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Identification of soluble WSX-1 not as a dominant-negative but as an alternative functional subunit of a receptor for an anti-Alzheimer's disease rescue factor Humanin

Yuichi Hashimoto, Megumi Kurita, Masaaki Matsuoka *

Department of Pharmacology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan

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ABSTRACT

Humanin (HN) inhibits Alzheimer's disease (AD)-relevant neuronal death and dysfunction, by interacting with a receptor (s) involving ciliary neurotrophic factor receptor α (CNTFR), WSX-1, and gp130. It remains unknown whether this complex is the sole HN receptor that mediates HN-induced anti-AD activity. We here report that an alternatively spliced WSX-1 isoform, encoding an extracellular 270-amino-acid region of WSX-1 with cytokine-binding regions (named soluble WSX-1; sWSX-1), is expressed in neuronal cells lacking function of full-length WSX-1 and enables HN to rescue AD-relevant death. This result suggests that CNTFR/soluble WSX-1/gp130 behaves as an alternative functional HN receptor.

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Introduction

Humanin (HN) was identified as an endogenous neurotrophic factor that inhibits neuronal death relevant to Alzheimer's disease (AD) *in vitro* [1,2]. Multiple studies have shown that potent HN derivatives inhibit memory impairment in mice, induced by intracerebroventricular administration of toxic Aβ peptides, intraperitoneal injection of muscarinic receptor antagonists [3], and transgenic mice overexpressing a familial AD-causative APP mutant [4]. These results have established that HN inhibits neuronal death and dysfunction relevant to AD. In addition, it has also been reported that Humanin exerts its protective activity on non-AD-related cell death, including ischemia-induced neuronal death [5], serum deprivation-induced death of PC12 pheochromocytoma cells [6], and DRPLA polyglutamine peptide-induced death [7].

HN inhibits AD-relevant neuronal cell death by binding to its putative receptor on the cell membrane [1] and by activating STAT3 [8]. We have recently found that HN acts by interacting with a putative heterotrimeric receptor (receptors) belonging to the IL-6 receptor family involving ciliary neurotrophic factor receptor α (CNTFR)/WSX-1/gp130 [9]. Cytokines, such as IL-6, IL-11, leukemia inhibitory factor (LIF), CNTF, oncostatin M, cardiotrophin-1, and IL-27, bind to heterodimeric or heterotrimeric receptors belonging to the "IL-6 receptor family" that contain gp130 as a common subunit and one or two gp130-related subunits. Binding of IL-6 or IL-11 to its receptor ultimately leads to "homodimerization" of the intracellular domains of gp130 while binding of other cytokines to their receptors leads to "heterodimerization" between the intracellular domains of gp130 and a gp130-related β subunit such as the LIF receptor β subunit, the oncostatin M receptor β subunit (OSMR), or IL-27 receptor β subunit WSX-1, given β subunits are defined as gp130-related receptor with intracellular signaling modules [10–12]. The HN receptor appears to belong to the latter-type IL-6 receptor family [9].

In this study, we show that soluble WSX-1, an alternatively spliced isoform of WSX-1 encoding an extracellular part of WSX-1, is expressed in neuronal cells and able to replace full-length WSX-1 for HN-mediated neuroprotection.

Materials and methods

WSX-1 knockout mice. Mice whose exon 8 or exons 3–8 of the WSX-1 gene were disrupted (named WSX-1-ΔE8 or WSX-1-ΔE3–8 mice) were kindly provided by Dr. Chris Saris and Amgen, Inc. (Palo Alto, CA) [13] and Dr. F.J. de Sauvage and Genetech., Inc. (South San Francisco, CA) [14], respectively.

Cloning of soluble WSX-1. A rapid amplification of 3′ cDNA end (3′-RACE) was performed to amplify WSX-1 cDNAs, generated from mRNAs derived from a brain of WSX-1-ΔE8 mouse, with appropriate forward primers (Supplementary materials and methods) using a 3′-RACE kit (Invitrogen).

Cells viability and death assay. The transient transfection and cell viability assay for SH-SY5Y human neuroblastoma cells was performed, as described [1,9]. At 48 h after transfection, cells were stained with 6 μM calcein AM and harvested for cell viability assays with Cell Counting kit-8 (Dojindo, Kumamoto) [9].

* Corresponding author. Fax: +81 3 3352 0316.
E-mail address: sakimatu@tokyo-med.ac.jp (M. Matsuoka).

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Peptides and recombinant cytokines. Synthetic HN and S14G-HN (HNG) peptides were from Peptide Institute, Inc. (Minoh, Osaka, Japan). Rat IL-6 and recombinant C-terminally 6×His-tagged human IL-27p28 (Phe^{29}-Pro^{243}) N-terminally fused to human EBI3 (recombinant human IL-27) were purchased from R&D Systems (Minneapolis, MN). Rat CNTF was from Pepro Tech EC Ltd. (London, UK).

Antibodies. Antibody to phoshoSTAT3 (Tyr^{705}) was purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to STAT3 (C-20) and mouse APP (22C11) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Chemicon. Two rabbit polyclonal antibodies to the N-terminal 16-amino acid region of human and mouse WSX-1, affinity-purified with an immunizing peptide, was described [9].

Detection of Tyr^{705}-phosphorylated STAT3. Primary cortical neurons (PCNs), obtained from day 14 embryos (E14) of ICR mice (control), WSX-1-ΔE8, and WSX-1-ΔE3–8 mice, were seeded in poly-L-lysine-coated 6-well plates (Sumitomo Bakelite) at 1.0×10^6 cells/well in Neuron Medium (Sumitomo Bakelite) [1]. Purity of PCNs was estimated to be more than 95% [1]. On the third day after PCN preparation (DIV3), the culture medium was replaced with DMEM with N2 supplement. At 48 h after the replacement of the media, the cells were treated with 100 ng/ml human CNTF, 1 μM human IL-27, or 100 nM HNG for 15 min in the presence of absence of neutralizing antibody to WSX-1 or control serum at 37 °C. Immunoblot analysis was performed as described.

RT-PCR analysis. RT-PCR was performed with mRNA purified from PCNs. The first cDNAs were synthesized with "Sensiscript" reverse transcriptase using oligo dT as a primer. PCR amplification of cDNAs was performed as described in Supplementary materials and methods.

Statistical analysis. Statistical analysis was performed with one-way factorial ANOVA followed by Fischer's PLSD.

Genes and vectors. pcDNA3-V642I-APP was previously described [1,9]. C-terminally FLAG-tagged full-length mouse WSX-1 and sWSX-1 were generated by inserting cDNA encoding these proteins into the pFLAG vector (Eastman Kodak, New Haven, CT, USA).

Results

STAT3 is phosphorylated by HNG and IL-27 in primary cortical neurons (PCNs) lacking the exon 8 of the WSX-1 gene

HN activates STAT3 [5] by binding to the HN receptor involving CNTFR/WSX-1/gp130 [9]. Because WSX-1 is essential for HN activity, homozygous disruption of the WSX-1 gene was thought to cancel the HN-induced phosphorylation of STAT3. To test this, we used homozygous mice with the WSX-1 gene whose exon 8 was replaced by a neomycin-resistant gene (named WSX-1-ΔE8) [13]. Unexpectedly, however, co-incubation with HNG, a 1000-fold potent HN derivative, induced the phosphorylation of STAT3 in PCNs derived from knockout mice as well as wild-type PCNs (Fig. 1). Treatment with IL-27, whose receptor is thought to consist of WSX-1/gp130 [12], similarly induced the phosphorylation of STAT3 in WSX-1-ΔE8 PCNs as well as wild-type PCNs (Fig. 1). In contrast, as already shown in an earlier study [9], an antibody to the N-terminal sixteen-amino-acid sequence of mouse WSX-1 nullified the HNG- and IL-27-induced phosphorylation of STAT3 in these cells (Fig. 1). These results indicate that HN- as well as IL-27-induced activation of STAT3 is not nullified by the disruption of exon 8 of the WSX-1 gene.

Identification of soluble WSX-1

By performing 3′-RACE with several forward primers from exon 1, we have isolated cDNA encoding a WSX-1 isoform whose 5′ part of the exon 6 (73 bases) is directly spliced to a newly identified exon named E7′, which is located between the exon 6 and the authentic exon 7 in the genome (Fig. 2A). There is a stop codon in frame in the middle of E7′ and a poly (A) stretch at the end of the cDNA (Fig. 2B). The newly isolated WSX-1 cDNA encodes a 290 amino-acid protein of the extracellular domain of WSX-1 with cytokine-binding regions and a WSxWS (sWSX-1) (Fig. 2B). Transfection of a C-terminally FLAG-tagged sWSX-1-encoding vector into COS7 cells resulted in an expression of FLAG-sWSX-1 with a molecular weight of 33 kDa (Fig. 2C). Note that sWSX-1 is secreted into the conditioned media (Fig. 2C, right panel). RT-PCR analysis confirmed that only sWSX-1 was expressed in WSX-1-ΔE8 PCNs while both sWSX-1 and WSX-1 are expressed in wild-type PCNs (Fig. 2D).

Soluble WSX-1 acts as a functional subunit of an alternative HN receptor

Because HN binding to CNTFR/sWSX-1/gp130 triggered the phosphorylation of STAT3, it is likely that sWSX-1 does not behave as an isoform of WSX-1 with dominant-negative or null effect on the HN receptor but as an alternative functional HN receptor subunit. Overexpression of V642I-APP, the London-type familial AD-causative gene, induced the death of SH-SY5Y human neuroblastoma cells and co-incubation with 10 μM HN inhibited V642I-APP-induced death (Fig. 3). Such HN rescue activity was completely inhibited by co-incubation with an antibody to the N-terminal 16-amino-acid sequence of human WSX-1 (Fig. 3) [9]. However, enforced expression of mouse sWSX-1 or full-length WSX-1, which is not recognized by the antibody (data not shown), restored HN-induced neuroprotection in SH-SY5Y cells treated

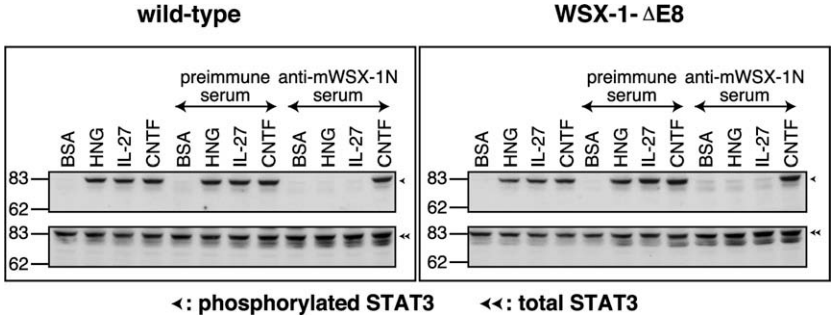

Fig. 1. Disruption of the exon 8 of the WSX-1 gene does not nullify HN- and IL-27-indued phosphorylation of STAT3. PCNs were preincubated in DMEM plus N2 supplement for 48 h (DIV3–5). After co-incubated with1 μM HNG, 1 μM human IL-27, 100 ng/ml rat CNTF, or 100 ng/ml BSA in the presence or absence of 1 μl of preimmune serum or an antibody to the N-terminal 16-amino-acid region of mouse WSX-1 (anti-mWSX-1 N) for 15 min at 37 °C in the same media, they were harvested for immunoblot analysis with the antibodies to phosphoSTAT3 and STAT3.

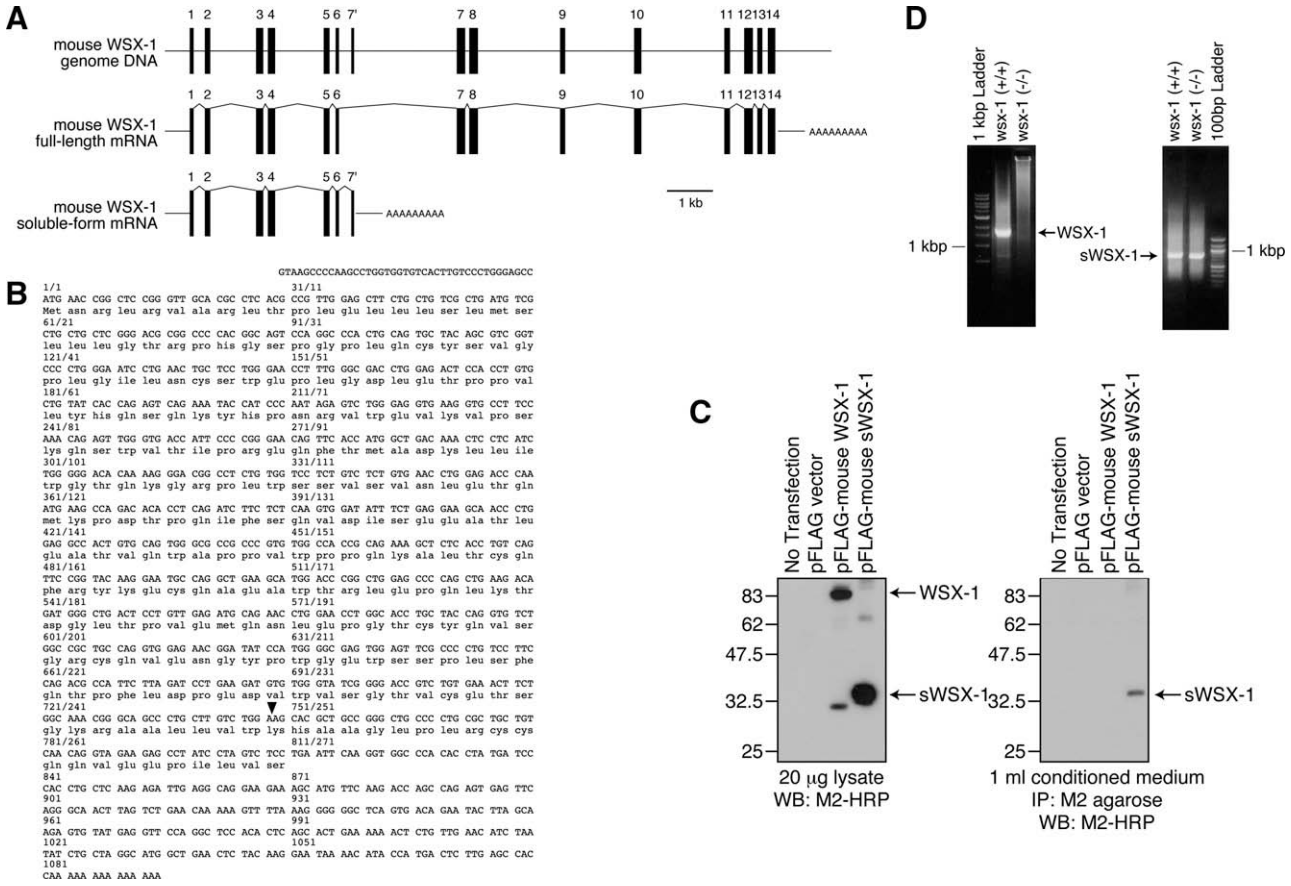

Fig. 2. Soluble WSX-1 is expressed in PCNs whose WSX-1-E8 is disrupted. (A) The structure of the mouse WSX-1 gene and two mRNAs, transcribed from the WSX-1 gene, are shown. (B) The sWSX-1 cDNA and the encoded amino acids. A triangle indicates the junction between the exon 6 and the exon 7′. (C) COS7 cells, transfected with vectors encoding C-terminally FLAG-tagged WSX-1 and sWSX-1, were harvested for immunoprecipitation with antibody to FLAG (M2) and immunoblot analysis with the same antibody. Culture supernatants were similarly analyzed (right panel). (D) RT-PCR analysis was performed with mRNA purified from wild-type WSX-1 and WSX-1-ΔE8 PCNs to detect expression of WSX-1 and sWSX-1.

with the antibody (Fig. 3). This result indicates that sWSX-1 is able to replace full-length WSX-1 as a functional subunit of the HN receptor. An immunofluorescence-based binding study indicated that the binding of HN to F11 cells was upregulated when sWSX-1 was overexpressed in F11 cells in a fashion similar to when full-length WSX-1 was overexpressed (Supplementary Fig. 1). The effective dose of HN for CNTFR/sWSX-1/gp130 is approximately equal to that for CNTFR/WSX-1/gp130 (data not shown).

Disruption of exons 3–8 of the WSX-1 gene nullifies HN-induced phosphorylation of STAT3

Using PCNs derived from homozygous knockout mice whose exons 3–8 of the WSX-1 gene were replaced with the neomycin-resistant gene, established by Chen et al. [14], we examined whether HN activated STAT3 in the absence of both WSX-1 and sWSX-1 expression. RT-PCR analysis confirmed that neither WSX-1 nor sWSX-1 was expressed in PCNs derived from the mice (Fig. 4A). Immunoblot analysis indicated that neither IL-27 nor HNG treatment induced the phosphorylation of STAT3 in these PCNs (Fig. 4B).

Discussion

Co-incubation with neutralizing antibody to the extracellular domain of CNTFR, WSX-1, or gp130 results in the disappearance of cellular responsiveness to HN [9]. siRNA-mediated knockdown of endogenous CNTFR or WSX-1, or co-incubation with a dominant-negative gp130 also nullifies cellular responsiveness to HN [9]. Because the WSX-1 antibody recognizes sWSX-1 as well as full-length WSX-1 and the WSX-1 siRNA reduces sWSX-1 as well as full-length WSX-1 expression simultaneously (data not shown), it is concluded that WSX-1 or sWSX-1 as well as CNTFR and gp130 are essential for HN activity, mediated by the activation of STAT3 [8]. As shown in Fig. 1, HN treatment induced the phosphorylation of STAT3 in WSX-1-ΔE8 PCNs where sWSX-1, but not full-length WSX-1, is expressed (Fig. 2). HN treatment inhibits V642I-APP-induced death in SH-SY5Y cells in which only mouse sWSX-1 is intact (Fig. 3). Taken altogether, it is concluded that sWSX-1 is able to replace full-length WSX-1 as a functional subunit of an alternative HN receptor, at least when mouse sWSX-1 is overexpressed.

Only gp130 has the intracellular signaling module in the alternative HN receptor consisting of CNTFR/sWSX-1/gp130. Because homodimerization is essential for gp130 to initiate the intracellular signal [10,11], HN-induced homodimerization of the intracellular domains of gp130 is speculated to trigger intracellular prosurvival signals. In support, our previous study showed that the co-incubation with IL-6 or IL-11 in association with soluble IL-6 receptor or soluble IL-11 receptor, respectively, but not the co-incubation with IL-6 or IL-11 only, mimicked co-incubation with HN in PCNs [15]. Similarly, G-CSF-induced homodimerization of the intracellular domains of gp130 fused to the extracellular domain of the G-CSF receptor that had been overexpressed by transfection, resulted in neuroprotection against AD-related toxicity in F11 neurohybrid cells (Supplementary Fig. 2). Together, these results have indicated that the upregulated homodimerization of

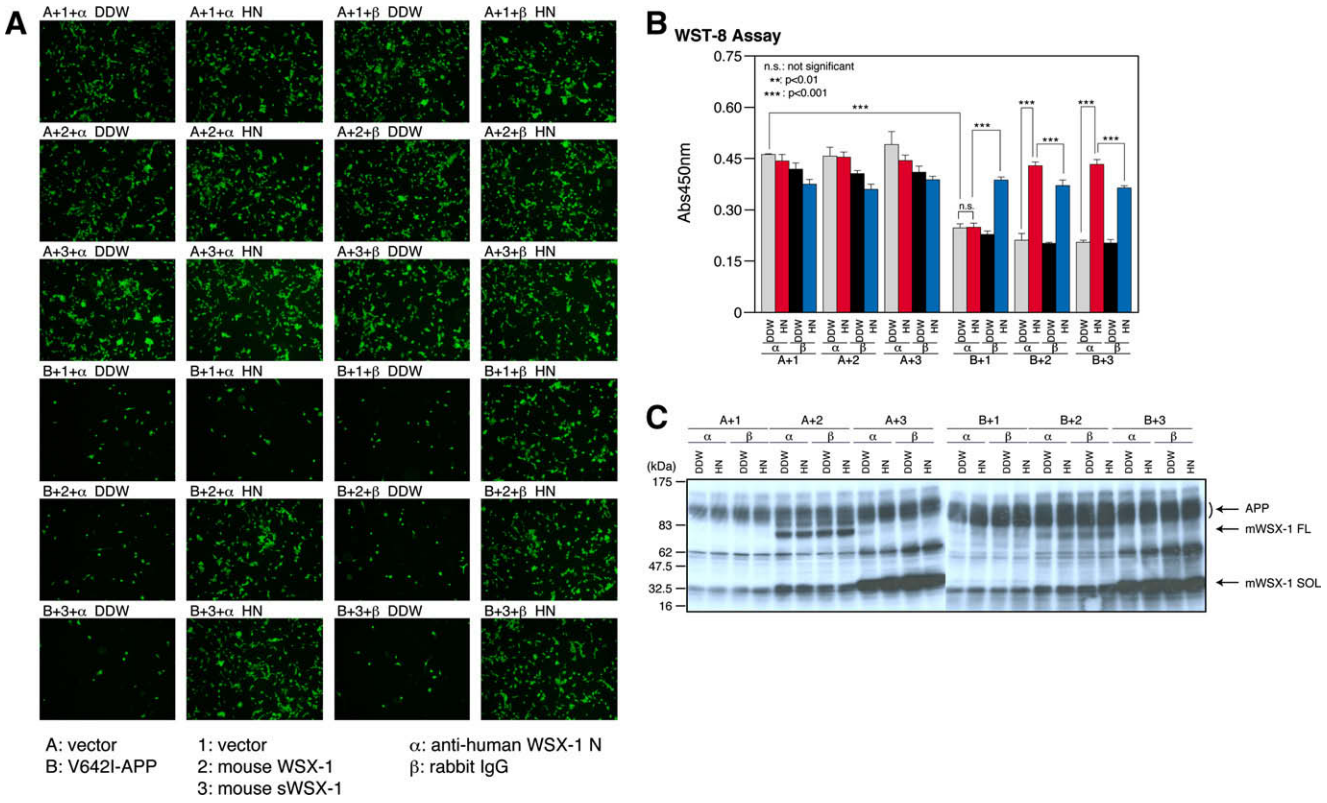

Fig. 3. Soluble WSX-1 acts as a functional subunit of an alternative HN receptor. SH-SY5Y cells, transfected with 0.5 μg of the pcDNA3 vector or pcDNA3-V642I-APP in association with 0.5 μg of the empty vector, a mouse WSX-1-FLAG-encoding vector, or a mouse sWSX-1-FLAG-encoding vector for 3 h in the absence of serum. They were then incubated in DMEM-10% FBS for 21 h. Media were replaced by DMEM containing N2 supplement with/without 10 μM HN in association with 2.5 μg of the affinity-purified antibody to human WSX-1 or control rabbit IgG (Santa Cruz Biotech.) at 24 h after the onset of transfection. After incubated in the same media for additional 48 h, they were stained with calcein AM (A) and harvested for WST-8 cell viability assay (B) and immunoblot analysis with antibody to APP (22C11) and antibody to FLAG (M2) for the detection of FLAG-tagged WSX-1 and sWSX-1 (C).

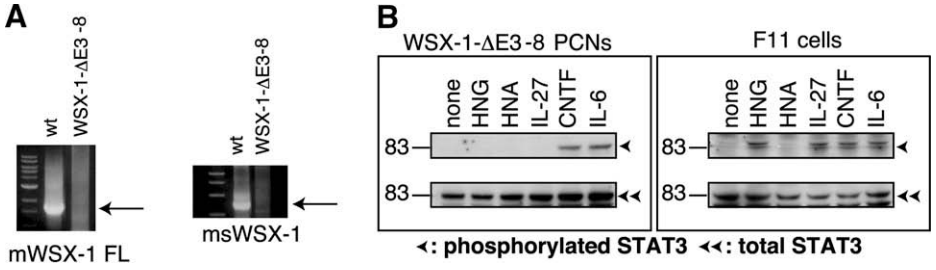

Fig. 4. Disruption of the exons 3–8 of the WSX-1 gene nullifies HN-induced STAT3 phosphorylation. (A) RT-PCR analysis was performed with mRNA purified from wild-type WSX-1 and WSX-1-ΔE3–8 PCNs to detect expression of WSX-1 and sWSX-1. (B) PCNs, derived from WSX-1-ΔE3–8 homozygous mice and F11 cells (2.0×10^5 cells/ 6 cm well) were incubated in DMEM plus N2 supplement for 48 h (DIV3–5). 1 μM HNG, 1 μM human IL-27, 100 ng/ml rat CNTF, or 100 ng/ml BSA was added and co-incubated for 15 min for at 37 °C in the same media. The cells were harvested for immunoblot analysis with antibodies to phosphoSTAT3 and STAT3.

gp130 triggers HN-like activity in these neuronal cells. HN binding to the alternative HN receptor is therefore speculated to induce the homodimerization of gp130 to an extent sufficient for HN to exhibit neuroprotective activity.

Additionally, this study has indicated that IL-27 induces the phosphorylation of STAT3 possibly by binding to sWSX-1/gp130 and speculatively inducing the homodimerization of gp130 (Fig. 1). IL-27 plays an essential role in the initiation of Th1 immunity [13,14], mainly mediated by interaction between the intracellular domain of WSX-1 and STAT1 [16–18]. IL-27 also suppresses IL-2 production from $CD4^+$ cells and IL-23-mediated differentiation of Th17 cells while it promotes the growth of IL-10-producing $CD4^+$ cells [19,20]. The latter activities of IL-27, mediated by the activation of STAT3 as well as STAT1, result in the inhibition of immune responses and inflammation. Altogether, these results suggest that IL-27 treatment might show a biological phenotype related to the latter activities of IL-27 by activating STAT3 in WSX-1-E8 knockout cells. This possibility should be carefully examined.

In conclusion, this study provides evidence that HN inhibits AD-relevant neuronal death by binding to an alternative HN receptor consisting of CNTFR/sWSX-1/gp130.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.095.

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