hI93M mice

UCH-L1<sup>I93M</sup> has reduced  $\alpha$ -helical content as compared with UCH-L1<sup>WT</sup> (Nishikawa et al., 2003), and UCH-L1<sup>I93M</sup> overexpression in COS7 cells results in more cells that contain cytoplasmic inclusions (Ardley et al., 2004). Thus, the presence of UCH-L1-positive inclusions in H-hI93M dopaminergic neurons led us to speculate whether UCH-L1<sup>I93M</sup> would be less soluble than the wild-type protein *in vivo*. To biochemically characterize the changes in UCH-L1 deposited in the brains of H-hI93M mice, we sequentially extracted frozen midbrain tissues with 5% SDS (soluble fraction) and 8 M urea/5% SDS (insoluble fraction) and analyzed each fraction by immunoblotting with anti-UCH-L1. As expected, immunoblots of insoluble fractions showed a modest but statistically significant increase in UCH-L1 in the midbrains of H-hI93M mice as compared with those from a non-Tg mouse (Fig. 5A and B), indicating increased insolubility of UCH-L1<sup>I93M</sup> *in vivo*, which might have resulted in dopaminergic neurotoxicity.

### 3.5. Decreased dopamine content in the striata of H-hI93M mice

Because the nigro-striatal pathway is severely affected in PD patients, and because our mice showed the degeneration of dopaminergic neurons in the substantia nigra, we evaluated the nerve terminals in the striatal pathway using

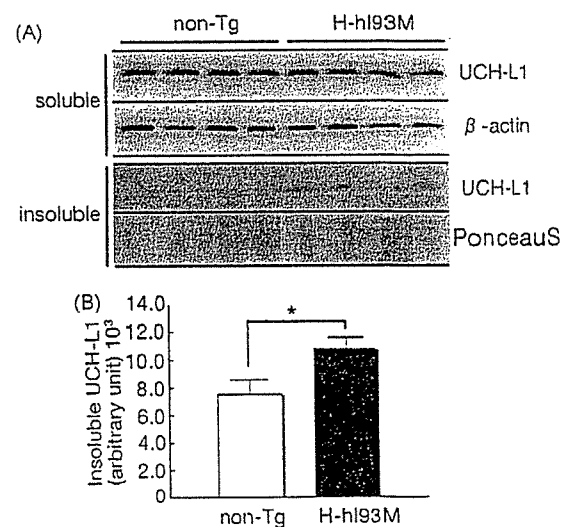


Fig. 5. Protein insolubility of UCH-L1 in H-hI93M Tg mice. (A) Immunoblotting analysis of UCH-L1 in soluble (5% SDS soluble) and insoluble (5% SDS insoluble and 8 M urea/5% SDS soluble) fractions from tissue containing the substantia nigra (11–13 weeks). Soluble fraction (5  $\mu$ g for each) was probed with anti-UCH-L1 or anti- $\beta$ -actin. Insoluble fraction (0.5  $\mu$ g for each) was probed with anti-UCH-L1. One microgram of each insoluble fraction was applied to dot-blotting and stained by Ponceau S to show that each fraction contained the same amount of total protein. A slight increase in the insolubility of UCH-L1 in the substantia nigra fraction from H-hI93M mice is seen as compared with that from non-Tg mice. (B) The experiment was done with H-hI93M mice and non-Tg littermates from five different litters, and the results of quantitative analyses in insoluble fraction is shown ( $n = 5$  mice for each group).

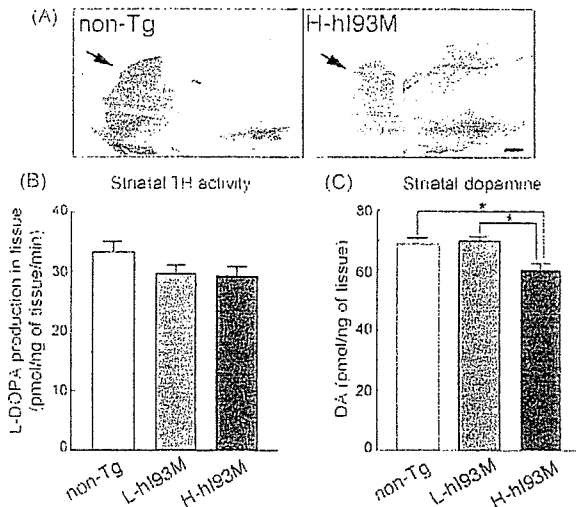


Fig. 6. H-hI93M mice show pathology in the striatum. Dopamine content and TH activity were lower in H-hI93M mice. (A) Sagittal sections from non-Tg and H-hI93M mice at 20 weeks of age were immunostained with the dopaminergic marker anti-TH. TH immunoreactivity is decreased in the nigro-striatal axons (arrows) of H-hI93M brains. Scale bar: 100  $\mu$ m. (B) TH activity and (C) dopamine content were measured following extraction and homogenization of the mouse striatum of non-Tg, L-hI93M and H-hI93M mice at 20 weeks of age ( $n = 4$ ; mean  $\pm$  S.E.). Significance was examined by a one-way ANOVA. \* $p < 0.05$ .

immunohistochemical and biochemical analyses. In agreement with the reduction of TH-positive dopaminergic neurons in the substantia nigra, nigro-striatal fibers in H-hI93M mice showed decreased immunoreactivity for TH as compared with that of non-Tg mice (Fig. 6A). TH activity, analyzed by determining L-DOPA production in the striatal tissues, also showed a tendency to decline in H-hI93M mice, although it was not significantly different (Fig. 6B). Loss of dopaminergic neurons in the substantia nigra and decreased TH activity in the striatum of H-hI93M mice prompted us to examine the concentration of striatal dopamine. Compared with non-Tg mice, H-hI93M mice showed a significant reduction of dopamine content in the striatum (Fig. 6C).

### 3.6. Decreased spontaneous, voluntary movements of H-hI93M mice

Given the prominent loss of dopaminergic neurons in the substantia nigra and the reduction in dopamine content in the striatum of H-hI93M mice, we next assessed the locomotor abilities of H-hI93M mice using a battery of well-established behavioral tests. Involuntary movement was analyzed by the rota-rod test (Goldberg et al., 2005) on 23–26-week-old mice. H-hI93M mice and non-Tg mice were similarly able to maintain their balance on the rotating rod during rod acceleration before falling off (Fig. 7A). We next analyzed spontaneous, voluntary movements with a locomotor activity test (Goldberg et al., 2005). Unexpectedly, 11–13-week-old H-hI93M mice showed significant hyperlocomotion during active periods (i.e., at night) as compared with non-Tg mice during home cage monitoring (Fig. 7B). However, 19–21-week-old H-

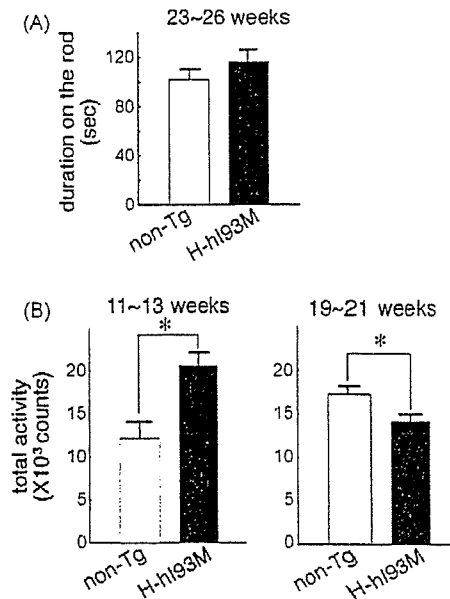


Fig. 7. H-hI93M transgenic mice show locomotor deficits. (A) Accelerated rota-rod analysis of H-hI93M and non-Tg mice ( $n = 6$  for non-Tg and  $n = 7$  for H-hI93M) at 23–26 weeks of age. Mice were placed on a rod, and their duration on the rod before falling off (mean value of three trials for each animal) was recorded. (B) Home cage monitor analysis of H-hI93M and non-Tg mice at 11–13 weeks of age (left;  $n = 4$  for each line) and at 19–21 weeks of age (right;  $n = 8$  for non-Tg and  $n = 10$  for H-hI93M). Note the significant hyperlocomotion of H-hI93M mice as compared with non-Tg mice at 19–21 weeks of age. Values are the mean  $\pm$  S.E.M. Significance was examined using the unpaired Student's  $t$ -test. \* $p < 0.05$ .

hI93M mice showed a modest but significant reduction in locomotor activity during active periods as compared with non-Tg mice (Fig. 7B). These results indicate that, in addition to the neuropathological changes, H-hI93M mice exhibit mild behavioral deficits related to PD.

## 4. Discussion

In this study, we characterized transgenic mice expressing hUCH-L1<sup>I93M</sup>, a mutation with presumptive association with familial PD, in the brain. Our previous attempt of making mouse UCH-L1<sup>WT</sup> Tg mice under various higher expressing promoters, such as EF1 $\alpha$ , resulted in an infertility of mice, thus it was impossible to maintain the lines. This failure resulted from the effect of overexpressing UCH-L1 in the testis/ovary leading to an increased apoptosis in these reproductive organs, although we did not find obvious morphological differences in the brain (Wang et al., 2006). Thus, we used *PDGF-B* promoter in this study to avoid massive expression of the transgene.

Two lines of hUCH-L1<sup>I93M</sup> Tg mice and one line of hUCH-L1<sup>WT</sup> Tg mice were viable and fertile without any predictable abnormalities. All of the three Tg lines expressed very limited levels of the human *UCHL1* gene with a maximum transcript ratio of about 1/100 as compared with the endogenous mouse *Uchl1*. However, immunohistological analysis indicated that higher level of hUCH-L1<sup>I93M</sup> expression could be detected in the large number of neurons in the substantia nigra of

H-hI93M/gad mice at 2 weeks of age. In addition, there is a difference in the morphology of hUCH-L1<sup>I93M</sup> expressing neurons, reminiscent of dying neurons, in the substantia nigra of H-hI93M/gad mice among 7 and 20 weeks of age. We also observed an eventual decline in the number of UCH-L1-positive neurons in H-hI93M/gad mice, as they age. Furthermore, the dopaminergic neurons in the substantia nigra of H-hI93M mice at 12 weeks of age showed silver staining-positive argylophilic grains, which represent neurons undergoing degeneration (Lo Bianco et al., 2004). Since we observed a loss of dopaminergic neurons in the substantia nigra and reduced dopamine content in the striatum of H-hI93M mice at 20 weeks of age, our results indicate the possibility that hUCH-L1<sup>I93M</sup> expressing dopaminergic neurons degenerate with age.

In addition to cell loss, several neuropathological features were observed in the substantia nigra of H-hI93M mice. Dopaminergic neurons had (1) electron dense-core vesicles in the perikarya, and (2) cytoplasmic inclusions that were positive for both UCH-L1 and ubiquitin. Despite these features, we did not observe eosinophilic or  $\alpha$ -synuclein-positive Lewy bodies at the substantia nigra in our morphological analyses. Thus, the mouse dopaminergic neurons expressing UCH-L1<sup>I93M</sup> may die prior to the formation of Lewy bodies, or those mice might form these structures at stages beyond the period of our study.

The mechanisms responsible for dopaminergic cell loss in the substantia nigra of H-hI93M mice remain elusive. The I93M mutation in UCH-L1 reduces its hydrolase activity by about 50%, which has been suggested as a cause for the pathogenesis of PD (Nishikawa et al., 2003). However, we have not found clear evidence for nigro-striatal dopaminergic pathology in *gad* mice (data not shown). Since expression of UCH-L1 is not detected in *gad* mice, the reduction of hydrolase activity alone would not be the cause of PD. In light of our finding here that transgenic expression of UCH-L1<sup>I93M</sup> results in dopaminergic pathology in mice, it would seem that this mutation elicits a gain of toxic function leading to the neuronal toxicity in the substantia nigra.

Our previous work using circular dichroism suggests that the I93M mutation reduces the  $\alpha$ -helical content of UCH-L1 (Nishikawa et al., 2003). Recently, we had also showed, using small-angle neutron scattering, that wild-type or I93M mutant UCH-L1 exists as a dimer in an aqueous solution. Moreover, their configuration differed; wild-type UCH-L1 has ellipsoidal shape where as I93M mutant has more globular shape (Naito et al., 2006). Cells expressing UCH-L1<sup>I93M</sup> are more prone to form inclusions (Ardley et al., 2004). Proteomic analysis of autopsied brains from PD patients and AD patients shows that UCH-L1 is extensively modified by carbonyl formation, methionine oxidation and cysteine oxidation in the diseased brains (Choi et al., 2004). These modifications are shown to result from oxidative stress (Choi et al., 2004). We show here that I93M mutation in UCH-L1 increases its insolubility *in vivo*. From the very limited expression of human UCH-L1 I93M, it is possible to speculate that endogenous mouse UCH-L1 might become insoluble in the presence of I93M UCH-L1. In addition, L-hI93M neurons were more susceptible than hWT or non-Tg neurons to MPTP, an inhibitor of complex I. This

observation suggests that UCH-L1<sup>I93M</sup> easily gains toxicity under oxidative stress. The conformational change and/or the additional methionine oxidation in UCH-L1 caused by I93M mutation may cause increased insolubility and lead to the gain of a toxic function.

In addition, our behavioral analysis revealed that H-hI93M mice exhibit very slight defects in spontaneous, voluntary movement, as shown by their hyperlocomotion at 11–13 weeks of age and by their hypolocomotion at 19–21 weeks of age in the home cage monitor test. Patients with PD exhibit no clinical symptoms until 70–80% of dopaminergic neurons are lost (Dauer and Przedborski, 2003). Thus, the level of dopaminergic neuronal loss seen in H-hI93M mice might not be sufficient to produce severe clinical phenotypes. It is difficult to explain the hyperlocomotion detected at 11–13 weeks of age, by simple changes in the nigro-striatal pathway. Other brain areas might be related to the locomotor changes seen in H-hI93M mice. We will need further analysis to connect the dopaminergic cell loss and defects in spontaneous, voluntary movement in H-hI93M mice.

In attempts to replicate neuropathological aspects of PD, several of the familial PD genes have been altered in mice. Up to date,  $\alpha$ -synuclein Tg mice with or without mutation (Fernagut and Chesselet, 2004), parkin knockout mice (Goldberg et al., 2003; Itier et al., 2003; Palacino et al., 2004; Perez and Palmiter, 2005; Von Coelln et al., 2004), and DJ-1 knockout mice (Chen et al., 2005; Goldberg et al., 2005; Kim et al., 2005) have been reported. Although these mice show some alterations in the function of dopaminergic neurons, none has dopaminergic neuron loss in the substantia nigra. Thus, we have developed the first mouse model with an alteration in a familial PD gene that leads to dopaminergic cell loss. Further analysis of these mice will help establish the role of UCH-L1 in PD, which may elucidate a common pathway for both familial and sporadic PD.

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## Identification of a novel regulatory mechanism for norepinephrine transporter activity by the IP<sub>3</sub> receptor

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### Abstract

The norepinephrine transporter (NET) plays a crucial role in noradrenergic neurotransmission and is a target of many antidepressants and psychostimulants. Intracellular Ca<sup>2+</sup> is reportedly involved in regulating NET activity, but the detailed mechanism is not clear. We employed a norepinephrine uptake assay using SH-SY5Y cells and found that the IP<sub>3</sub> receptor inhibitors, 2-aminoethoxydiphenyl borate and xestospongine C, reduced the NET V<sub>max</sub>. These reductions were accompanied by the decreased cell surface expression of NET. Our findings suggest that intracellular Ca<sup>2+</sup> mobilized by IP<sub>3</sub> receptor is required for the maintenance of NET activity. This adds another pathway involving Ca<sup>2+</sup> for the regulation of NET to other known mechanisms providing intracellular Ca<sup>2+</sup>.

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**Keywords:** Norepinephrine; Transporter; Calcium; Membrane traffic

### 1. Introduction

Norepinephrine is an important neurotransmitter that controls alertness, attention, mood, and learning/memory in the central nervous system (Foote et al., 1983). It was suggested that noradrenergic neuron dysfunctions may cause many disorders, such as depression (Brunello et al., 2002), attention deficit hyperactivity disorder (ADHD) (Biederman and Spencer, 1999) and posttraumatic stress disorder (PTSD) (Hageman et al., 2001). Indeed, alterations of regional norepinephrine function may underlie these norepinephrine-related disorders (Tanaka, 1999; Thomas et al., 1994). Noradrenergic neurons are localized in brain stem nuclei A1–7 and project extensively to

the cortex, amygdala, hypothalamus, hippocampus and septum (Foote et al., 1983). Norepinephrine is released at the terminals within each brain region, and it has been reported that norepinephrine concentration at each target is regulated in a region-specific manner. For example, regional variations of norepinephrine concentration are observed in the noradrenergic terminals of different cortical regions such as the prefrontal, somatosensory, and visual cortex (Brown et al., 1979; Levitt et al., 1984). However, the mechanisms that establish variations in norepinephrine concentration at each terminal are not clear. The norepinephrine transporter (NET) regulates norepinephrine concentration (Xu et al., 2000a). NET is a 12-transmembrane, Na<sup>+</sup>/Cl<sup>-</sup>-dependent neurotransmitter transporter with cytoplasmic N and C termini (Pacholczyk et al., 1991). The characteristics of NET are shared with the glycine transporter (GLYT), dopamine transporter (DAT) and serotonin transporter (SERT). NET is a target of tricyclic antidepressants and psychostimulants. Dysfunction of NET induces abnormal extracellular neurotransmitter concentrations at the prefrontal

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cortex, hippocampus and cerebellum (Xu et al., 2000a). On the other hand, NET activity in SH-SY5Y cells is regulated by intracellular signaling factors such as protein kinase C (PKC) (Apparsundaram et al., 1998b) and protein phosphatase 2A (PP2A) (Bauman et al., 2000), which modulate the cell surface NET. However, the molecular mechanism of NET regulation at each terminal site of noradrenergic neurons is not clear.

NET activity is reduced in the presence of the membrane-permeable  $\text{Ca}^{2+}$  chelator, 1,2-bis (o-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM) (Apparsundaram et al., 2001). Although PKC is  $\text{Ca}^{2+}$ -dependent, its activation reduces NET activity (Apparsundaram et al., 1998b), suggesting that another  $\text{Ca}^{2+}$ -dependent factor may potentiate NET activity. Activation of PKC requires both  $\text{Ca}^{2+}$  and diacylglycerol. Both diacylglycerol and inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) are products of the breakdown of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC).  $\text{IP}_3$  is a major inducer of intracellular  $\text{Ca}^{2+}$  release. Since intracellular  $\text{Ca}^{2+}$  signaling is strictly regulated to affect on site-specific signaling (i.e., via  $\text{Ca}^{2+}$  microdomains) (Becherer et al., 2003; Delmas et al., 2002; Olivos Ore and Artalejo, 2004; Zheng, 2000), the  $\text{IP}_3$  receptor may be critical for regional regulation of NET at each noradrenergic terminal.

Here, we examined whether intracellular  $\text{Ca}^{2+}$  mediated by  $\text{IP}_3$  receptor affects NET activity in the SH-SY5Y cells, which are human neuroblastoma cell lines expressing native NET. We found that the  $\text{IP}_3$  receptor- $\text{Ca}^{2+}$  pathway plays a critical role for the maintenance of NET activity. Our findings suggest that  $\text{IP}_3$  receptor acts as a link between  $\text{Ca}^{2+}$  microdomains and NET regulation to modulate norepinephrine concentration.

## 2. Materials and methods

### 2.1. Cell culture

SH-SY5Y cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin in 5%  $\text{CO}_2$  at 37 °C and plated in Falcon 100-mm dishes (Becton Dickinson, Franklin Lakes, NJ). Only three specific passages were used for the assay.

### 2.2. Materials

Reagents used to modify receptors and second messengers were obtained from the following sources: 2-aminoethoxydiphenyl borate (2APB), (Calbiochem, San Diego, CA); protease inhibitor cocktail (Roche, Mannheim, Germany); xestospongins C and  $\text{LaCl}_3$  (Wako, Osaka, Japan); norepinephrine (= arterenol), ryanodine, cyclopiazonic acid (CPA), desipramine, BAPTA, BAPTA-AM and DMEM (Sigma, St. Louis, MO); [ $^3\text{H}$ ] norepinephrine (L-[7,8- $^3\text{H}$ ] noradrenaline), (Amersham Biosciences Corp., Piscataway, NJ); 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) and Calcium Kit-Fluo 3 (Dojindo, Tokyo, Japan); West Dura Extended Duration Substrate, horseradish peroxi-

dase (HRP)-conjugated anti-mouse and anti-rabbit IgG, monomeric avidin beads and EZ-Link sulfo-NHS-LC-Biotin (Pierce, Rockford, IL); antibody against hNET (NET 1-17, Mab technology, Stone Mountain, GA); antibody against calnexin (Stressgen, Victoria, Canada). Other reagents were of analytical purity and were obtained from standard sources.

### 2.3. Norepinephrine uptake assay

NET activity was assessed as the uptake of [ $^3\text{H}$ ] norepinephrine (velocity per total protein). The cells were collected by trypsinization, plated at  $5 \times 10^4$  cells/well in collagen-coated 96-well dishes 24 h before experiments. For norepinephrine uptake studies, the culture medium was removed from 96-well plates, and the cells in each well were washed with Krebs-Ringer-HEPES (KRH) buffer (125 mM NaCl, 4.8 mM KCl, 1.3 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 5.6 mM glucose, 25 mM HEPES, 100  $\mu\text{M}$  ascorbic acid, 50  $\mu\text{M}$  pargyline, pH 7.4) at room temperature. To analyze NET kinetics, a norepinephrine uptake assay was performed using [ $^3\text{H}$ ] norepinephrine and non-radiolabeled norepinephrine to obtain final concentrations of 0.2–7  $\mu\text{M}$ . In other norepinephrine uptake analyses, cells were treated with each reagent for the signal transduction factor prior to the addition of 500 nM [ $^3\text{H}$ ] norepinephrine. Cells were incubated at 37 °C for 10 min, and the assay was terminated by three rapid washes with ice-cold KRH buffer. The cells were then lysed in 0.1 N NaOH, and the lysate was applied to a Luma plate (Perkin Elmer, Wellesley, MA) and dried. The radioactivity was quantified using a Microbeta counter (Perkin Elmer). Nonspecific [ $^3\text{H}$ ] norepinephrine uptake was determined using 1  $\mu\text{M}$  desipramine and was subtracted from the total uptake to yield NET-specific uptake. An aliquot of extract was used to determine protein concentration using the Bio-Rad Protein Assay. Nonlinear curve fits of saturation data were calculated using the Eadie-Hofstee model,  $V_0 = -K_m \times V_0 / S + V_{\text{max}}$ . All the reagents used in our experiments affected the lactic acid dehydrogenase (LDH) assay for cytotoxicity by less than 5%.

### 2.4. Intracellular $\text{Ca}^{2+}$ measurements

Intracellular  $\text{Ca}^{2+}$  was measured using the Calcium Kit-Fluo 3 (Dojindo). Briefly,  $5 \times 10^4$  cells were incubated with Fluo-3 AM for 90 min and then placed in  $\text{Ca}^{2+}$ -free KRH buffer containing 0.1% dimethyl sulfoxide (DMSO; control) or one of the  $\text{IP}_3$  receptor inhibitors, 20  $\mu\text{M}$  2APB or 10  $\mu\text{M}$  xestospongins C. After 10 min, carbachol was added to yield a final concentration of 10  $\mu\text{M}$  to initiate intracellular  $\text{Ca}^{2+}$  release. Fluorescence was monitored in a 1420 Multilabel counter (Perkin-Elmer, Turku, Finland) at 485 nm (excitation) and 530 nm (emission). Fluorescence data were acquired at a frame interval of 0.5 s.

### 2.5. Cell surface biotinylation

SH-SY5Y cells were plated at  $5 \times 10^6$  cells into collagen or poly-L-ornithine coated 60-mm dish (Falcon) for 24 h. After

treatment with reagents for 15 min, norepinephrine (500 nM) was added and incubated for 10 min in KRH buffer. Next, cells were washed with ice-cold phosphate buffered saline/ $\text{Ca}^{2+}/\text{Mg}^{2+}$  (PBS/ $\text{Ca}^{2+}/\text{Mg}^{2+}$ ; 138 mM NaCl, 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 9.6 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{CaCl}_2$ , pH 7.3) and then treated with EZ-Link Sulfo-NHS-LC-Biotin (1.5 mg/ml, Pierce) at 4 °C for 1 h in PBS/ $\text{Ca}^{2+}/\text{Mg}^{2+}$ . The biotinylating reagents were removed and washed twice with 100 mM glycine in PBS/ $\text{Ca}^{2+}/\text{Mg}^{2+}$ . The biotinylation reaction was quenched by incubation in 100 mM glycine in PBS/ $\text{Ca}^{2+}/\text{Mg}^{2+}$  at 4 °C for 30 min, and washed three times with PBS/ $\text{Ca}^{2+}/\text{Mg}^{2+}$ . Cells in each dish or well were solubilized by Radioimmunoprecipitation Assay (RIPA) buffer (20 mM Tris pH 7.5, 137 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate) containing protease inhibitor cocktail (Roch) and shaken at 4 °C for 1 h. Extracts were centrifuged at 20,000  $\times g$  at 4 °C for 30 min. Aliquots of supernatants (total lysate) were removed and preserved in other tubes. Monomeric avidin beads (Pierce) that were washed in RIPA buffer were added to the remainder of the samples and shaken for 1 h at room temperature. The unbound fractions (non-biotinylated lysate) were removed by centrifugation and preserved in other tubes. The beads were washed three times with RIPA buffer and the absorbed proteins were eluted by Laemmli loading buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5%  $\beta$ -mercaptoethanol and 0.1% bromophenol blue) with shaking for 30 min at room temperature. Total lysates and non-biotinylated lysates were diluted with 2 $\times$  Laemmli loading buffer. All samples were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), prior to transfer to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). After blocking with 5% nonfat dried milk in PBS containing 0.1% Tween 20 (PBST), blots were probed with primary antibody. Following washes with PBST, proteins reacting with HRP-conjugated anti-mouse or rabbit IgG (secondary antibody, 1:20,000, Pierce) were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce). Values of NET in total, non-biotinylated (intracellular) fraction, and the biotinylated (cell surface) fraction were normalized by the levels of calnexin immunoreactivity in total fraction.

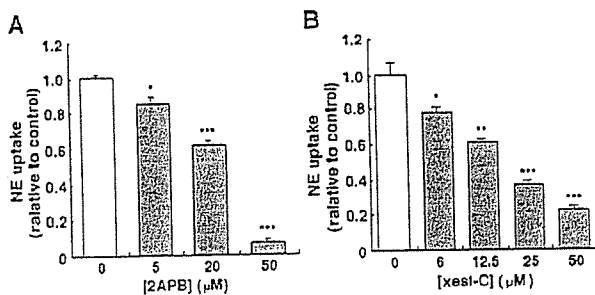


Fig. 1. The  $\text{IP}_3$  receptor inhibitors 2APB and xestospingon C decreased NET activity. (A, B) Cells were incubated with vehicle or each reagent (37 °C, 15 min) followed by incubation with [ $^3\text{H}$ ] norepinephrine (500 nM). The nonspecific signal was defined using 1  $\mu\text{M}$  desipramine. Data represent means  $\pm$  S.E.M. ( $n=3$ ; \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , vs. controls, ANOVA followed by the Dunnett's method).

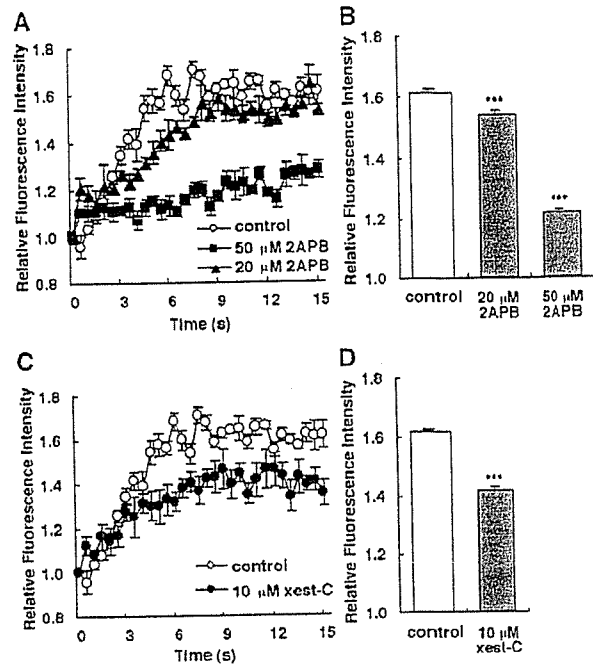


Fig. 2. The  $\text{IP}_3$  receptor inhibitors 2APB and xestospingon C blocked the elevation of intracellular  $\text{Ca}^{2+}$  concentration. (A–D) Intracellular  $\text{Ca}^{2+}$  was monitored using the  $\text{Ca}^{2+}$ -sensitive fluorescent indicator, Fluo-3 AM. Cells were incubated with Fluo-3 AM for 90 min and then incubated in  $\text{Ca}^{2+}$ -free KRH buffer (compensated with 10 mM  $\text{MgCl}_2$ ) containing 0.1% DMSO (control) or 20  $\mu\text{M}$ /50  $\mu\text{M}$  2APB (A) or 10  $\mu\text{M}$  xestospingon C (C), also in 0.1% DMSO. After 10 min, 10  $\mu\text{M}$  (final concentration) carbachol was added to initiate intracellular  $\text{Ca}^{2+}$  release. The effects of 2APB and xestospingon C on intracellular  $\text{Ca}^{2+}$  release are presented as means of relative fluorescence intensity after the plateau (8–15 s, B, D). Data represent means  $\pm$  S.E.M. ( $n=3$ ; \*\*\* $P<0.001$ , vs. controls, ANOVA followed by the Dunnett's method (B) and Student's  $t$  test (D)).

## 2.6. Data analyses

Statistical analyses were performed by comparing mean values or mean percentages derived from each experiment. The Student's  $t$  test was used for statistical analysis of data involving comparisons between two groups. One-way analysis of variance (ANOVA) followed by the Bonferroni method was used for deducing statistical differences in experiments involving multiple comparisons. The Dunnett's test was used for comparison of a single control group to all other groups. All analyses were performed using PRISM version 3.0 (GraphPad Software, Inc., San Diego, CA).

## 3. Results

### 3.1. Effects of $\text{IP}_3$ receptor inhibitors on NET activity and intracellular $\text{Ca}^{2+}$ concentration

To establish whether  $\text{IP}_3$  receptor is involved in regulating NET activity, we examined the effect of two structurally different  $\text{IP}_3$  receptor inhibitors, 2-aminoethoxydiphenyl borate (2APB) and xestospingon C, on NET activity in the human neuroblastoma cells, SH-SY5Y. Treatment with both  $\text{IP}_3$

receptor inhibitors produced the reduction of NET activity (Fig. 1A, B).

Next, we tested whether IP<sub>3</sub> receptor inhibitors block the elevation of intracellular Ca<sup>2+</sup> concentration induced by IP<sub>3</sub> receptor stimulation under our experimental condition. SH-SY5Y cells are reported to express muscarinic receptors (Kukkonen et al., 2001). Carbachol, a specific agonist for the muscarinic receptor, induced intracellular Ca<sup>2+</sup> elevation via IP<sub>3</sub> generation (Kukkonen et al., 2001; Xu et al., 2000b). Thus we measured carbachol-stimulated Ca<sup>2+</sup> elevation in SH-SY5Y cells with the fluorescent Ca<sup>2+</sup> indicator, Fluo-3. As shown in Fig. 2A–D, the treatment of IP<sub>3</sub> receptor inhibitors significantly reduced intracellular Ca<sup>2+</sup> elevation under extracellular Ca<sup>2+</sup>-free condition (0–50 μM 2APB, 0–10 μM xestospongine C). Kukkonen et al. (2001) reported that 2APB failed to reduce the intracellular Ca<sup>2+</sup> elevation in SH-SY5Y cells stimulated by carbachol under their experimental conditions. Although our

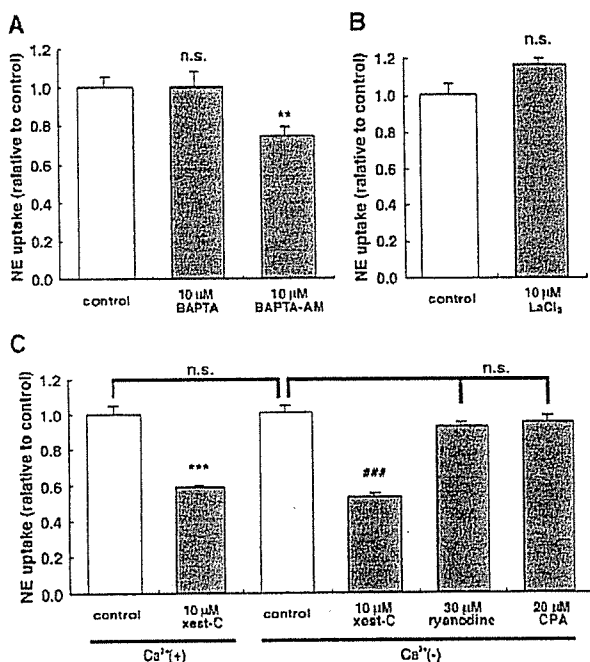


Fig. 3. Effects of intracellular and extracellular Ca<sup>2+</sup> on NET activity. (A) Effect of the membrane-permeable Ca<sup>2+</sup> chelator BAPTA-AM, and the non-membrane permeable chelator, BAPTA, on NET activity. Cells were incubated with 10 μM BAPTA or 10 μM BAPTA-AM in KRH buffer containing 0.1% bovine serum albumin (BSA) for 2 h and then assayed for NET activity. The nonspecific signal was defined using 1 μM desipramine. Data represent means ± S.E.M. (*n* = 3, n.s., not significant, \*\**P* < 0.01, ANOVA followed by the Bonferroni method). (B) Effect of the SOC channel inhibitor, LaCl<sub>3</sub> on NET activity. Cells were incubated with 10 μM LaCl<sub>3</sub> for 15 min and then assayed for NET activity. Data represent means ± S.E.M. (*n* = 3, n.s., not significant, Student's *t* test). (C) Effect of extracellular Ca<sup>2+</sup>, ryanodine receptor and xestospongine C (xest-C) on NET activity. Cells were treated with the IP<sub>3</sub> receptor inhibitor, 10 μM xestospongine C, the ryanodine receptor agonist, ryanodine (30 μM), or the ryanodine receptor antagonist, CPA (20 μM), for 15 min in KRH buffer with or without Ca<sup>2+</sup> and then assayed for NET activity. The nonspecific signal was defined using 1 μM desipramine. Data represent means ± S.E.M. (*n* = 3, n.s. not significant, \*\*\**P* < 0.001 vs. control in the presence of extracellular Ca<sup>2+</sup>, Student's *t* test; ###*P* < 0.001 vs. control in the extracellular Ca<sup>2+</sup>-free buffer, ANOVA followed by the Bonferroni method).

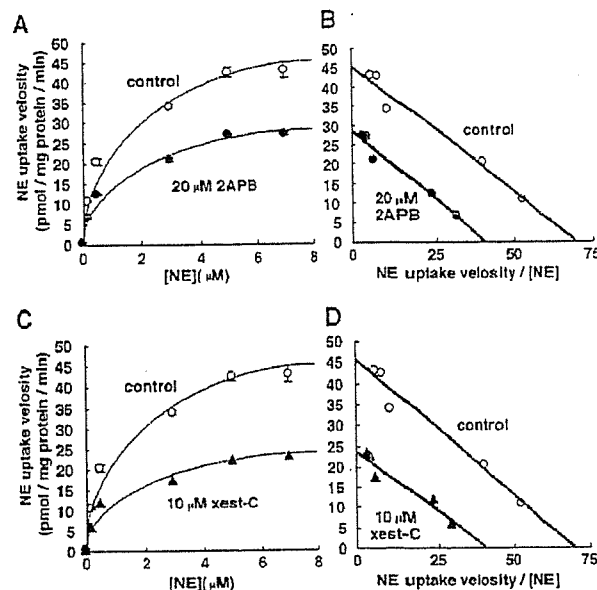


Fig. 4. Effects of IP<sub>3</sub> receptor inhibitors on NET activity are accompanied by a reduction in *V*<sub>max</sub>. (A–D) Effects of IP<sub>3</sub> receptor inhibitors on NET activity. Cells were treated with or without 20 μM 2APB or 10 μM xestospongine C (xest-C) for 15 min and then assayed for NET activity (A, C). The data were plotted using the Eadie–Hofstee format, and the *V*<sub>max</sub> and *K*<sub>m</sub> values were calculated by linear regression analysis (B, D). The nonspecific signal was defined using 1 μM desipramine.

data differed from the report, this discrepancy may be caused by the different 2APB incubation period or the extracellular Ca<sup>2+</sup> concentration. Taken together, these results indicated that the elevation of intracellular Ca<sup>2+</sup> concentration mediated by IP<sub>3</sub> receptor links with NET activity.

### 3.2. IP<sub>3</sub> receptor-dependent release of intracellular Ca<sup>2+</sup> specifically regulates NET activity

Intracellular Ca<sup>2+</sup> reportedly has a facilitatory role for NET activity, and chelation of intracellular Ca<sup>2+</sup> by BAPTA-AM reduces NET activity in SK-N-SH cells (Apparsundaram et al., 2001). We examined the effect of 10 μM BAPTA-AM on NET activity in SH-SY5Y cells. BAPTA-AM reduced NET activity, whereas BAPTA, which is not membrane permeable, did not at an equivalent concentration (Fig. 3A). These results indicate that intracellular Ca<sup>2+</sup> has a facilitatory role on NET activity in SH-SY5Y cells. Conversely, IP<sub>3</sub> receptor inhibitors block extracellular Ca<sup>2+</sup> entry via the store-operated Ca<sup>2+</sup> (SOC) channel (Ma et al., 2000; Van Rossum et al., 2000). The SOC channel becomes activated to replenish intracellular Ca<sup>2+</sup> stores (Putney, 1986). Thus, the effect of IP<sub>3</sub> receptor inhibitors on NET activity could be a consequence of SOC channel inhibition. To exclude this possibility, we measured norepinephrine uptake in SH-SY5Y cells in the presence of another SOC inhibitor, LaCl<sub>3</sub> (Baba et al., 2003). As shown in Fig. 3B, LaCl<sub>3</sub> did not alter the NET activity. Moreover, the effect of IP<sub>3</sub> receptor inhibitor was not altered in the presence or absence of extracellular Ca<sup>2+</sup> (Fig. 3C). These results suggest that the effects of IP<sub>3</sub> receptor inhibitors on NET activity are SOC

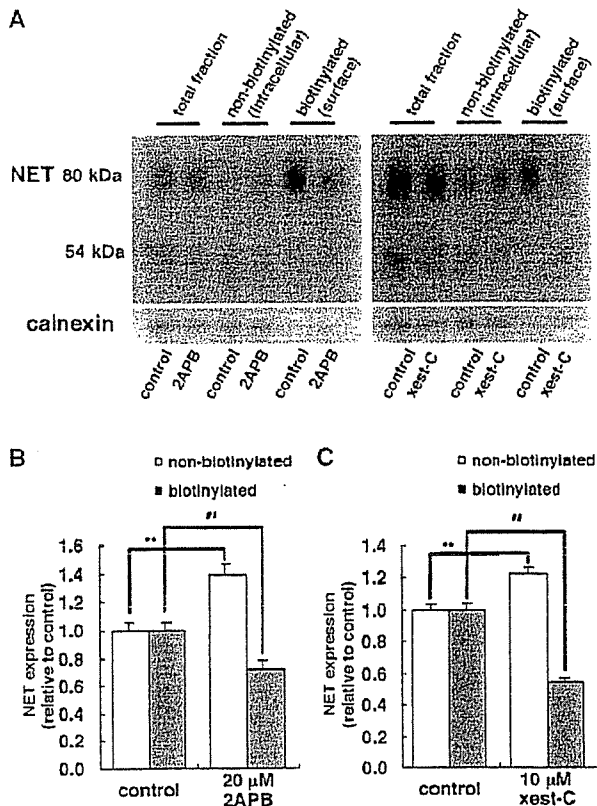


Fig. 5. Effects of IP<sub>3</sub> receptor inhibitors on NET activity are accompanied by a reduction in the surface expression of NET. (A) Analysis of cell surface expression of NET by biotinylation. Following the incubation IP<sub>3</sub> receptor inhibitors, 2APB and xestospongin C, for 15 min, norepinephrine was added (final 500 nM) and incubated for 10 min in KRH buffer prior to biotinylation with sulfo-NHS-LC-biotin (1.5 mg/mL). Cells were lysed with RIPA buffer, and the biotinylated fraction was captured with monomeric avidin beads. Total fraction, non-biotinylated fraction (intracellular), and biotinylated fraction (cell surface) were immunoblotted with anti-NET (1:1000) and anti-calnexin (1:4000). (B, C) Densitometric analysis of non-biotinylated (intracellular) and biotinylated (cell surface) NET expression (80 kDa) shown in A. Data represent means ± S.E.M. (*n* = 4 (B), *n* = 3 (C), \*\**P* < 0.01 and ##*P* < 0.01 v.s. each control, Student's *t* test).

channel-independent. We also tested whether ryanodine and the ryanodine receptor, another system for intracellular Ca<sup>2+</sup> release (Berridge et al., 2000), are involved in regulating NET activity. However, treatment with the ryanodine receptor agonist, ryanodine, or antagonist, cyclopiazonic acid (CPA), did not affect NET activity (Fig. 3C), indicating that the release of intracellular Ca<sup>2+</sup> via IP<sub>3</sub> receptor but not ryanodine receptor is required for the maintenance of NET activity in SH-SY5Y cells.

### 3.3. The effects of IP<sub>3</sub> receptor inhibitors on NET activity are mediated by a reduction in both *V*<sub>max</sub> and surface expression of NET

We analyzed the kinetics of norepinephrine transport in SH-SY5Y cells in the presence or absence of IP<sub>3</sub> receptor inhibitors. Eadie–Hofstee plots showed that treatment with 20 μM 2APB or 10 μM xestospongin C significantly reduced *V*<sub>max</sub> from the control level of 45.3 ± 1.35 to 28.5 ± 0.34 pmol/mg protein/min

(\*\*\**P* < 0.001, Student's *t* test) and 23.4 ± 0.52 pmol/mg protein/min (\*\*\*) *P* < 0.001, Student's *t* test), respectively (Fig. 4A–D). However, the IP<sub>3</sub> receptor inhibitors did not significantly affect *K*<sub>m</sub> values (679.6 ± 6.3 nM to 740.0 ± 35.4 and 620.1 ± 56.5 nM, not significant, Student's *t* test). The decreased *V*<sub>max</sub> values suggest that cell surface expression of functional NET was reduced.

To address this possibility, we assayed for hNET in the total fraction, non-biotinylated fraction (intracellular NET) and biotinylated fraction (NET surface expression) by western blotting using an antibody against hNET. We detected both 80- and 54-kDa forms of hNETs in total and non-biotinylated fraction (Fig. 5A). It is reported that these two forms depend on *N*-glycosylation of hNET protein (Melikian et al., 1994). Because glycosylation is required for the expression of NET at the cell surface (Nguyen and Amara, 1996), we analyzed 80-kDa of NET. Treatment of SH-SY5Y cells with IP<sub>3</sub> receptor inhibitors increased the expression of intracellular NET and reduced the cell surface expression of NET (Fig. 5A–C). These results suggest that intracellular Ca<sup>2+</sup> modulation by IP<sub>3</sub> receptor is required to maintain the surface expression of NET. To monitor the efficacy of biotinylation of intracellular proteins, we used calnexin, one of the endoplasmic reticulum marker (Wada et al., 1991). The membrane was reprobed with the anti-calnexin antibody after stripping the anti-NET antibody. The results showed the decreased density in biotinylated fraction compared to the total or non-biotinylated fraction (Fig. 5A, lower panel), suggesting that the fractionation was done successfully.

## 4. Discussion

Although a previous study reported that intracellular Ca<sup>2+</sup> is involved in NET regulation (Apparsundaram et al., 2001), the detailed mechanism has not been defined. We demonstrated that IP<sub>3</sub> receptor, which controls intracellular Ca<sup>2+</sup> concentration, regulates NET activity. This is relevant to the function of Ca<sup>2+</sup> microdomains, which could achieve localized regulation of noradrenergic neurons mediated by NET regulation. We also showed that the IP<sub>3</sub> receptor pathway is involved in regulating the cell surface expression of NET.

IP<sub>3</sub> receptor is activated in parallel with PKC downstream of PLC. It has been reported that PKC activation reduces NET activity (Apparsundaram et al., 1998b). In the present study, the IP<sub>3</sub> receptor inhibitors, 2APB and xestospongin C, reduced NET activity in a dose-dependent manner (Fig. 1), suggesting that the IP<sub>3</sub> receptor–Ca<sup>2+</sup> system has facilitatory effects on NET activity. The fact that the effect of IP<sub>3</sub> receptor on NET activity is opposite to that of PKC raises the possibility that a balance between these PLC-related signaling pathways is critical for the regulation of NET activity. Another inducer of intracellular Ca<sup>2+</sup> releaser, ryanodine receptor, as well as an influx of extracellular Ca<sup>2+</sup> mediated by SOC channels, did not alter NET activity (Fig. 3B,C). Moreover, it has been reported that intracellular Ca<sup>2+</sup> signaling activated by the muscarinic M<sub>3</sub> receptor reduces NET activity (Apparsundaram et al., 1998a), whereas an influx of extracellular Ca<sup>2+</sup> induced by the activation of the insulin receptor increases NET activity

(Apparsundaram et al., 2001). These data indicate that the effect of intracellular  $\text{Ca}^{2+}$  on NET activity is distinct from the sources of  $\text{Ca}^{2+}$ . Our results suggest that the elevation of NET activity is a consequence of specific intracellular  $\text{Ca}^{2+}$  release mediated by  $\text{IP}_3$  receptor, implying that a specific  $\text{Ca}^{2+}$  microdomain regulates NET activity. This hypothesis is supported by a report that a  $\text{Ca}^{2+}$  microdomain involved in  $\text{IP}_3$  receptor– $\text{Ca}^{2+}$  signaling via the muscarinic  $\text{M}_1$  receptor differs from the  $\text{Ca}^{2+}$  microdomain affected by the bradykinin  $\text{B}_2$  receptor in a single cell (Delmas et al., 2002). In fact, the  $\text{IP}_3$  receptor inhibitor blocked the elevation of carbachol-stimulated  $\text{Ca}^{2+}$  concentration (Fig. 2) without decreasing basal (pre-carbachol stimulation) intracellular  $\text{Ca}^{2+}$  concentration (data not shown). Because intracellular  $\text{Ca}^{2+}$  is maintained at a low level (about 100 nM) by channels and  $\text{Ca}^{2+}$ -binding proteins (Berridge et al., 2000), it may be impossible for  $\text{IP}_3$  receptor inhibitors to decrease intracellular  $\text{Ca}^{2+}$  concentration further. On the other hand, these results suggest the possibility that the concentration of  $\text{Ca}^{2+}$  in a specific  $\text{Ca}^{2+}$  microdomain relevant to NET activity, which is blocked by  $\text{IP}_3$  receptor inhibitors, does not reflect overall intracellular  $\text{Ca}^{2+}$  concentration. In our study,  $\text{IP}_3$  receptor inhibitors reduced NET activity by about 90% (Fig. 1). However, we could not confirm whether BAPTA-AM can abolish NET activity since BAPTA-AM concentrations over 10  $\mu\text{M}$  induced SH-SY5Y cell death (data not shown).

Noradrenergic neurons project over a wide range of the brain, including the cortex, hippocampus, cerebellum and spinal cord (Schroeter et al., 2000). Norepinephrine concentration varies in different regions of the brain (Tanaka et al., 2000). Although localized regulation of each noradrenergic neuron has been suggested (Foote et al., 1983), this mechanism is not clear. NET activity and/or expression level is altered by stimulation of the insulin receptor (Apparsundaram et al., 2001), muscarinic receptor (Apparsundaram et al., 1998a) and nerve growth factor (NGF) receptor (Ikeda et al., 2001).  $\text{IP}_3$  receptor functions at the downstream of G-protein-coupled receptors (including the muscarinic receptor) or receptor tyrosine kinases (including the insulin and NGF receptors). These results suggest that extracellular input or the environment alters NET activity, consequently contributing to localized noradrenergic neurotransmission.

We have shown for the first time that  $\text{IP}_3$  receptor-mediated intracellular  $\text{Ca}^{2+}$  has facilitatory effects on NET activity, suggesting that  $\text{IP}_3$  receptor– $\text{Ca}^{2+}$  signaling functions between NET regulation and signals mediated by cell surface receptors. Since intracellular  $\text{Ca}^{2+}$  is integral to establishing both temporal and spatial regulation of intracellular signal transduction systems, an  $\text{IP}_3$  receptor-mediated  $\text{Ca}^{2+}$  microdomain may contribute to region-specific regulation of noradrenergic neurons. These findings provide new insights into the regulation of transporters, and the elucidation of the mechanisms underlying the modulation of transporters may lead to novel therapeutic strategies for norepinephrine-related disorders.

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## Research Report

## Identification and functional characterization of mouse TPO1 as a myelin membrane protein

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## ABSTRACT

TPO1 is a member of the AIGP family, a unique group of proteins that contains 11 putative transmembrane domains. Expression of the rat TPO1 gene is upregulated in cultured oligodendrocytes (OLs) during development from pro-oligodendroblasts to postmitotic OLs. However, the distribution of native TPO1 protein in cultured OLs and in the brain has not been elucidated. We investigated the distribution and cellular function of TPO1 in myelinating cells of the nervous system. In mice, TPO1 gene expression was detected in the central (CNS) and peripheral (PNS) nervous systems and was markedly upregulated at postnatal days 10–20, an early phase of myelination in the mouse brain. To investigate TPO1 localization, we generated affinity-purified antibodies to synthetic peptides derived from mouse TPO1. Immunohistochemical analysis showed that TPO1 was expressed in OLs and Schwann cells but not in neurons and astrocytes. Schwann cells from trembler mice, which lack PNS myelin, had significantly decreased TPO1 expression and an altered localization pattern, suggesting that TPO1 is a functional myelin membrane protein. In OL lineage cell cultures, TPO1 was detected in A2B5(+) bipolar early progenitors, A2B5(+) multipolar Pro-OLs, GalC(+) immature OLs and MBP(+) mature OLs. The subcellular localization of TPO1 in OL lineage cells was mapped to the GM130(+) Golgi in cell bodies and Fyn(+) cell processes and myelin-like sheets. Furthermore, TPO1 selectively colocalized with non-phosphorylated Fyn and promoted Fyn autophosphorylation in COS7 cells, suggesting that TPO1 may play a role in myelin formation via Fyn kinase activation in the PNS and CNS.

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**Abbreviations:**

aa, amino acid  
AIGP, axotomy-induced glycosylated/Golgi-complex protein  
CNS, central nervous system  
DMEM, Dulbecco's modified Eagle' medium  
EEA1, early endosome antigen1  
EGFP, enhanced green fluorescent protein  
ER, endoplasmic reticulum  
GalC, galactocerebroside  
GAPDH, glyceraldehyde-3-phosphate dehydrogenase  
GFAP, glial fibrillary acidic protein  
HRP, horseradish peroxidase  
Lamp-1, lysosome-associated membrane glycoprotein-1  
MAG, myelin-associated glycoprotein  
Map-2, microtubule-associated protein-2  
MBP, myelin basic protein  
nt, nucleotide  
MOG, myelin oligodendrocyte glycoprotein  
MyTI, myelin transcriptional factor I  
OL, oligodendrocyte  
OPC, oligodendrocyte precursor cell  
ORF, open reading frame  
PO, myelin protein zero  
PBS, phosphate-buffered saline  
PLP, proteolipid protein  
PMP22, peripheral myelin protein-22  
PNS, peripheral nervous system  
T-TBS, Tris-buffered saline containing 0.1% (v/v) Tween-20

## 1. Introduction

Schwann cells and oligodendrocytes (OLs) produce myelin in the PNS and CNS, respectively. Myelin is composed of lamellar membranes and ensheaths axons and is essential for nervous system function, as demonstrated by the severe neurological symptoms observed in various myelin diseases (Baumann and Pham-Dinh, 2001; Shy et al., 2002). A number of myelin membrane proteins have been identified to date and have been shown to play pivotal roles in myelin construction and maintenance (Baumann and Pham-Dinh, 2001). Myelin-associated glycoprotein (MAG) is a single transmembrane protein and has multiple immunoglobulin domains, and MAG-deficient mice show delayed myelin compaction (Li et al., 1994). Myelin OL glycoprotein (MOG) is a CNS myelin-specific, single-transmembrane protein that induces experimental autoimmune encephalomyelitis (Scolding et al., 1989; Weissert et al., 1998). Proteolipid protein (PLP) is the most abundant myelin membrane protein, comprising 50% of total myelin protein, and is a member of the tetraspan protein family. Studies in

jumpy mice and human Pelizaeus–Merzbacher disease have shown that PLP is essential for stabilizing the myelin membrane (Klugmann et al., 1997; Baumann and Pham-Dinh, 2001). Peripheral myelin protein-22 (PMP22), another member of the tetraspan protein family, and myelin protein zero (PO), a single-transmembrane domain protein, specifically localize to PNS myelin membranes, and the genes encoding these proteins are among those causing Charcot–Marie–Tooth disease (Sakamoto et al., 1987; Lupski et al., 1991; Suter et al., 1992; Suh et al., 1997; Sakai et al., 1999).

AIGP1 (axotomy-induced glycosylated/Golgi complex protein 1) is a neuronal membrane protein that specifically localizes to Golgi membranes in neurons. AIGP1 has eleven potential transmembrane helices. Previously, we reported the possible involvement of AIGP1 in axotomy-associated neuronal cell death (Aoki et al., 2002). The AIGP family genes are evolutionarily conserved from yeast to mammals and, in mammals, consist of three members AIGP1, TMS-2 (Grossman et al., 2000; Aoki et al., 2002) and TPO1 (Krueger et al., 1997). The rat TPO1 gene was originally identified by

differential gene screening, and its expression was shown to be upregulated during OL differentiation (Krueger et al., 1997). Pfeiffer and coworkers also showed that the TPO1 gene is expressed in cultured OLs and rat brain (Krueger et al., 1997). However, the lack of availability of TPO1-specific antibodies has precluded elucidation of the natural distribution and function of this protein. Similarly to other proteins in the AIGP family, TPO1 is also a putative eleven-span membrane protein. However, the putative TPO1 amino acid sequence also has features that distinguish it from other AIGP family members; these features include an extremely polarized structure (N-terminal basic and C-terminal acidic composition), a sequence homologous to myelin transcription factor I (MyTI) and a potential C-terminal palmitoylation sequence (Krueger et al., 1997). Accordingly, the cell type-specific expression patterns of TPO1 are distinct from those of the AIGP1 and TMS-2 genes in the nervous system; AIGP1 and TMS-2 are specifically expressed in neurons (Grossman et al., 2000; Aoki et al., 2002), whereas the rat TPO1 gene is expressed in OL lineage cells (Krueger et al., 1997). AIGP1 has been suggested to function as a cell death regulator in axotomized neurons (Aoki et al., 2002), but the biological function of TPO1 is not known.

In this study, we used TPO1-specific antibodies to investigate the localization of TPO1 and to characterize its function in the nervous system. We show that TPO1 is a myelin membrane protein that possibly promotes autophosphorylation of Fyn kinase. Our results suggest that TPO1, a glial subtype of the AIGP family, may play a role in myelin formation and maintenance by regulating Fyn kinase activity.

## 2. Results

### 2.1. Identification of mouse TPO1

While screening for molecules involved in neuronal regeneration and death, we previously identified a mouse axotomy-induced glycosylated/Golgi complex protein, AIGP1 (Grossman et al., 2000; Aoki et al., 2002). Database searches suggested that the mouse or rat genome encodes three AIGP1 homologs: AIGP1, TMS-2 (GenBank accession no. AF181685) and TPO1 (GenBank accession no. L20319) (Fig. 1A). In mice, the AIGP1 and TMS-2 genes are expressed in neurons in the cortex, hippocampus, cerebellum and hypoglossal nucleus (Grossman et al., 2000; Aoki et al., 2002). On the other hand, in rats, the TPO1 gene has been reported to be expressed in OL lineage cells *in vitro* (Krueger et al., 1997). Since the cell type-specific expression pattern of TPO1 suggests distinct functionality, we were interested in the molecular function of TPO1 in the nervous system. The mouse TPO1 cDNA sequence (GenBank accession no. AB029501, (Aoki et al., 2002)) contains a 1416-bp open reading frame (ORF) that is homologous to the rat TPO1 cDNA sequence (57.7%). The ORF encodes 462 aa with 90% similarity to rat TPO1. Mouse AIGP1 and TMS-2 are less similar (36.8% and 39%, respectively) to mouse TPO1 (data not shown). The sequence of TPO1 is less conserved compared with AIGP1 and TMS-2 (Grossman et al., 2000; Aoki et al., 2002). Compu-

tational analyses with the transmembrane prediction software SOSUI (<http://sosui.proteome.bio.tuat.ac.jp>) suggest that TPO1 has eleven putative transmembrane helices (Fig. 1B). The second and fourth extracellular loops and fifth intracellular loop have relatively longer structures than the other loops (Fig. 1B), but the amino acid sequences of the long loops are not well conserved between TPO1 and other family members (data not shown). Two cysteine-rich zinc finger-like motifs, CX<sub>5</sub>CX<sub>6-10</sub>CX<sub>2</sub>C (residues 6–27, similar to aminoacyl tRNA synthetases) and CX<sub>5</sub>CX<sub>1</sub>CX<sub>4</sub>H (residues 100–123, similar to transcription factor MYT1; (Hirayama et al., 2003), and a potential C-terminal palmitoylation sequence (residues 453–454) were also found in the TPO1 sequence (Fig. 1B, Krueger et al., 1997). These motifs are highly conserved between rats and mice, but are not present in mouse AIGP1 and TMS-2 (data not shown). Two potential N-linked glycosylation sites (Asn-X-Thr) were identified in both AIGP1 and TMS-2 (Grossman et al., 2000; Aoki et al., 2002), but such sites were not found in the amino acid sequence of TPO1 (data not shown). Two potential phosphorylation sites for protein kinase C were identified in the second and third intracellular loops, and one potential target site for cAMP-dependent protein kinase was found in the fifth intracellular loop of TPO1 (data not shown). Phylogenetic analysis of the AIGP family members showed that mouse and rat TPO1 form a cluster that is completely separate from the AIGP1 and TMS-2 clusters (Fig. 1A).

### 2.2. TPO1 expression in the PNS and CNS

Previous studies (Krueger et al., 1997) have shown that the rat TPO1 gene is expressed in the CNS and in OL lineage cells. To examine gene expression levels in the PNS and CNS precisely, we performed SYBR green-based real-time RT-PCR analysis with the mouse CNS and PNS myelin-specific markers MOG (Scolding et al., 1989) and PO (Sakamoto et al., 1987; Lupski et al., 1991) (Fig. 1C, top). We examined TPO1 gene expression in the mouse dorsal root ganglion and brain. Both PNS and CNS tissues expressed TPO1 mRNA at considerable levels, which were remarkably higher than expression levels observed in the E14 brain (Fig. 1C, top). In the postnatal CNS, TPO1 gene expression markedly increased after P10 and reached a maximum level at P20. This level of expression was maintained throughout adult life (Fig. 1C, bottom).

### 2.3. Production and characterization of antibodies to mouse TPO1

We prepared specific antibodies against relatively hydrophilic peptide sequences of the fifth intracellular and second extracellular loops of mouse TPO1 (EP05 and EP06, respectively, [EP; epitope], Fig. 1B). Immunoblot analysis using mouse cultured OL cell lysates showed that anti-TPO1 (ABEP05; against EP05) recognized a ~48-kDa band (Fig. 2A, lane 2) that was slightly smaller than the expected molecular mass (51.8 kDa), probably because of its hydrophobicity (Fig. 2A, lane 2). No signal was detected when the blot was probed with control rabbit IgG (Fig. 2A, lanes 3 and 4). TPO1 was detected in the membrane fraction but not in the soluble fraction (Fig. 2A, lanes 1 and 2).