Upregulation of neuronal astrocyte elevated gene-1 protects nigral dopaminergic neurons in vivo

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Abstract

The role of astrocyte elevated gene-1 (AEG-1) in nigral dopaminergic (DA) neurons has not been studied. Here we report that the expression of AEG-1 was significantly lower in DA neurons in the postmortem substantia nigra of patients with Parkinson’s disease (PD) compared to age-matched controls. Similarly, decreased AEG-1 levels were found in the 6-hydroxydopamine (6-OHDA) mouse model of PD. An adeno-associated virus-induced increase in the expression of AEG-1 attenuated the 6-OHDA-triggered apoptotic death of nigral DA neurons. Moreover, the neuroprotection conferred by the AEG-1 upregulation significantly intensified the neurorestorative effects of the constitutively active ras homolog enriched in the brain [Rheb(S16H)]. Collectively, these results demonstrated that the sustained level of AEG-1 as an important anti-apoptotic factor in nigral DA neurons might potentiate the therapeutic effects of treatments, such as Rheb(S16H) administration, on the degeneration of the DA pathway that characterizes PD.

Introduction

Astrocyte elevated gene-1 (AEG-1), also known as metadherin, was originally identified as a human immunodeficiency virus-1- and tumor necrosis factor-alpha-inducible gene in human fetal astrocytes, and its upregulation is a well-established important oncogenic event in various types of human cancer. The downregulation of neuronal AEG-1 has recently been shown to reduce the viability of motor neurons in a mouse model of amyotrophic lateral sclerosis (ALS) by activating apoptotic signaling pathways via inhibition of the phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/Akt) signaling pathway.

The aberrant activation of apoptotic signaling pathways in the adult brain is a well-known neurotoxic event that is associated with neuronal loss, such as that observed in neurodegenerative diseases, including Parkinson’s disease (PD) and Alzheimer’s disease (AD), and the PI3K/Akt/mammalian target of rapamycin complex 1 (mTORC1) signaling pathway has been shown to elicit neuroprotective effects on the survival and growth of neurons in the nigrostriatal dopaminergic (DA) system. However, little is known about the neuroprotective role of AEG-1 in PD.

Here we found that the loss of DA neurons in postmortem substantia nigra (SN) tissue from patients with...
PD were associated with significant decreases in the levels of expression of AEG-1 in nigral DA neurons of patients with PD compared to age-matched controls. These findings suggested that the relationship between AEG-1 downregulation and the pathogenesis of PD are clinically relevant. To investigate the role of AEG-1 as a survival factor in nigral DA neurons in the adult brain, we examined the effects of the adeno-associated virus (AAV)-mediated overexpression of AEG-1 on these neurons in the 6-hydroxydopamine (6-OHDA)-treated animal model of PD\(^9,10,12\). Additionally, we examined whether the neuroprotection conferred by AEG-1 overexpression, which might be a therapeutic intervention, contributed to the neurorestorative effects on the in vivo nigrostriatal DA system of treatment strategies, such as the administration of constitutively active ras homolog enriched in brain (with a S16H mutation) [Rheb(S16H)], which induces axonal regrowth in damaged DA neurons\(^9,10\).

**Results**

**Decreased levels of AEG-1 expression in the SN of patients with PD and a neurotoxin-based model of PD**

To investigate the alterations in the levels of AEG-1 expression in the SN of patients with PD (Fig. 1a), we performed immunohistochemical staining of the expression patterns (Fig. 1b) and quantified the changes using western blotting (Fig. 1c). AEG-1-positive immunoreactivity (blue) was clearly reduced in neuromelanin-positive DA neurons (brown) in the SN of patients with PD compared to age-matched controls (Fig. 1b). Western blot analyses revealed significant decreases in the levels of AEG-1 and tyrosine hydroxylase (TH, a marker of DA neurons) in the SN of the patients with PD compared to age-matched controls (Fig. 1c; *p = 0.033 and *p = 0.022 for AEG-1 and TH, respectively, vs. CON). However, decreased AEG-1 expression was not observed in the hippocampus of patients with AD compared to age-matched controls, even though there was a significant loss of neuronal nuclei (NeuN, a marker of neurons) in that region in the patients compared to controls (Fig. 1f, g; *p = 0.001 vs. CON). The reduction of AEG-1 (brown) was specific to the SN pars compacta of 1 day post-lesion 6-OHDA-treated mice (Fig. 1d), which is a well-known neurotoxin-based model of PD\(^9,10,12-14\). Western blot analyses similarly showed a significant decrease in the levels of AEG-1 expression in the SN after 6-OHDA administration, compared to untreated controls, 1 day post-lesion (Fig. 1e; *p = 0.024 vs. CON), even though the levels of TH were not significantly decreased in the SN (Fig. 1e).

**Decreased levels of apoptotic signaling molecules by AEG-1 transduction of mature neurons in the SN**

To determine whether the upregulation of AEG-1 affected the apoptotic signaling pathways in nigral DA neurons, we evaluated the in vivo effects of AEG-1 overexpression on the basal levels of apoptotic markers, such as cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase 1 (PARP-1) in nigral DA neurons. Mice were sacrificed 4 weeks after intranigral injections of AAV-AEG-1 or the control vector AAV-green fluorescent protein (GFP), and the transduction of DA neurons was confirmed by the patterns of GFP expression and the immunoperoxidase staining of the hemagglutinin (HA) epitope in the AAV-AEG-1 vector, respectively (Fig. 2a). HA- and GFP-positive cells were clearly colocalized with TH-positive DA neurons (Fig. 2b) but not with glial fibrillary acidic protein (GFAP)-positive astrocytes or ionized calcium binding adaptor molecule 1 (Iba1)-positive microglia in the SN (Fig. 2c). Upregulation of AEG-1, which showed no neurotoxicity (Fig. 2d–f), resulted in a significant decrease in the basal levels of cleaved caspase-3 and cleaved PARP-1 in the SN compared to noninjected and GFP controls (Fig. 2g; *p = 0.005 vs. CON).

Similar to previous reports that have implicated apoptosis in the loss of DA neurons in patients with PD\(^6,8\), western blot analyses revealed significant increases in the levels of caspase-3, cleaved caspase-3, and cleaved PARP-1 were observed in the SN of patients with PD compared to age-matched controls (Fig. 3a; &p = 0.014, &&p = 0.019, and ###p = 0.009 vs. CON, respectively). As shown in the experimental schematic (Fig. 3b), the double immunofluorescence staining of TH (red) and cleaved caspase-3 (green) and of TH and cleaved PARP-1 (green) (Fig. 3c), and western blot analyses (Fig. 3d) showed that the levels of both cleaved caspase-3 and cleaved PARP-1 significantly increased 2 days post-lesion in the TH-positive DA neurons in the SN of mice injected with 6-OHDA only\(^15,16\). However, the upregulation of AEG-1 significantly inhibited the cleavage of both caspase-3 and PARP-1 in the nigral DA neurons following the 6-OHDA injections compared to injections of 6-OHDA alone (Fig. 3c, d; ***p = 0.009 and **p = 0.002, respectively, vs. 6-OHDA alone). The anti-apoptotic effects of AEG-1 on the 6-OHDA-induced neurotoxicity in DA neurons were confirmed with western blot analyses of the B-cell lymphoma 2/Bcl-2-associated X protein (Bcl-2/Bax) ratio (Supplementary Figure S1).

**Neuroprotective effects of AEG-1 upregulation against 6-OHDA neurotoxicity**

We evaluated the neuroprotective effects of AAV-AEG-1 in the 6-OHDA-treated mouse model of PD (Fig. 4a). 6-OHDA administration clearly caused neurotoxicity in the nigrostriatal DA projections (Fig. 4b, c), and the transduction of DA neurons with AEG-1 but not GFP effectively mitigated the 6-OHDA-induced neurotoxicity in the SN compared to the effects of treatment with 6-OHDA alone (Fig. 4b; *p = 0.026 vs. 6-OHDA alone).
Fig. 1 (See legend on next page.)
Western blot analyses also showed that AEG-1 transduction significantly preserved the levels of TH expression following 6-OHDA-induced neurotoxicity compared to treatment with 6-OHDA alone (Fig. 4d; \( p = 0.002 \) vs. 6-OHDA alone) in the SN but not in the striatum (STR). Similar to the limited neuroprotective effects observed using immunostaining and western blotting, the results obtained with reversed-phase high-performance liquid chromatography (HPLC) analyses indicated that the levels of striatal dopamine and its metabolites, including 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), did not significantly differ between the mice treated with 6-OHDA following AEG-1 transduction and those treated with 6-OHDA alone (Supplementary Figure S2).

To evaluate the effects of glial AEG-1 in the nigrostriatal DA system, we unilaterally injected adenovirus (Ad)-AEG-1 (Ad-AEG-1) or Ad-null, which was a control vector, in the SN of healthy mice and examined whether any neuroprotective effects were observed against 6-OHDA neurotoxicity. Four weeks after intranigral injections of Ad-AEG-1, immunohistochemical staining of the HA epitope tag for Ad-AEG-1 indicated the site-specific transduction of SN microglia with Ad-AEG-1 (Supplementary Figures S3a to c), and no neurotoxicity was observed in the nigrostriatal DA projections in the brains of the healthy mice (Supplementary Figures S3d and e). However, we did not observe neuroprotective effects of the overexpression of microglial AEG-1 against 6-OHDA-induced neurotoxicity at 1 week post-lesion (Fig. 4e, f).

Lack of activation of the Akt/mTORC1 signaling pathway in nigral DA neurons by AEG-1 up regulation

The results of previous studies suggested that the Akt/mTORC1 signaling pathway could be regulated by changes in AEG-1 expression\(^3\)–\(^5\),\(^17\),\(^18\), and the activation of Akt/mTORC1 signaling pathway could regulate the autophagy–lysosomal pathway (ALP), which is associated with axonal degeneration in PD\(^12\),\(^19\). Moreover, the sustained activation of the Akt/mTORC1 signaling pathway induces axonal regeneration in damaged neurons\(^9\),\(^10\),\(^20\),\(^21\). However, western blot analyses showed that the overexpression of AEG-1 alone did not alter the phosphorylation statuses of the Thr37/46 residues of 4E-BP1, which are indicative of mTORC1 activity (Supplementary Figure S4). Similarly, AEG-1 overexpression did not induce significant increases in the levels of p-Akt, which is associated with activation of mTORC1, compared to the levels in noninjected controls (Supplementary Figure S4). Additionally, no significant changes were observed in the levels of microtubule-associated protein 1A/1B-light chain 3 (LC3)-I and II, which are used as an indicator of autophagosome formation\(^12\),\(^19\),\(^22\), following injections of AAV-AEG-1 in the SN of healthy mice compared to those in noninjected controls (Supplementary Figure S4).

In the SN of patients with PD, we observed significant increases in the levels of LC3-II and p62, which are well-known markers of ALP\(^12\),\(^19\),\(^22\),\(^23\), compared to those in age-matched controls (Supplementary Figure S5a). Consistent with the increase in the levels of LC3-II and p62, a significant decrease in the levels of p-4E-BP1 expression was also observed in the SN of patients with PD (Supplementary Figure S5a). These observations suggested that the suppression of aberrant ALP through the activation of the mTORC1 signaling pathway might also be associated with neuroprotection of the nigrostriatal DA system. However, AEG-1 overexpression in DA neurons did not suppress the aberrant accumulation of autophagic components, such as LC3-II and p62, and the decrease in mTORC1 activity following 6-OHDA neurotoxicity (Supplementary Figure S5b).

Application of AEG-1-induced neuroprotection to the functional recovery of the disrupted nigrostriatal DA system

To determine the importance of sustaining the increased levels of neuronal AEG-1 in the adult...
nigrostriatal DA system and, consequently, the potential of AEG-1 overexpression as a therapeutic approach for PD, we examined the effects of AEG-1 overexpression following post treatment with AAV-Rheb(S16H) on the functional recovery of nigral DA neurons and induction of axonal regeneration in damaged DA neurons. As shown in the experimental schematic (Fig. 5a), treatment with 6-OHDA alone induced significant reductions in the motor performance, which was measured using the open-field test (Fig. 5b, c) and rotarod test (Fig. 5d), compared to the control group.
to that in noninjected controls (*p < 0.001 vs. intact controls). Similar to the results described in our previous report, Rheb(S16H) overexpression rescued the motor impairments induced by 6-OHDA neurotoxicity compared to mice injected with 6-OHDA alone (##p = 0.016 and ##p = 0.007 for open-field test and rotarod test, respectively, vs. 6-OHDA + AAV-Rheb(S16H)]. Consistent with these results, the immunohistochemical staining of TH demonstrated that Rheb(S16H) overexpression following 6-OHDA administration induced axonal regeneration in damaged DA neurons (Fig. 5f, h; *p < 0.001 vs. 6-OHDA alone)\(^9\)\(^{10}\)\(^\)\(^\)\(^\)\(^\), and Rheb(S16H) overexpression in the presence of increased levels of AEG-1 significantly restored the density of DA fibers in the STR compared to the effects in the absence of AEG-1 [Fig. 5f, h; *p = 0.003 vs. 6-OHDA + AAV-Rheb(S16H)]. Moreover, the depleted levels of striatal dopamine following 6-OHDA administration, which were measured using HPLC, were greatly restored following Rheb(S16H) overexpression in the presence of virally
Fig. 4 (See legend on next page.)
overexpressed AEG-1 compared to the levels in its absence [Fig. 5i; \( p = 0.024 \) vs. 6-OHDA + AAV-Rheb (S16H)]. Similarly, the levels of the metabolites of dopamine, including DopAC and HVA, were restored by Rheb (S16H) overexpression, and the effects were more obvious in the presence of overexpressed AEG-1 than in its absence (Supplementary Figure S6).

Rheb(S16H) administration did not affect the levels of expression of AEG-1 in the SN of the mice (Supplementary Figure S7), which suggested that the neurorestorative effects from this administration might be independent of AEG-1 expression and that its upregulation might activate the Akt/mTORC1 signaling pathway as a supplementary mechanism in the presence of AEG-1. These data suggested that AEG-1, which is significantly reduced in the SN of patients with PD, is an important endogenous factor that protects nigral DA neurons from neurotoxicity and that this protection by the anti-apoptotic effects of neuronal AEG-1 enhances the restoration of the disrupted nigrostriatal DA system (Fig. 6), as shown by the effects of Rheb(S16H) administration in the neurotoxin model of PD (Fig. 5).

### Discussion

Under physiological conditions, apoptosis is an essential homeostatic mechanism that maintains the cell population in healthy tissue and protects cells from injury\(^{24,25}\). However, aberrant apoptosis, which is one of the neurotoxic events in the adult brain, may inevitably be related to neurodegenerative diseases, such as PD and Alzheimer's disease\(^{6,8,26}\). Moreover, consistent with the results of studies on patients with PD\(^{6,8}\), increases in the levels of apoptotic markers, such as cleaved caspase-3 and cleaved PARP-1, are observed in in vivo and in vitro models of PD\(^{15,16}\). As reported previously, the inhibition of apoptotic pathways protects DA neurons against neurotoxin treatment\(^{27,30}\). Thus, these results suggest that the expression and maintenance of endogenous anti-apoptotic factors are beneficial for the survival of nigral DA neurons in the adult brain.

The downregulation of AEG-1 contributes to the apoptosis of motor neurons by inhibiting the PI3K/Akt signaling pathway in in vivo and in vitro models of ALS\(^5\). Although the role of AEG-1 in the pathogenesis of PD was unknown, these results suggest that AEG-1 might be critical for the survival of DA neurons in the SN of patients with PD. Here, we observed decreased expression of AEG-1 in damaged DA neurons in the adult brain in an immunohistochemical analysis of the SN of patients with PD and 6-OHDA-treated mice (Fig. 1b, d). However, no significant reductions in AEG-1 were observed in the hippocampus of patients with AD compared to age-matched controls (Fig. 1g). Thus, these observations suggested that the decreased levels of AEG-1 were a specific event that occurred in damaged DA neurons and AEG-1 downregulation and the loss of nigral DA neurons in PD might be clinically correlated.

Here, AAV-mediated overexpression of AEG-1 in DA neurons decreased the levels of apoptotic markers, including cleaved caspase-3 and cleaved PARP-1, following 6-OHDA administration (Fig. 3c, d)\(^{15,16}\), resulting in neuroprotection in the SN (Fig. 4b, d). The AEG-1-induced anti-apoptotic effects were confirmed by western blot analyses of the Bcl-2/Bax ratio (Supplementary Figure S1). An increase in the levels of apoptotic signaling molecules, which was similar to the results obtained in the mouse model (Fig. 3c, d), were also observed in the SN of patients with PD (Fig. 3a). These results suggested that AEG-1 plays a role as a negative regulator of apoptosis in adult DA neurons and the sustained levels of neuronal AEG-1 following neurotoxic events may confer neuroprotection to nigral DA neurons in vivo. Moreover, the overexpression of microglial AEG-1 in the 6-OHDA-based mouse model of PD did not confer neuroprotection (Fig. 4e, f). Therefore, consistent with the effects of reduced AEG-1 expression in motor neurons in an ALS mouse model\(^5\), our results showed that the sustained increased levels of neuronal AEG-1 were important for attenuating the vulnerability of nigral DA neurons in the SN of adult brain.

Despite the significant anti-apoptotic effects of AEG-1, its overexpression was not sufficient to protect the whole nigrostriatal DA projection against 6-OHDA-induced...
Fig. 5 (See legend on next page.)
neurotoxicity (Fig. 4c, d and Supplementary Figure S2). These observations indicated limitations in the protective effects of AEG-1 against 6-OHDA-induced neurotoxicity. One explanation for the observation that AEG-1 overexpression in DA neurons was insufficient to protect the DA system seems related to the mTORC1 pathway. The mTOR kinase, which plays a central role in the integration of responses to various environmental conditions, exists in two complexes, mTORC1 and mTORC2. mTORC1 is an important mediator of Akt. mTORC2 can activate Akt, which in turn can act on mTORC1. Activation of the Akt/mTOR signaling pathway enhances the activity of cell survival pathways under various conditions, including trophic factor withdrawal, ischemic shock, and oxidative stress. Moreover, recent reports have showed that the activation of neuronal mTORC1, which is a key biomolecule for neurotrophic support, by either the delivery of a specific gene or the direct administration of trophic factors induces protective effects against neurodegeneration in animal models of PD. We recently demonstrated that the Rheb(S16H) delivery-induced activation of mTORC1 in DA neurons protects and reconstructs the damaged nigrostriatal DA projections in a mouse model of PD, which suggested that the activation of the Akt/mTORC1 signaling pathway might be a promising therapeutic strategy for the functional recovery of DA neurons. Here, however, our results indicated that AEG-1 upregulation in nigral DA neurons did not enhance Akt/mTORC1 signaling in healthy mice (Supplementary Figure S4). Moreover, the activation of the Akt/mTORC1 signaling pathway suppressed the initiation of autophagy and prevented the aberrant accumulation of autophagic components, which might inhibit normal lysosomal degradation, such as the removal of expended macromolecules and organelles, in the nigrostriatal DA system. Although autophagy is physiologically important for preserving cellular homeostasis and inducing protective effects, such as the suppression of apoptosis and axonal degeneration, its aberrant activity promotes neurodegeneration in the SN of patients with PD. Therefore, these observations suggest that autophagic stress that is caused by the aberrant accumulation of autophagic components might be one of the
critical mechanisms that induces the loss of DA neurons in PD.

AEG-1 is known to induce autophagy, which results in the survival of cancerous cells under metabolic stress and apoptosis resistance, and these results may underlie its considerable cancer-promoting properties. However, our results indicated that AEG-1 overexpression in DA neurons had no effect on the levels of LC3-II in healthy brains (Supplementary Figure S4), and on the aberrant accumulation of LC3 and p62, which are critical for ALP following 6-OHDA administration (Supplementary Figure S5b). Moreover, the decrease in the activity of mTORC1 following 6-OHDA neurotoxicity was not inhibited by the presence of AEG-1 in DA neurons in vivo (Supplementary Figure S5b). Therefore, to potentiate the beneficial effects of AEG-1 in nigral DA neurons, it may be necessary to activate the Akt/mTORC1 signaling pathway, which suppresses the aberrant accumulation of autophagic components as a supplementary protective mechanism in the presence of AEG-1 and consequently results in enhanced neuroprotection of the nigrostriatal DA projection in the adult brain.

The overexpression of Rheb(S16H), which is a constitutively active form of Rheb and activates mTORC1, suppresses the induction of the abnormal autophagy signaling pathway by 6-OHDA treatment. To further examine if AEG-1 overexpression strengthened the neuroprotective and neurorestorative effects of therapeutic agents, such as Rheb(S16H), AAV-Rheb(S16H) was injected into the SN 3 weeks post-lesion, which is when the maximum neurodegeneration is observed after 6-OHDA treatment, in the absence or presence of virally overexpressed AEG-1 (Fig. 5a). Our results demonstrated that Rheb(S16H) upregulation in the presence of increased levels of AEG-1 induced synergistic neurorestorative effects, such as restored motor functions, in the nigrostriatal DA system disrupted by 6-OHDA neurotoxicity (Fig. 5b–i). As shown in Supplementary Figure S7, the Rheb(S16H) transduction of DA neurons did not affect the levels of AEG-1 in the SN of the mice, suggesting that the Rheb(S16H)-induced neurorestoration was independent of AEG-1 upregulation and that the AEG-1-induced neuroprotection against 6-OHDA neurotoxicity augmented the beneficial effects of Rheb(S16H) in the lesioned nigrostriatal DA system in vivo (Fig. 5).

In conclusion, our findings suggested that AEG-1 functioned as an anti-apoptotic factor in nigral DA neurons of the adult brain and the decrease in AEG-1 might be involved in the loss of DA neurons, which is one of the key pathological features in PD. However, the overexpression of AEG-1 in DA neurons was not sufficient to protect the whole nigrostriatal DA projection in the animal model of PD owing to its limited protective effects as it did not affect the aberrant accumulation of autophagic components and Akt/mTORC1 activity following 6-OHDA administration, which could contribute to the neurotoxic effects on the nigrostriatal DA system. To overcome this limitation of AEG-1, we further transduced the Akt/mTORC1 activator Rheb(S16H) into AEG-1-overexpressing DA neurons. Surprisingly, the synergistic effects of the two factors restored the nigrostriatal DA system that was disrupted by 6-OHDA administration, and the effects were more obvious in the presence of AEG-1 than in its absence (Figs. 5 and 6). Therefore, we concluded that AEG-1 was an important endogenous factor for protecting nigral DA neurons from aberrant apoptotic signaling pathway in the adult brain and that the maintenance of increased levels of AEG-1 in nigral DA neurons in patients with PD, in combination with therapeutic agents, such as an Akt/mTORC1 signaling activator, may be a highly promising therapeutic strategy to maximize the functional recovery of the damaged nigrostriatal DA system (Fig. 6).

Materials and methods

Ethics statement

All animal experiments were performed in accordance with the approved animal protocols and guidelines established by the Animal Care Committee at Kyungpook National University (Number: KNU 2016-42). Experiments involving human tissue were approved by the Bioethics Committee, Institutional Review Board Kyungpook National University Industry Foundation (IRB Number: KNU 2014-0007 and 2016-0011).

Human brain tissue

Frozen and paraffin-fixed brain tissues were obtained from the Victorian Brain Bank Network (VBBN), supported by the Florey Institute of Neuroscience and Mental Health, The Alfred, and the Victorian Forensic Institute of Medicine, and funded by Australia’s National Health & Medical Research Council and Parkinson’s Victoria. Frozen and paraffin-fixed brain tissues were used in quantitatively analyzing the level of proteins and in observing the expression pattern of target molecule, respectively.

Materials

Materials were purchased from the following companies: 6-OHDA (Sigma, St Louis, MO), desipramine (Sigma), 1-ascorbic acid (Sigma), rabbit anti-TH (Pel-Freez, Brown Deer, WI), mouse anti-TH (R&D Systems, Minneapolis, MN), rabbit anti-Iba1 (Wako Pure Chemical Industries, Osaka, Japan), rabbit anti-GFAP (Millipore, Billerica, MA), rabbit anti-AEG-1 (Invitrogen, Camarillo, CA), rabbit anti-GFP (Millipore), mouse anti-HA (Cell Signaling, Beverly, MA), rabbit anti-HA (Cell Signaling), rabbit anti-FLAG (Sigma), rabbit anti-caspase-3 (Cell
ampli
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carrying AEG-1 with a HA-encoding sequence at the
bed17,42. AEG-1-HA obtained from pcDNA3.1-AEG-1-
AEG-1 (pcDNA3.1-AEG-1-HA), as previously descri-

lateral injection of AAV or Ad into the right SN (AP:
CA) with a mouse adapter. Each mouse received a uni-
placed in a stereotaxic frame (Kopf Instruments, Tujunga,
were anesthetized with chloral hydrate solution and

WPRE10. Constitutively activated Rheb was also cloned
utilizes the chicken
HA was cloned into an AAV packaging construct that
formed before viral vector production. Viral vectors
were produced as described previously, with some mod-
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cations10,17,41,42. Ad viral vectors were supplied by S.G.
Lee. Briefly, for the production of AAV viral vectors
carrying AEG-1 with a HA-encoding sequence at the
3’-end (AEG-1-HA), AEG-1 cDNA tagged with HA was
amplified from the mammalian expression plasmid of
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HA was cloned into an AAV packaging construct that
utilizes the chicken β-actin promoter and contains a 3’
WPRE10. Constitutively activated Rheb was also cloned
into the same AAV packaging construct10. All nucleotide
sequences in the AAV packaging construct were con-
firmed before viral vector production. AAVs were
produced at the University of North Carolina Vector Core.
The genomic titer of AAV-AEG-1 and AAV-Rheb(S16H)
were 9.4 × 10^{12} viral genomes/ml and 3.6 × 10^{12} viral
genomes/ml, respectively. Enhanced GFP, used as a con-
trol, was subcloned into the same AAV viral backbone,
and viral stock was produced at a titer of 2.0 × 10^{12} viral
genomes/ml. Genomic titers of both Ad-AEG-1 and Ad-
null viral stocks were 2.0 × 10^{9} infectious unit/ml.

Production of viral vectors

The two types of viral vectors used in the present study
were AAV serotype 1 and Ad serotype 5. Viral vectors
were produced as described previously, with some mod-
ifications10,17,41,42. Ad viral vectors were supplied by S.G.
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carrying AEG-1 with a HA-encoding sequence at the
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null viral stocks were 2.0 × 10^{9} infectious unit/ml.

Intranasal AAV and Ad injection

Adult (8- to 10-week-old) male C57BL/6 mice were
obtained from Daehan Biolink (Eumseong, Korea). As
previously described, with some modifications9,10 mice
were anesthetized with chloral hydrate solution and
placed in a stereotaxic frame (Kopf Instruments, Tujunga,
CA) with a mouse adapter. Each mouse received a uni-
lateral injection of AAV or Ad into the right SN (AP:
−0.35 cm, ML: −0.11 cm, DV: −0.37 cm, relative to
bregma) using a 30-gauge Hamilton syringe attached to
an automated microinjector41. Viral vector suspension
in a volume of 2.0 μl was injected at a rate of 0.1 μl/min over
20 min. After injection, the needle was left in place for an
additional 5 min before being slowly retracted.

Intrastratal 6-OHDA injection

The intrastratal 6-OHDA model was induced as previ-
ously described9,10. Mice were intraperitoneally injected
with desipramine (25 mg/kg in 0.9% NaCl), and then
anesthetized with chloral hydrate. Anesthetized mice
were placed in a stereotaxic frame, and a solution of 6-OHDA
(5 mg/ml in 0.9% NaCl/0.02% ascorbic acid), with a final
volume of 3.0 μl was injected by Hamilton syringe at a rate
of 0.5 μl/min. The injection was performed into the right
STR at coordinates (AP: +1.09 cm; ML: −0.22 cm; DV:
−0.25 cm, relative to bregma)43. The needle was with-
drawn slowly after 5 min. Animals were sacrificed and
analyzed at the indicated time points for each exper-
iment9,10.

Behavioral tests

Open-field test

The open-field test was performed as described pre-
viously, with some modifications13. Briefly, 11 weeks after
the 6-OHDA injection, mice were placed individually
in the corner of a test chamber (40 × 40 × 40 cm) enclosed
with white acrylic walls. After a 1 min adaptation period,
animal behaviors such as the total distance traveled (in
cm) and velocity (in cm/sec) were recorded for 5 min
using a video camera. The change in locomotor activity
was analyzed offline by video-tracking software (SMART,
Panlab, Barcelona, Spain). The test chamber was cleaned
between trials with 70% ethyl alcohol. To minimize stress
levels, tests were performed under conditions of low
illumination.

Rotarod test

The rotarod test was performed at 11 weeks post-lesion,
using a previously described procedure with some mod-
ifications13. Before 6-OHDA treatment, all mice were pre-
trained on the rotarod apparatus (3 cm rod diameter;
Scitech Inc., Seoul, Korea) at 10 revolutions per min (rpm)
for 10 min, and the training was performed for 3 con-
secutive days. Eleven weeks after the 6-OHDA injection,
performance on the rod was evaluated at a constant
acceleration rate of 4–40 rpm in 300 sec. Two consecutive
trials were performed at 60 min intervals.

Immunohistochemical staining

Postmortem brain tissues were processed for immuno-
histochemistry as described previously44. Briefly, the
human SN sections were deparaffinized and subjected to
citrate-based antigen retrieval, and then washed in cold PBS and blocked with blocking solution. The sections were incubated with primary antibodies against AEG-1 (1:500) at 4 °C overnight, and then incubated with biotinylated secondary antibodies for 1 h at room temperature, followed by addition of the avidin-biotin reagent (Vectastain ABC kit, Vector Laboratories) for 1 h at room temperature. The SN sections were visualized using a 3,3′-diaminobenzidine (DAB; Sigma) peroxidase substrate solution [0.05% DAB, 0.05% Cobalt Chloride (Sigma), 0.05% Nickel Ammonium Sulfate (Sigma) and 0.015% H2O2 in PBS, pH 7.2]. Each section was covered with a thin glass coverslip and analyzed under a bright-field microscope (Carl Zeiss, Oberkochen, Germany).

As previously described9,10,44, mice were transcardially perfused and fixed, and the brains were dissected out, frozen, and cut into 30-μm-thick coronal sections using a cryostat microtome (Thermo Fisher Scientific). Briefly, the brain sections were washed in PBS and blocked with blocking buffer, and then incubated at 4 °C for 2 days with the following primary antibodies: rabbit anti-TH (1:2000), rabbit anti-AEG-1 (1:500), rabbit anti-Iba1 (1:2000), rabbit anti-cleaved caspase-3 (1:1000), rabbit anti-cleaved PARP-1 (1:1000), rabbit anti-cleaved caspase-3 (1:1000), rabbit anti-FLAG (1:3000), rabbit anti-Akt (1:1000), rabbit anti-p-Akt (1:2000), rabbit anti-p62/SQSTM1 (1:1000), rabbit anti-Akt (1:1000), mouse anti-Bcl-2 (1:1000), mouse anti-Bax (1:1000), rabbit anti-LC3B (1:1000), rabbit anti-p-4E-BP1 (1:1000), and rabbit anti-p-4E-BP1 (1:1000). Subsequently, the membranes were incubated with secondary antibodies for 1 h at room temperature, and the bands were finally detected using Western-blot detection reagents (Thermo Fisher Scientific, Rockford, IL). For quantitative analyses, the density of each band was measured using a Computer Imaging Device and accompanying software (Fuji Film, Tokyo, Japan), and the levels were quantitatively expressed as the density normalized to the housekeeping protein band for each sample.

Stereological estimation

As previously described10,44, the total number of TH-positive neurons was counted in the various animal groups using the optical fractionator method. Counting of TH-positive neurons in the SN was performed on a bright-field microscope (Olympus Optical, BX51, Tokyo, Japan) using Stereo Investigator software (MBF Bioscience, Williston, VT). This unbiased stereological method of cell counting is not affected by either the reference volume (SN pars compacta) or the size of the counted elements (neurons).

Quantitative determination of striatal TH immunoperoxidase staining

Densitometric analysis of the mouse STR was carried out as previously described10,44. Briefly, an average of 6 coronal sections of the STR that gathered according to the bregma of the brain atlas43 was imaged at a ×1.25 magnification. The density of striatal TH-positive fibers was measured using the Science Lab 2001 Image Gauge (Fujiﬁlm, Tokyo, Japan). To control for variations in background illumination, the density of the corpus callosum was subtracted from the density of STR for each section. The density in both the contralateral and ipsilateral sides was expressed by comparing with the average density of TH-positive fiber innervating the contralateral side.

Measurement of dopamine and its metabolites in the STR

As previously described44, the levels of striatal dopamine and its metabolites were measured by high performance liquid chromatography (HPLC, 1260 Infinity system, Agilent Technologies, Santa Clara, CA) using an
design the viral constructs; S.R.K., S.-G.L., P.B.F., and N.K. contributed the viral vectors; C.M. contributed the human brain samples; Y.-S.O., K.J.L., Y.S.C., K.S.A., B. KJ, D.W.K., J.M.L., S.G.L., and S.R.K. supervised the analysis of the data obtained with the human brain samples; S.R.K. supervised the whole project and wrote the paper. All of the authors contributed to the data analysis and preparation of the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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