The Gtx Homeodomain Transcription Factor Exerts Neuroprotection Using Its Homeodomain

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Certain cases of familial Alzheimer’s disease are caused by mutants of amyloid-β precursor protein (AβPP), including V642I-AβPP, K595N/M596L-AβPP (NL-AβPP), A617G-AβPP, and L648P-AβPP. By using an unbiased functional screening with transfection and expression of a human brain cDNA library, we searched for genes that protect neuronal cells from toxicity by V642I-AβPP. One protective clone was identical to the human Gtx, a neuronal homeobox gene. Human Gtx (hGtx) inhibited caspase inhibitor-sensitive neuronal progenitors in ventral spinal cord and inhibits V0 neurons from oligodendrocytes with its potential role for myelinization (2, 3). This paper is available online at http://www.jbc.org

Gtx, a neuronal homeobox gene, has been isolated from the postnatal mouse brain (1) and is expressed in differentiated pattern formation in embryonic development. Molecular control of differentiation depends on lineage-restricted transcription factors that regulate expression of tissue-specific genes. Among the genes governing the development of Drosophila, sequential activation of homeotic and segmentation genes controls the identity, polarity, and number of segments. Many such genes, including the Antennapedia (Antp), Engrailed (En), and Paired (Prd) families, contain a characteristic -60-amino acid sequence motif, termed a homeobox (7). Consistent with the notion that these genes act as sequence-specific transcription factors, Gtx binds to (AT)TAAATGA as an optimal binding sequence and represses the transcription of the downstream genes (8).

Alzheimer’s disease (AD)1 is the most prevalent neurodegenerative disease, for which a curative therapy has not yet been developed. Three kinds of known mutant genes cause early onset familial AD (FAD): mutants of amyloid-β precursor protein (AβPP), presenilin1 (PS1), and PS2, each of which has been shown to cause neuronal cell death (9–15). Investigations using an edcsyne (Ecd)-inducible system (16) revealed that four different FAD mutants (V642I-AβPP, K595N/M596L-AβPP (NL-AβPP), M146L-PS1, and N141I-PS2) trigger different combinations of distinct toxic mechanisms in an expression-dependent manner (13–15). Considering that these FAD mutants cause only a small fraction of total AD cases, such mechanistic complexity suggests that it may be difficult to identify a single anti-AD neuroprotective reagent solely based on the underlying mechanisms.

We thus took advantage of a new approach, called “death trap” screening developed by D’Adamio et al. (17), as an unbiased functional screening of molecules that allow dying cells to survive. We applied this method to V642I-AβPP-inducible neuronal cells (18) using an expression cDNA library constructed from an occipital lobe of an AD patient brain. Here we report that one of the obtained genes was human Gtx (hGtx), which has inhibitory activity against neuronal cell death by AD-relevant insults through its homeodomain.

MATERIALS AND METHODS

Cell and Genes—V642I-AβPP, NL-AβPP, A617G-AβPP, L648P-AβPP, M146L-PS1, and N141I-PS2 cDNAs, subcloned in pcDNA or pIIND plasmids, were described previously (9, 13, 19–22). G8SR superoxide dismutase 1 (G8SR-SOD1) cDNA in pEF-BOS plasmid, kindly provided by Drs. Takashi Koide and Shoji Tsuji, was described previously (20). F11 cells, the hybrid cells of a rat embryonic primary neuron and a mouse neuroblastoma (23), were grown in Ham’s F-12 (Invitrogen) plus 18% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1 The abbreviations used are: AD, Alzheimer’s disease; AβPP, amyloid-β precursor protein; FAD, familial AD; ORF, open reading frame; Z, benzoyloxy carbonyl; fmk, fluoromethyl ketone; Ecd, edcsyne; CMV, cytomegalovirus; IGF, insulin-like growth factor; PS, presenilin1; FBS, fetal bovine serum; EcR, Ecd receptor; h, human; m, murine; HN, Humanin.

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and expression of each mutant was detected by anti-(His)6 antibody/P.

Transfection Protocols (F11 Cells)—For transient transfection of F11 cells with pcDNA, pEF-BOS, or pEF4/His plasmids, F11 cells were seeded at 7 x 10^4 cells/well in a 6-well plate and cultured in Ham’s F-12 medium supplemented with 18% FBS for 12–18 h, and were transfected with the plasmids (0.75 µg of pcDNA plasmid, 0.75 µg of pEF plasmid, 3 µl of LipofectAMINE, and 6 µl of LipofectAMINE PLUS for each well; Invitrogen) in the absence of serum for 3 h. After subsequent 2-h incubations with Ham’s F-12 plus 18% FBS, cells were cultured with Ham’s F-12 plus 10% FBS. Cell mortality was measured by trypan blue exclusion at 48 h after the onset of transfection (1.5 µg of protocol). Some experiments were performed with 0.5 µg of pcDNA, 0.5 µg of pEF4-hGtx, 2 µl of LipofectAMINE, and 4 µl of LipofectAMINE PLUS and cell mortality measurement 72 h after transfection (0.5-µg protocol). In multiple studies (13–15, 20, 22, 24), it has been indicated that the 0.5-µg protocol with F11 cells mimics expression in the F11/EcR cell system of the coding protein with 1 µg of pIND and 10 µm EcD treatment; and the 1.5-µg protocol with F11 cells mimics expression of the coding protein with 1 µg of pIND transfection and 40 µm EcD treatment in F11/EcR cells.

Transfection Protocol (F11/EcR Cells)—For transient transfection of the pIND plasmids, F11/EcR cells were seeded at 7 x 10^4 cells/well in a 6-well plate and cultured in Ham’s F-12 for 12–18 h, and were transfected with EcD-inducible pIND plasmids (1 µg of pIND, 1 µg of pEF4/His (pEF4-hGtx), 4 µl of LipofectAMINE, and 8 µl of LipofectAMINE PLUS) in the absence of serum for 3 h (pIND protocol). After subsequent incubation with Ham’s F-12 plus 18% FBS for 16 h, cells were cultured with Ham’s F-12 plus 10% FBS for 2 h, and EcD was then added to the media, as described previously (13–15).

Death Trap Screening—F11/EcR/V642I cells, in which V642I-AβPP is overexpressed by treatment with EcD, were described previously (18). By using these cells, in which death occurred in a vast majority of EcD-treated cells for a few days (18), we performed the death trap screening with our modifications. We first transfected F11/EcR/V642I cells with human expression cDNA library in pEF-BOS plasmid constructed from an occipital cortex of a necropsy-diagnosed AD patient, treated cells with EcD for 72 h, and recovered the plasmids from surviving cells. The recovered plasmids were again transfected to F11/EcR/V642I cells, which were then treated with EcD for 72 h. This procedure was repeated for three rounds, and we randomly picked 250 clones of plasmids. Using dot blot hybridization with randomly selected clones, we classified them into 36 independent groups.

Subcloning of hGtxs and Constructions of Deletion Mutants—To construct the tagged hGtx open reading frame (ORF) cDNA, we digested the DT236 by EcoRI and XbaI; the EcoRI site is 39 nucleotides upstream of the ATG start codon, and the XbaI site is in the untranslated upstream region of hGtx; and subcloned each fragment into pEF4/His. To construct pEF4-hGtxX, we first performed PCR (using Phu turbo-polymerase from Stratagene, La Jolla, CA) with the following primers: the sense primer for pEF4-hGtxH was 5’-TTTTCTAGATGGGAG-3’ and the antisense primer for pEF4-hGtxH was 5’-TTTTTTGTGTGGTGGAATTCCGGAT-3’. The antisense primer for pEF4-hGtxH was 5’-TTTTTTGATTTCCAAGAGACCGTCGGCAGCCCATCTC-3’, and the sense primer for pEF4-hGtxX was the same as the antisense primer for hGtxX. Each amplified fragment was subcloned into pEF4/His at the EcoR site and the XbaI site. The sequences of deletion mutants were confirmed, and expression of each mutant was detected by anti-(His)6 antibody (Sigma, St. Louis, MO). In brief, cells were suspended by pipetting gently, and 50 µl of 0.4% trypan blue solution (Sigma) was mixed with 200 µl of cell suspension (final trypan blue concentration was 0.08%) at room temperature.

Stained cells were counted within 3 min after being mixed with the trypan blue solution. The mortality of cells was then determined as a percentage of trypan blue-stained cells in total cells. The cell mortality thus assessed represents the population of dead cells in total cells, including both adhesive and floating cells, at the termination of experiments. Cell viability assay (Cell Counting Kit-8, Wako Pure Chemical Industries, Osaka, Japan) was performed using WST-8 (2-(2-methoxy-4-(3-[4-(2-hydroxyethyl)-1-piperazinyl]ethyl)amino)-2-nitrophenyl)-5-(2,4-disulfophenyl)-3H-tetrazolium, monosodium salt), as described previously (25). It has been demonstrated that in the F11 systems, the cell mortality assessed by trypan blue exclusion assay correlates excellently with the cell viability assessed by WST-8 assay (25). Aβ3 was purchased from Oncogene Science (Cambridge, MA), and anti-IOF-I-specific neutralizing antibody was from R & D Systems (Minneapolis, MN).

Antibody and Immunoblot Analysis—The immunoblot analysis (20 µg lane) of AβPP, PS1, PS2, and SOD1 was performed as described previously (13 15,20–22), using anti-AβPP antibody 22C11 (2.5 µg/ml; Roche Diagnostics), anti-PS1 N-terminal fragment antibody (1/2000 dilution; Chemicon, Temecula, CA), anti-PS2 C-terminal fragment antibody 2192 (1/500 dilution; Cell Signaling Technology, Beverly, MA), or anti-SOD1 antibody (2 µg/ml; MBL, Nagoya, Japan). For the blotting in Fig. 4B, anti-PS2 N-terminal fragment antibody (1/500 dilution), kindly provided by Drs. Taisuke Tomita and Takeshi Iwatsubo (University of Tokyo, Tokyo, Japan), was used.

Statistical Analysis—All experiments described in this study were repeated at least three times with independent transfections. Statistical analysis was performed with one-way analysis of variance followed by post hoc test, in which p < 0.05 was assessed as significant.

RESULTS

Disease-based Death Trap Screening—F11/EcR/V642I cells, in which V642I-AβPP was inducibly overexpressed by EcD treatment, were described previously (18). By using these cells, in which death occurred in a vast majority of EcD-treated cells within a few days (18), we performed the death trap screening with a unique modification, termed disease-based death trap screening. In the original screening by Vito et al. (26), a normal tissue cDNA library was transfected to Jurkat cells, which were killed by T cell receptor antibody; library fragments were then recovered from surviving cells. In contrast, our approach was unique in that we employed an expression cDNA library constructed from an occipital lobe of an autopsy-diagnosed AD patient brain, as we reasoned that neuroprotective genes must be expressed in an occipital lobe of the AD brain. We first transfected F11/EcR/V642I cells with AD-1 library (human AD occipital cDNA library in pEF-BOS), treated cells with EcD for 72 h, and recovered the plasmids from surviving cells. This procedure was repeated for three rounds, and we randomly picked 250 clones from the recovered plasmids. We classified all 250 clones into 36 independent groups by dot blot hybridization. One such gene was DT236.

By using a simple transient transfection system with F11 neurohybrid cells (9), we confirmed the antagonistic activity of DT236 against neuronal cell death by V642I-AβPP (Table 1). When F11 cells were transfected with V642I-AβPP cDNA in pcDNA, 50–60% of cells underwent death by 72 h. As the transfection efficiency was 65–70% under the employed conditions (13), it followed that most cells expressing this FAD gene were dead 72 h after the onset of transfection. When F11 cells were co-transfected with DT236, cell death by V642I-AβPP was significantly suppressed. This result suggests that DT236 does inhibit neuronal cell death by V642I-AβPP.

The DT236 cDNA sequence had an ORF encoding a polypeptide consisting of 277 amino acids. This ORF sequence turned out to be identical to human GTX (GenBank™ accession number AAK13251) and showed an extreme homology to murine Gtx (mGtx, GenBank™ accession number L08074). Comparing human and murine GTX genes, the homology was 88.6% in the nucleotide level and 97.5% in the amino acid level. The hGtx homoeomain exhibited high similarity to that of Nkx6.1 (27), indicating that Nkx6.1 is a close relative of Gtx (Fig. 1). Gen
After F11 cells were transfected with or without wtAPP, V642I-APP, NL-APP, M146L-PS1, or N141I-PS2 cDNA (in pcDNA) with pEF-BOS or DT236 (in pEF-BOS), cell mortality was measured by trypan blue exclusion assay.

|          | none          | pcDNA         | wtAPP         | V642I-APP     | NL-APP       | M146L-PS1    | N141I-PS2    |
|----------|---------------|---------------|---------------|---------------|--------------|--------------|--------------|
| none     | 7.4 ± 1.1%    | N.D.          | N.D.          | N.D.          | N.D.         | N.D.         | N.D.         |
| pEF-BOS  | N.D.          | 18.5 ± 1.0%   | 29.8 ± 1.3%*  | 48.9 ± 0.5%   | 51.5 ± 1.5%  | 49.1 ± 0.4%  | 56.3 ± 1.9%  |
| DT236    | N.D.          | N.D.          | N.D.          | 35.9 ± 1.2%*  | 45.6 ± 4.5%  | 46.6 ± 2.0%  | 52.9 ± 1.5%  |

* Co-transfection result with pEF4/His vector instead of pEF-BOS.

** p < 0.01 versus cytotoxicity by V6421-APP with pEF-BOS.

**Fig. 1. Schematic illustration of DT236 and its homeodomain.** The upper illustration indicates the schematic structure of DT236 cDNA. The lower sequences indicate similarities of the hGtx homeodomain to other homeodomains. The residue identical to that of hGtx is indicated by a dash. The percent identity with hGtx is given at the right. The consensus (CONS.) is derived from known homeoboxes (30). Symbols at the top stand for the putative interaction sites with DNA bases (closed circles) and with the DNA sugar phosphate backbone (open circles), based on the crystal structure of the DNA homeodomain complex of En and MATa2 (31, 32). This information suggests that hGtx functions as a nuclear transcription factor, not only as a homeobox protein but also as other types of DNA-binding proteins.

**hGtx Inhibits Neuronal Cell Death by Antibody**—To confirm that the cytoprotective effect of hGtx was not due to hGtx-mediated inhibition of V6421-APP expression, we examined whether hGtx can also inhibit F11 neurohybrid cell death caused by extracellularly added Aβ1–43. Aβ is the major constituent of senile plaques, extracellular deposits characteristic for AD, and has been implicated in the pathogenesis of AD. Whereas F11 cells per se were not killed by Aβ1–43, as reported previously (9, 19), cell death was induced by Aβ1–43 when F11 cells were transfected with the p75 neurotrophin receptor p75NTR (Table II). This is in agreement with the notion that p75NTR mediates Aβ neurotoxicity, as has been shown by multiple studies (33–35). When p75NTR-transfected F11 cells were co-transfected with hGtx, cell death by 25 μM Aβ1–43 was drastically suppressed (Table II). Expression of p75NTR was not inhibited by co-transfection of hGtx (data not shown). hGtx could thus inhibit Aβ neurotoxicity in F11 cells, suggesting that neuroprotection by hGtx would not be due to the inhibition of FAD gene expression.

**hGtx Inhibits Neuronal Cell Death by Low Expression of NL-αβPP**—With the 1.5-μg protocol used here, 0.75 μg of FAD gene (in pcDNA) with 0.75 μg of pEF4-hGtx (in pEF4/His) was transfected to F11 neurohybrid cells, and cell mortality was measured 48 h after transfection. In the pIND system, 1 μg of FAD gene (in pIND) with 1 μg of pEF4-hGtx (in pEF4/His) was transfected to F11/EcR cells; cells were then treated with EcD;
and cell mortality was measured 72 h after the onset of transfection. The neurotoxicity by V642I-AβPP and NL-AβPP in pcDNA transfected by the 1.5-μg protocol was quantitatively comparable with neurotoxicity by these genes in pIND transfected by the pIND protocol (1 μg of pIND plasmid transfection and 40 μM EcD treatment) in the pIND system, as shown in multiple studies (13–15, 20–22, 24). In this pIND system, high induction of NL-AβPP causes DEVD-resistant non-apoptotic
cell death different from DEVDS-sensitive apoptotic cell death by its low induction, whereas low to high induction of V642I-AβPP solely causes DEVDS-sensitive cell death (13). Given that hGtx inhibited DEVDS-sensitive cell death by V642I-AβPP, it was possible that hGtx could also inhibit neuronal cell death by low expression of NL-AβPP.

We thus examined the effect of hGtx on neuronal cell death by low expression of NL-AβPP using the pIND system, in which cell death by low and high induction is best analyzed. In this system, F11/EcR cells were transfected with NL-AβPP cDNA in EcD-inducible pIND plasmid with or without co-transfection of pEF4-hGtx and treated with various concentrations of EcD. Hashimoto et al. (13–15) demonstrated the following: (i) enhanced green fluorescent protein cDNAs in pIND express the enhanced green fluorescent protein in a manner proportional to EcD doses; (ii) wild-type (wt)AβPP is expressed in a relationship linear to treated EcD doses; and (iii) V642I-AβPP or NL-AβPP in the presence of DEVDS or glutathione-ethyl-ester (GEE) is expressed in almost the same linear EcD dependence as that observed for wtAβPP. In F11/EcR cells transfected with pIND-wtAβPP cDNA, 10 and 40 μM EcD caused 1.5- and 6-fold expression of transfected wtAβPP relative to the expression of endogenous AβPP (total amounts of AβPP were 2.5- and 7-fold that of endogenous AβPP, respectively), as described previously (13). Expression of V642I-AβPP or NL-AβPP in the absence of DEVDS reached saturation at 10 μM EcD, and its expression at >10 μM EcD stayed at the level of expression observed at 10 μM EcD. This feature of the expression profile is due to the feedback loop comprised of mutant AβPP-induced activation of DEVDS caspases and the DEVDS-caspase-induced proteolysis of the expressed AβPP holoprotein (36), as discussed in detail previously (13). The vehicle of EcD was ethanol, which provided no induction of AβPP proteins. Hence, the observed effects of treated EcD were attributed to the expressed AβPP proteins.

In this pIND system, NL-AβPP neurotoxicity elicited by 10 μM EcD was almost fully suppressed by co-expression of hGtx (Fig. 2B). In contrast, NL-AβPP neurotoxicity elicited by 40 μM EcD was not affected by hGtx. The curve of NL-AβPP neurotoxicity in the presence of 100 μM Z-VAL-fmk virtually overlapped its curve in the presence of co-expression of hGtx (Fig. 2B). Co-expression of hGtx did not inhibit expression of NL-AβPP encoded in pIND (Fig. 2B, inset). As noted above and reported previously (13), final expression of NL-AβPP reached saturation at 10 μM EcD, and its expression at >10 μM EcD stayed at the level of expression observed at 10 μM EcD. In contrast, expression of NL-AβPP almost linearly increased in the presence of co-expressed hGtx. This is consistent with the previous report (13) that expression of NL-AβPP linearly increases when intracellular caspase activity is suppressed. These data indicate that hGtx exerts an effect similar to that of Z-VAL-fmk and inhibits apoptotic component of NL-AβPP neurotoxicity.

We also examined whether and how hGtx inhibits V642I-AβPP neurotoxicity by low (10 μM) or higher (40 μM) EcD induction (Fig. 2C). As expected from pCDNA data, co-expression of hGtx inhibited V642I-AβPP neurotoxicity elicited by both 10 and 40 μM EcD (Fig. 2C). The curve of V642I-AβPP neurotoxicity in the presence of co-expression of hGtx became virtually identical to the V642I-AβPP neurotoxicity curve in the presence of 100 μM Z-VAL-fmk. As was the case with expression of NL-AβPP, expression of V642I-AβPP was not attenuated by co-expression of hGtx in the pIND system (Fig. 2C, inset). Although hGtx appeared to exert a slight but significant negative effect on the expression of the constructs in pcDNA, a plasmid driven by a CMV promoter, hGtx, had no inhibitory effect on the expression of the pIND constructs driven by the heat shock protein (HSP) promoter. It is therefore reasonable to assume that EcR expression potentially inhibited by expressed hGtx (EcR of pVgKRX is driven by the CMV promoter) was sufficient to mediate the EcD effect on the expression of the pIND constructs. These data indicate, again, that hGtx suppresses caspase inhibitor-sensitive apoptotic neuronal cell death by V642I-AβPP, which concurs with the notion that hGtx effectively suppresses the apoptotic component of neurotoxicity by FAD mutants of AβPP.

hGtx Inhibits Cell Death by L648P-AβPP and Low Expression of A617G-AβPP—A617G-AβPP is known as a Flemish mutant of FAD (37) and L648P-AβPP as an Australian mutant (38), both of which have been shown to cause neuronal cell death in vitro (22, 38). By using the 1.5-μg protocol allowing for overexpression of FAD genes, we examined the effect of hGtx on neuronal cell death by A617G-AβPP and by L648P-AβPP. Fig. 3A shows that overexpression of either FAD mutant caused neuronal cell death as efficaciously as V642I-AβPP and NL-AβPP. hGtx inhibited neurotoxicity by L648P-AβPP to the level of wtAβPP-induced toxicity or to the level of V642I-AβPP-induced toxicity in the presence of hGtx co-expression (Fig. 3A). In contrast, hGtx did not at all inhibit neuronal cell death by overexpressed A617G-AβPP. Under the same condition, hGtx did not critically affect expression of A617G- or L648P-AβPP (Fig. 3A, inset). These data suggest that hGtx inhibits neurotoxicity by overexpressed L648P-AβPP, but not by overexpressed A617G-AβPP and, that these two FAD mutants cause cell death through different mechanisms, as is the case with V642I-AβPP and NL-AβPP (13).

It should also be noted that in the pIND system, low induction of NL-AβPP caused DEVDS/hGtx-sensitive neuronal cell death, whereas high induction of NL-AβPP additionally caused DEVDS/hGtx-resistant cell death (Fig. 2B). We thus reasoned that there is a possibility that neuronal cell death by low expression of A617G-AβPP and L648P-AβPP is inhibited by hGtx. We examined this possibility by using the 0.5-μg protocol, which allows for low expression of the FAD genes. Transfection of F11 cells with wtAβPP cDNA in pcDNA by this protocol resulted in 1–1.5 times higher expression of wtAβPP than expression of endogenous AβPP (Fig. 3A, inset; the total AβPP expression resulted in 2–2.5 times higher than that of endogenous AβPP), which was equivalent to the expression of wtAβPP in F11/EcR cells transfected with wtAβPP cDNA (in pIND) by the pIND protocol and treated with 10 μM EcD for 72 h. Both A617G-AβPP and L648P-AβPP, transfected by the
Fig. 3. Effect of pEF4-hGtx on neuronal cytotoxicity by A617G- or L648P-AβPP. A, effect of pEF4-hGtx on neuronal cytotoxicity by A617G-AβPP or L648P-AβPP by the 1.5-μg protocol. F11 cells were transfected with empty pcDNA (pcDNA) or each of pcDNA-encoded V642I-AβPP, A617G-AβPP, or L648P-AβPP with pEF4-hGtx (hGtx) or empty pEF4/His (pEF). Cell mortality was measured 48 h after transfection. *, significantly different. n.s., no significant difference. Inset, effect of pEF4-hGtx on expression of A617G-AβPP or L648P-AβPP. Using the same
Fig. 4. Effect of pEF4-hGtx on neuronal cell death by M146L-PS1 or N141I-PS2. A and B, effects of pEF4-hGtx on F11/EcR cell death by EcD-induced expression of M146L-PS1 (A) or N141I-PS2 (B) in the pIND system. F11/EcR cells were transfected with pIND-encoded M146L-PS1 or N141I-PS2 with pEF4-hGtx or empty pEF4/His vector. Nearly 24 h after transfection, cells were treated with or without increasing concentrations of EcD. EcD treatment did not affect transfection efficiency. Cell mortality was measured by trypan blue exclusion 72 h after EcD treatment. The negative controls of cell mortality without transfection or with empty pIND plus empty pEF4/His vector were similar to those in Fig 2. Insets in A and B, effect of pEF4-hGtx on expression of M146L-PS1 (A) or N141I PS2 (B) in the pIND system. F11/EcR cells were transfected with pIND-encoded M146L-PS1 or N141I-PS2 with pEF4-hGtx (hGtx) or empty pEF4/His (−). Cells were then treated without or with increasing concentrations of EcD. Cellular expression of PS proteins was measured by immunoblot analysis 72 h after transfection. The upper and lower bands in each lane indicate the expressed holoproteins of PS mutants and actin, respectively.

Effects of hGtx on Neurotoxicity by M146L-PS1 and N141I-PS2—We next examined whether hGtx inhibits neuronal cell death by low and high induction of M146L-PS1 or N141I-PS2 in the pIND system. Both FAD mutants cause or enhance neuronal cell death in various cell systems, including F11 neurohybrid cells (10–12, 14, 15, 20, 39–43). As shown in Fig. 4, A and B, co-expression of hGtx had no inhibitory effect on neuronal cell death by low to higher induction of M146L-PS1. In contrast, co-expression of hGtx, but not with empty pEF4/His, drastically inhibited neuronal cell death by low induction of N141I-PS2. Without effect on neuronal cell death by higher induction of N141I-PS2. No effect of hGtx on neurotoxicity by high induction of M146L-PS1 or N141I-PS2 is consistent with the F11 cell transfection data using the 1.5-μg protocol (data not shown). These results concur with the notion that hGtx selectively inhibits DEVD-sensitive apoptotic neurotoxicity by mutant PS1/2, because (i) low induction of N141I-PS2 (achieved by the pIND protocol transfection and 10 μM EcD treatment) induces DEVD-sensitive apoptotic neurotoxicity; (ii) high induction (achieved by the pIND protocol transfection and 40 μM EcD treatment) of N141I-PS2 additionally causes DEVD-resistant non-apoptotic neurotoxicity; and (iii) low to high induction of M146L-PS1 solely causes DEVD-resistant non-apoptotic neurotoxicity (14, 15).

Neuroprotective Function Is Located in the Homeodomain of hGtx—To specify the region of hGtx responsible for the observed cytoprotective action, we constructed multiple deletion mutants of hGtx (hGtxNT, hGtxCT, hGtxΔP, and hGtxH) (Fig. 5A). hGtxNT consists of the N-terminal domain (residues 1–35) and the Gly-rich domain (residues 36–94); hGtxCT consists of the Pro-rich domain (residue 95–147), the homeodomain (residues 148–207), and the Polar (amino acid)-rich domain (residues 208–277); and hGtxΔP, consisting of residues 1–207, is the hGtx only lacking the polar-rich domain.

Again using the 1.5-μg protocol, we transfected F11 neurohybrid cells with each of these deletion mutants with V642I-AβPP cDNA (in pcDNA), and we assessed whether cell death was inhibited. Both hGtxCT and hGtxΔP inhibited neuronal cell death by V642I-AβPP as significantly as full-length hGtx. In contrast, hGtxNT did not inhibit it at all. hGtxCT, hGtxΔP, and hGtxNT were expressed similarly to hGtx (Fig. 5B, upper panel). Hence, the homeodomain and/or the Pro-rich domain would be responsible for the neuroprotective action of hGtx. We therefore examined the effect of hGtxH, the homeodomain (residues 148–207) of hGtx. hGtxH inhibited neuronal cell death by V642I-AβPP and by L648P-AβPP as significantly as full-length hGtx did (Fig. 5D). Expression of these FAD mutant

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1.5-μg protocol, F11 cells were transfected with either A617G-AβPP or L648P-AβPP (each in pcDNA) with pEF4-hGtx (hGtx) or empty pEF4/His (pEF4/His). Cellular expression of AβPP was measured by immunoblot analysis 48 h after transfection. An arrowhead indicates the holoproteins of expressed AβPPs. B, effect of pEF4-hGtx on neuronal cytotoxicity by V642I-AβPP, L648P-AβPP, NL-AβPP, or A617G-AβPP by the 0.5-μg protocol. F11 cells were transfected with pEF4-hGtx (hGtx) or empty pEF4/His (pEF4/His). At 72 h after transfection, cell mortality and viability were measured by trypan blue exclusion (left panels) and WST-8 assay (right panels), respectively. As negative controls, transfection with pcDNA alone (vec) or wtAβPP cDNA in pcDNA (wtAβPP) was performed in parallel. *, significantly different. n.s., no significant difference.
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domain would be common for other homeodomain-containing proteins, that of hGtx, the neuroprotective function of the hGtx homeodomain being against FAD mutant A/PP. By using the same 1.5-μg protocol, F11 cells were transfected with either V642I-A/PP or L648P-A/PP. The domains of hGtx are denoted as follows: N-terminal domain (Met1–Pro95), glycine-rich domain (Gly96–Gly306), proline-rich domain (Pro307–Lys145), homeodomain (Lys146–His207), and polar-rich domain (Ala208–Gln35), glycine-rich domain (Gly36–Gly94), proline-rich domain (Pro95–Lys145), homeodomain (Lys146–His207), and polar-rich domain (Ala208–Leu247).

B, upper panel, immunoblot analysis of expressed hGtx and its deletion mutants. Lysates of F11 cells transfected with hGtx or its deletion mutants were submitted to SDS-PAGE and immunoblot analysis with anti-(His)_6 antibody (lane 1, no transfection; lane 2, pEF vector transfection; lane 3, pEF-hGtx; lane 4, pEF hGtxNT; lane 5, pEF-hGtxCT; lane 6, pEF-hGtxΔP; lane 7, pEF-hGtxH; lane 8, DLX4). Representative results are indicated. Lower panel, effect of pEF4-hGtx on expression of V642I-A/PP or L648P-A/PP. By using the same 1.5-μg protocol, F11 cells were transfected with either V642I-A/PP or L648P-A/PP (each in pcDNA) with empty pEF4/His, pEF4-hGtx, hGtx mutant, or DLX4. Cellular expression of A/PP was measured by immunoblot analysis 48 h after transfection. C, effects of deletion mutants of hGtx on neuronal cytotoxicity by V642I-A/PP and L648P-A/PP. F11 cells were transfected with empty vector (pcDNA) or pcDNA-encoded V642I-A/PP or L648P-A/PP with pEF4/His vector (pEF) or either hGtx construct (pEF4-hGtx, pEF-hGtxNT, pEF-hGtxCT, pEF-hGtxΔP) using the 1.5-μg protocol. Cell mortality was measured 48 h after transfection. D, effect of hGtxH on neuronal cytotoxicity by V642I- or L648P-A/PP. Under the same conditions as in C, F11 cells were transfected with empty vector (pcDNA) or pcDNA-encoded V642I- or L648P-A/PP with empty pEF4/His vector (pEF) or either pEF-hGtx or pEF-hGtxH. Cell mortality was similarly measured. E, effect of DLX4 on neuronal cytotoxicity by V642I-A/PP. Under the same conditions as in C, F11 cells were transfected with empty vector (pcDNA) or pcDNA-encoded V642I-A/PP with either empty pEF4/His vector (pEF), pEF4-hGtxH, or DLX4. Cell mortality was similarly measured. C–E. * indicates cytotoxicity significantly lower than that by V642I-A/PP plus pEF4/His; and ** indicates cytotoxicity not significantly different from that by V642I-A/PP plus pEF4/His. ○ indicates cytotoxicity by V642I-A/PP plus pEF4/His, which is significantly augmented relative to the cytotoxicity by pcDNA plus pEF4/His.

AβPPs in cells co-transfected with hGtxCT, hGtxAP, hGtxNT, or hGtxH was not significantly inhibited, as compared with the expression of the AβPP mutants in cells co-transfected with empty pEF4/His (Fig. 5B, lower panel). Therefore, it was most likely that the neuroprotective function of hGtx against FAD mutants of AβPP is commonly located in its homeodomain.

Effect of DLX4 on Neuronal Cell Death by V642I-A/PP—We further tested whether other homeodomain-containing proteins inhibit neuronal cell death by overexpression of V642I-A/PP. For this purpose, we examined the effect of DLX4. Because the homeodomain of DLX4 is only 38.3% homologous to that of hGtx, the neuroprotective function of the hGtx homeodomain would be common for other homeodomain-containing proteins, if DLX4 inhibits neuronal cell death by V642I-A/PP. As shown in Fig. 5E, co-expression of DLX4 inhibited neurotoxicity by V642I-A/PP as significantly as hGtxH and full-length hGtx did. Immunoblot analysis with (His)_6 tag antibody revealed that DLX4 was expressed similarly to hGtxH (Fig. 5B, upper panel). CMV-promoter-driven expression of V642I-A/PP was virtually the same with hGtxH co-transfection and with DLX4 co-transfection (Fig. 5B, lower panel). It was therefore likely that homeodomains would be able to cause neuroprotection against FAD mutant AβPP.

Effect of hGtx Homeodomain on Aβ Neurotoxicity—We examined whether the homeodomain of hGtx suppresses Aβ-induced neurotoxicity. As noted above, F11 neurohybrid cells became sensitive to Aβ neurotoxicity when cells were transfected with p75NTR cDNA (Table II). As shown in Table II, not only hGtxCT but also hGtxH inhibited neuronal cell death by Aβ as strongly as full-length hGtx, whereas hGtxNT could not inhibit Aβ neurotoxicity. hGtx constructs were expressed similarly (data not shown). Expression of p75NTR was not affected by co-transfection of hGtx and its fragments (data not shown). Again, these data confirm that (i) hGtx can suppress Aβ neurotoxicity; and (ii) the homeodomain of hGtx is responsible for the neuroprotective function of hGtx. Table II also indicates that Aβ-induced neurotoxicity was DEVD-sensitive apoptosis, as has been reported by multiple groups (44–47). This finding is in good agreement with the notion that hGtx is effective in inhibiting apoptotic neuronal cell death.

Effect of hGtx on ApoE4-induced Neurotoxicity—To further confirm that neuroprotection by hGtx is not due to inhibition of the FAD gene expression, we examined whether hGtx inhibits F11 neurohybrid cell death caused by extracellularly added apolipoprotein E4 (apoE4). ApoE4 is the established insults relevant to AD and causes apoptotic cell death, when F11 neurohybrid cells are treated with apoE4 (21). As shown in Fig. 6A, apoE4 robustly induced cell death, as assessed with both trypan blue exclusion mortality assay and WST-8 cell viability assay. When cells had been transfected with hGtx, apoE4-induced toxicity was greatly suppressed, as assessed by both assays. Furthermore, hGtx-mediated suppression of apoE4-induced neuronal cell death was reproduced by hGtxH but not by hGtxNT. These Gtx constructs were expressed similarly
These data provide definite support to the notion that hGtx inhibition of AD insult-induced neurotoxicity is due to direct suppression of the relevant toxic pathways.

Mediation by IGF-I of hGtx-induced Neuroprotection—We noticed that hGtx transfection totally suppressed apoE4-induced cell death, despite the fact that the transfection effi-
iciency was ~65–70%. It was therefore reasonable to assume that a soluble rescue factor is secreted from hGtx-transfected cells. We have shown so far that IGF-I inhibits apoptotic neuronal cell death by V642I-AβPP (48) and does not affect DEVD-resistant cell death by NL-AβPP, N141I-PS2, or M141L-PS1 (22). As shown in Fig. 6B, IGF-I inhibited the F11 cell death by apoE4. Under the same conditions, the neuroprotective activity of hGtx was virtually antagonized by the addition of anti-IGF-I-specific antibody. The expression of hGtx did not change in the presence or absence of anti-IGF-I antibody. This spectrum of the effectiveness of IGF-I and its functional specificity against an apoptotic component of cell death resembles such a soluble rescue factor potentially secreted from hGtx-transfected cells.

We thus examined the effect of specific neutralizing anti-IGF-I antibody added to the culture medium. In fact, IGF-I antibody was able to completely inhibit hGtx-mediated suppression of apoE4-induced neuronal apoptosis without down-regulating expression of hGtx (Fig. 6D). In addition, as shown in Fig. 6C, not only anti-IGF-I neutralizing antibody but also αIR3, which is a neutralizing antibody against IGF-I receptor (48), antagonized this hGtx-induced neuroprotective effect. In the case of Humamin (HN), which is an anti-AD peptide (20–22), both neutralizing antibodies had no effect on neuroprotective activity of HN. The neuroprotective activity of DLX4 was also antagonized by both anti-IGF-I antibody and αIR3 (Fig. 6C). Finally, we confirmed that expression of IGF-I was actually induced in F11 cells and secreted into culture medium by transfection of hGtx, hGtxH, and DLX4 but not by transfection of hGtxNT (Fig. 6D). Taken these findings together, we concluded that IGF-I is the actual mediator for hGtx-induced neuroprotection.

To determine whether Gtx and DLX4 can actually enhance the secretion of IGF-I, we performed immunoblotting. As shown in Fig. 6D, hGtx, hGtxH, and DLX4 increased the production and the secretion of IGF-I. From these results, there are some possibilities that homeobox-containing genes can enhance the production of growth factors to modulate development, cell death, and survival.

**DISCUSSION**

We have shown here that hGtx has a novel function that inhibits neuronal cell death by FAD mutants of AβPP. In multiple systems, hGtx significantly inhibited neurotoxicity by Aβ, by low and high expression of V642I- and L648P-AβPP, and by low expression of NL-AβPP, A617G-AβPP, and N141I-PS2. On the contrary, hGtx did not significantly inhibit neuronal cell death by M146L-PS1, by high expression of NL-AβPP, A617G-AβPP, or N141I-PS2. Neurotoxicity by G85R-SOD1, a familial amyotrophic lateral sclerosis-linked mutant SOD1, is not suppressed by Gtx. The former hGtx-suppressive neurotoxicity is sensitive to caspase inhibitors, and the latter hGtx-resistant neurotoxicity is caspase-inhibitor-insensitive, indicating that hGtx specifically inhibits apoptotic death. This study has further specified the neuroprotective region of hGtx to be its homeodomain. As DLX4, which has a homeodomain only 38.3% homologous to the homeodomain of hGtx, has a similar cyto-protective function, it is conceivable that such a function is broadly associated with homeodomains.

The function of hGtx to promote neuronal cell survival is novel but not surprising, because (i) mGtx does not bind to the sequences recognized by Antp-, Eve-, and Prd-type homeobox proteins but binds to the MEF-2-binding sequence (1); and (ii) neuronal activity-dependent cell survival is mediated by MEF-2 (49). Whereas the relevance of this novel function of hGtx is not clear at present, this study does indicate interesting cross-talk among developmentally regulated machinery and AD-causative mechanisms. Some functional linkage has so far been postulated between them, based on the regulatory roles of PS proteins in activation of Notch (50–52) as well as the gene-targeting studies showing the essential roles of PS1 in skeletal and neuronal development (53, 54) and PS2 in pulmonary development (55). Inappropriate expression of developmentally regulated genes in AD has also been reported (56, 57). Hong et al. (58) reported that FAD-associated mutant PS1 plays a role in the differentiation of neuronal precursors. It has also been reported that mouse homeobox gene Hox-3.1 negatively regulates the AβPP mRNA level (59).

In this study, we identified IGF-I to be the mediator for hGtx-mediated neuroprotection. Expression of Gtx up-regulates expression of IGF-I. It has been known that Nkx family transcription factors including Gtx act as transcriptional repressors (1). Therefore, there is a possibility that Gtx down-regulates expression of the unknown genes that are suppressors or silencers for the IGF-I gene, whereas the precise mechanism of Gtx-mediated induction of IGF-I expression remains still unclear at this moment.

As a neuroprotective factor, IGF-I has been already known to antagonize apoptotic neuronal cell death by V642I-AβPP (48). We additionally obtained two important insights regarding biological and therapeutic roles of IGF-I by this study. First, some homeobox-containing transcription factors such as Gtx and DLX4 regulate expression of IGF-I, indicating that IGF-I plays a important role not only in neuronal cell protection but also in embryonic development. Second, as a therapeutic agent for a certain type of AD, IGF-I seems to be more promising than previously because it is considered to be a putative physiological mediator for Gtx-mediated neuroprotection from AβPP-relevant insults.

In summary, this study provides a molecular clue toward understanding neuroprotection against AβPP-relevant insults and suggests a novel linkage between neural development and inhibition of AD mechanisms. This study may open a new horizon in the functions of homeodomain-containing proteins.

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The Gtx Homeodomain Transcription Factor Exerts Neuroprotection Using Its Homeodomain
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