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Synphilin-1 exhibits trophic and protective effects against Rotenone toxicity

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Abstract

Synphilin-1 is a cytoplasmic protein with unclear function. Synphilin-1 has been identified as an interaction partner of α-synuclein. The interaction between synphilin-1 and α-synuclein has implications in Parkinson's disease. In this study, we stably overexpressed human synphilin-1 in mouse N1E-115 neuroblastoma cells. We found that overexpression of synphilin-1 shortened cell growth doubling time and increased neurite outgrowth. Knockdown of endogenous synphilin-1 caused neuronal toxicity and shortened neurite outgrowth. We further found that synphilin-1 increased activation of the extracellular signal-regulated kinases (ERK1/2) and mediated neurite outgrowth. Rotenone, mitochondrial complex I inhibitor, has been shown previously to induce dopaminergic neurodegeneration and Parkinsonism in rats and *Drosophila.* We found that Rotenone induced apoptotic cell death in N1E-115 cells via caspase-3 activation and PARP cleavage. Overexpression of synphilin-1 significantly reduced Rotenone-induced cell death, caspase-3 activation and PARP cleavage. The results indicate that synphilin-1 displays trophic and protective effects in vitro, suggesting that synphilin-1 may play a protective role in PD pathogenesis and may lead to a potential therapeutic target for PD intervention.

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Keywords

Parkinson's disease; N1E-115 neuroblastoma cells; neuroprotection; caspase-3; ERK1/2

Parkinson's disease (PD) is a common neurodegenerative movement disorder that is characterized by selective loss of dopaminergic neurons and the presence of Lewy bodies (LB). The pathogenesis of PD remains incompletely understood, but appears to involve aging, genetic susceptibility and environmental factors (Hardy et al., 2006; Ross and Smith, 2007; Thomas and Beal, 2007). Familial parkinsonism is associated with pathogenic mutations in α-synuclein, parkin, DJ-1, PINK1, LRRK2 and other related genes (Hardy et al., 2006; Ross and Smith, 2007; Thomas and Beal, 2007). α-Synuclein mutations were the first identified cause of autosomal dominant early onset PD (Polymeropoulos et al., 1997; Hardy et al., 2006). In studies of α-synuclein cell biology, Synphilin-1, a cytoplasmic protein, shows predominant neuronal expression and has been identified as an interaction partner of α-synuclein by a yeast two-hybrid approach (Engelender et al., 1999), but the normal functions of synphilin-1 remain poorly understood (Kruger, 2004; Szargel et al., 2008).

Several studies suggest that synphilin-1 may be of relevance to PD pathology. Synphilin-1 interacts with two proteins linked to familial PD: α-synuclein and parkin (Engelender et al., 1999; Chung et al., 2001), and is detected in 80–90% of the Lewy bodies in PD brain samples (Wakabayashi et al., 2000). *In vitro* studies have shown that co-expression of α-synuclein and synphilin-1 favor the formation of cytoplasmic inclusions that resemble Lewy bodies *in vivo* (Engelender et al., 1999; Wakabayashi et al., 2002; Smith et al., 2005b). Mutation analysis of the synphilin-1 gene in familial and sporadic German PD patients allowed the identification of the R621C mutation in two sporadic PD patients, suggesting a putative role of synphilin-1 in PD (Marx et al., 2003).

Epidemiological studies have suggested that PD could be caused by environmental toxins such as Rotenone. Rotenone is a mitochondrial complex I inhibitor and a commonly used natural pesticide. *In vitro* studies show that Rotenone can induce apoptosis in cultured cells (Newhouse et al., 2004; Watabe and Nakaki, 2007). Chronic systemic exposure to rotenone in rats and *Drosophila* has been shown to induce dopaminergic neurodegeneration and Parkinsonism (Betarbet et al., 2000; Coulom and Birman, 2004). *In vitro* studies demonstrate that Rotenone causes apoptosis though oxidative damage and activation of caspase-dependent pathway (Kitamura et al., 2002; Grivennikova and Vinogradov, 2006). Rotenone-based models are often used to study the putative pathogenesis and potential therapeutics of PD.

In this study, we used mouse N1E-115 neuroblastoma cells (Roth et al., 2002) and generated a stable pool cell line that overexpressed human synphilin-1. We found that overexpression of synphilin-1 shortened the cell growth doubling time and increased neurite outgrowth. Knockdown of endogenous synphilin-1 causes neuronal toxicity and shorten neurite outgrowth. We further found that synphilin-1 increased activation of the extracellular signal-regulated kinases (ERK1/2) and mediated neurite outgrowth. Overexpression of synphilin-1 protected against Rotenone-induced cell death via reducing caspase-3 activation and poly

(ADP-ribose) polymerase (PARP) cleavage. The results indicate that synphilin-1 displays trophic and protective effects in vitro, suggesting that synphilin-1 may play a protective role in PD pathogenesis.

Experimental procedures:

Materials:

Cell culture media and antibiotics were from Invitrogen (Carlsbad, CA, USA). Anti-PARP antibodies was purchased from BD PharMingen (San Diego, CA, USA); anti-cleaved PARP, anti-phosphorylated ERK1/2 and anti-ERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-human synphilin-1 polyclonal antibody was made against the human synphilin-1 fragment (34–500 aa) and had cross reactivity with rodent synphilin-1 as previously described (Engelender et al., 1999). Anti-actin antibody and Rotenone were from Sigma (St. Louis, MO, USA).

Cell Culture and Transfection:

N1E-115 cells were purchased from ATCC and grown in Dulbecco's modified Eagle's medium (DMEM; high glucose; Invitrogen) with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (100units/ml penicillin, 100μg/ml streptomycin and 2,5μg/ml Fungizone) at 37°C under 5% CO_2/95% air. Differentiation was induced in the DMEM media with 0.5% FBS and 1.5% dimethylsulfoxide (DMSO; Sigma) as previously described (Roth et al., 2002).

Generation of stable pool cells expressing human synphilin-1: The plasmid, pRK5-Synphilin-1 contains full-length cDNA of synphilin-1 under cytomegalovirus (CMV) promoter as described previously (Engelender et al., 1999). Transfections were performed with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. N1E-115 cells were co-transfected with pRK5-synphilin-1 and pcDNA3.1(+) vector (Invitrogen) which has the Geneticin (G418) selected marker at a 20:1 molar ratio.

Pooled cells stably expressing human synphilin-1 were selected in media containing 300mg/ml G418 (Invitrogen) for 4 weeks. Western blot analysis and immunostaining were employed to confirm expression of human synphilin-1 using an anti-human synphilin-1 antibody.

Assessment of cell viability and apoptosis assays:

Cell viability was evaluated using Trypan blue exclusion—counting the number of dead (blue) and live cells using 0.4% trypan blue. Doubling time was calculated by the following formula: T(double time) = time duration × log 2/log (newly harvested cells) – log (number of cells originally plated) (Liu et al., 2005). Hoechst/propidium iodide (PI) labeling of cells was used to detect apoptotic and necrotic cell death as described previously (Wei et al., 2002). Briefly, fresh media containing 10 μM Hoechst 33342 and 10 μM PI were added for 20 min before the cells were photographed by fluorescence microscopy. Apoptotic cells were identified by the appearance of condensed and fragmented nuclei.

Measurements of neurite outgrowth:

Digital images were transferred into image analysis software (NIH Image J) for neurite morphometric analyses as previously described (Kamishina et al., 2009). Primary neurites were defined as processes directly emerging from the cell body which usually have a thicker diameter than branching neurites. All primary and branching neurites were manually traced on the digital images. The following parameters were measured: 1) total neurite length/neuron, 2) mean length of primary neurite/neuron, and 3) mean number of primary neurites/neuron. In each experimental group, neurites of 50–100 cells were measured in six randomly selected fields. The experiments were repeated three to five times.

Real time RT-PCR and Western blot analysis:

Cells were harvested, and the total RNA was extracted and subjected to real time RT-PCR to detect endogenous mouse synphilin-1 mRNA by a standard protocol described previously (Gao et al., 2007). The primers for mouse synphilin-1 were 5'-CTCAAGACCATCCCAGCACT −3' and 5'-TCAGTGGAGAAACTCGCTTCA −3'. For western blot analysis, cells were harvested in lysis buffer (20 mM HEPES, pH 7.4,2 mM EGTA, 50 mM ß-glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na_3VO_4 and 5 mM NaF). Lysates were resolved on 4–12% NuPAGE Bis-Tris gels (30 μg/lane) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk and probed with different primary and secondary antibodies. Proteins were detected by using enhanced chemiluminescence reagents (NEN Life Science, Boston, MA, USA).

Caspase-3 activity assay:

Cells were harvested in cell lysis buffer (50 mM HEPES, pH 7.4, 1 mM DTT, 0.1 mM EDTA, 0.1% CHAPS and 0.1% Triton X-100). DEVD-p-nitroanilide was used as a substrate for caspase-3. The experiments were performed according to the manufacturer's protocol (Biosource International).

Synphilin-1 siRNA knockdown:

The siRNA targeting mouse endogenous synphilin-1 and the corresponding scrambled RNA oligonucleotide sequence (negative control) were ordered from Dharmacon (Chicago, IL). Transfection of siRNA into N1E-115 cells was performed with lipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. After 4 h transfection, cells were grown in normal growth media for 3 days. Cell toxicity and caspase-3 activity were measured. The expression level of synphilin-1 mRNA was detected by real time RT-PCR and western blot using anti-synphilin-1 antibodies. For measurements of neurite outgrowth, post-transfection cells were differentiated for 3 days and cell images were captured for assessment.

Data analysis:

Quantitative data were expressed as arithmetic mean ± SE based on at least three separate experiments. The difference between two groups was statistically analyzed by Student's *t*-test or an analysis of variance (one-way-ANOVA). A *P*-value <0.05 was considered significant.

Results:

Synphilin-1 promotes N1E-115 cells proliferation.

To generate stable pool cells expressing human synphilin-1, we co-transfected pRK5-CMV-synphilin-1 plasmids (containing human synphilin-1 cDNA) with pcDNA3.1(+) empty vector (containing a G418 selected marker for stable expression) into N1E-115 cells at 20:1 molar ratio. After 24h transfection, we added G418 and selected for a month to generate a stable pool cells expressing human synphilin-1 (Fig. 1A). To detect synphilin-1 protein expression, we employed western blot analysis using an anti-human synphilin-1 antibody which we developed. Human synphilin-1 was overexpressed in N1E-115 stable cells (Fig. 1A) and maintained stable expression level over the passage generations (Fig. 1B). Immunostaining showed that human synphilin-1 was diffusely expressed in cytosol in N1E-115 cells (data not shown), consistent with the distribution of synphilin-1 expression in other systems (Engelender et al., 1999). We found that stable expression of synphilin-1 shortened the cell growth doubling time and significantly increased N1E-115 cell proliferation after passing for 5 generations (Fig. 1B). The doubling times of cells expressing synphilin-1 and empty vector were 23.5±1.1 h and 27.1±1.2 h, respectively.

Synphilin-1 regulates neurite outgrowth in N1E-115 cells.

To assess the effect of synphilin-1 on neurite outgrowth, we differentiated N1E-115 cells for 3 days in the DMEM supplemented with 0.5% serum and 1.5% DMSO as previously described (Roth et al., 2002). We quantified the number and the length of neurites using microscopy and NIH ImageJ software. We found that cells expressing synphilin-1 had longer neurite outgrowth than vector control cells (Fig. 2). There was no significant difference in mean number of primary neurites per neuron between the cells expressing vector and synphilin-1.

Knockdown of synphilin-1 increased neuronal toxicity and shortened neurite outgrowth.

To further determine the role of synphilin-1, we transfected the siRNA targeting mouse synphilin-1 into normal N1E-115 cells to knock down endogenous synphilin-1 expression. Synphilin-1 mRNA and protein expression reduced more than 90% after 2 days transfection of siRNA targeting mouse synphilin-1 (Fig. 3A). This reduced level of expression maintained for 4 days post transfection. We further found that knockdown of synphilin-1 deceased cell viability by Trypan blue exclusion method (Fig. 3B), and increased caspase-3 activity (Fig. 3C).

After 4 h transfection with synphilin-1 targeting siRNA, we added differentiation media for 3 days. We found that knockdown of synphilin-1 significantly shortened neurite outgrowth

in N1E-115 cells compared with that of cells transfected with control RNA (Fig. 3D). Taken together, these results indicated that overexpression of synphilin-1 exhibited a trophic effect.

Synphilin-1 increased ERK1/2 phosphorylation and mediates neurite outgrowth.

Activation of ERK pathways have been shown to promote cell survival, proliferation and differentiation after growth factor stimulation and to play a protective role after oxidant treatment (Robinson, et al., 1997; Fukunaga et al., 1998). To study the mechanisms underlying syphilin-1-linked neurotrophic effects, we employed western blot analysis using anti-phosphorylated and total ERK1/2 antibodies. We found that synphilin-1 increased ERK 1/2 phosphorylation but it did not alter the total ERK1/2 levels after differentiation (Fig. 4A and 4B). We further found that treatment of 4 μM U0126 (an inhibitor of ERK pathway) abolished synphilin-1-induced neurite outgrowth in N1E-115 cells (Fig. 4C). These results demonstrate that activation of ERK1/2 mediates synphilin-1-induced differentiation.

Synphilin-1 protects against Rotenone toxicity via reduced caspase-3 and PARP cleavage.

To investigate whether synphilin-1 has a protective role in PD-related insult, we used Rotenone (a classical mitochondrial complex I inhibitor) as a model system since Rotenone causes significant oxidative stress and apoptosis in cell culture (Kitamura et al., 2002; Grivennikova and Vinogradov, 2006). Treatment with Rotenone for 24 h period induced apoptotic cell death in vector control cells at concentrations of 20 to 50 nM (Fig. 5A). In contrast, cells stably expressing synphilin-1 significantly protected against Rotenone-induced apoptotic cell death (Fig 5A).

Caspase-3 is a key execution caspase in apoptotic cell death (Strasser et al., 2000). Previous report shows that Rotenone can induce caspase-3 activation and cleave a downstream effector, PARP (Pei et al., 2003; Li et al., 2005). To further study the protective mechanisms of synphilin-1, we conducted a caspase-3 activity assay. We found that synphilin-1 significantly reduced the basal level of caspase-3 activation and further reduced Rotenone-induced caspase-3 activation compared to cells with vector alone (Fig. 5B). To assess the downstream effector of caspase-3, PARP, we performed Western blot analysis using anti-PARP antibodies. We found that synphilin-1 reduced Rotenone-induced PARP cleavage compared with empty vector cells (Fig. 5C). Taken together, these results indicate that synphilin-1 plays a protective role against Rotenone toxicity.

Discussion

The main findings in this study are that expression of synphilin-1 shortens N1E-115 cell division doubling time, promotes neurite outgrowth, and protects against Rotenone-induced toxicity. Knockdown of synphilin-1 induced cell toxicity and attenuated neurite outgrowth. This is the first demonstration that synphilin-1 displays a neurotrophic effect *in vitro,* suggesting that synphilin-1 may play a neuroprotective role in PD pathogenesis.

Synphilin-1, a cytoplasmic protein, is ubiquitously expressed in all tissues, with enriched expression in neurons. Synphilin-1 interacts with alpha-synuclein (Engelender et al., 1999; Wakabayashi et al., 2002; Smith et al., 2005b), parkin (Chung et al., 2001), seven in absentia homologs (siah) and dorfin (Ito et al., 2003; Nagano et al., 2003; Liani et al., 2004). The

functional similarity of parkin, siah, and dorfin suggests physiological redundancy and multiple pathways to link synphilin-1 to the ubiquitin-mediated protein degradation. Synphilin-1 also interacts with proteasomal protein S6 (Kruger, 2004) . S6 ATPase is a regulatory subunit of the 19S proteasome responsible for the degradation of ubiquitinated proteins including abnormal toxic proteins in the cell. Synphilin-1 is present in LB at the late stages of the pathology. R621C mutation in the synphilin-1 protein has been identified in PD. In vitro studies demonstrate that mutant R621C synphilin-1 increases cell susceptibility to cellular stress (Marx et al., 2003). However, the function of synphilin-1 is poorly understood. Our results showed that expression of synphilin-1 gradually shortened the cellular doubling time when cultured in 10% FBS-containing DMEM. We found statistically significant changes in doubling time when comparing cells expressing synphilin-1 and vector control cells for 5 generations of culture (20 days). In contrast, we found that synphilin-1 increased neurite outgrowth in the differentiation culture condition (0.5 % FBS, 1.5 % DMSO DMEM) after culture for only 2-3 days. Under these differentiation conditions, there was no difference in cell numbers by expressing synphilin-1 compared with vector control cells. Our results indicated that the promotion of proliferation by synphilin-1 was a separate process from the enhancement of differentiation by synphilin-1; two processes occurred in distinct experimental conditions. These findings suggested that synphilin-1 displayed a neurotrophic effect. It is well known that activation of the ERK pathway plays an important role in regulating differentiation and survival in neurons after extracellular stimuli such as neurotrophins, and growth factors (Fukunaga et al., 1998). To study the mechanisms underlying synphilin-1-induced neurotrophic effect, we found that synphilin-1 increased ERK1/2 phopshorylation (activation). We further found that U0126, an inhibitor of ERK pathway reduced synphilin-1-induced neurite outgrowth. This result indicate that synphilin-1 enhances neurite outgrowth via activation of ERK signaling pathway.

Rotenone, an inhibitor to mitochondrial complex I, has been shown to produce some pathological features of PD in rat and *Drosophila* model (Betarbet et al., 2000; Coulom and Birman, 2004). It has been reported that rotenone can induce dopaminergic neuron apoptosis through activation of caspase-dependent pathway (Pei et al., 2003; Li et al., 2005). Consistent with these findings, we also found that caspase-3 and PARP, two main effectors of apoptotic pathways were activated and cleaved by Rotenone in N1E-115 cells.

Overexpression of synphilin-1 significantly protected against Rotenone-induced apoptotic cell death. Moreover, synphilin-1 reduced Rotenone-induced caspase-3 activation and PARP cleavage, which was beneficial for neuronal survival in N1E-115 cells. This is the first demonstration that synphilin-1 displays protective effects against Rotenone toxicity, suggesting that synphilin-1 may act as a negative regulator in PD pathogenesis. PD-affected brains exhibit selective loss of substantia nigra pars compacta dopaminergic neurons. Dopaminergic neuronal death is apparently associated with exacerbated oxidative stress and apoptosis. Synphilin-1 may alter the ultimate fate of dopaminergic neurons to survive in the face of these toxic insults. Consistent with this idea, a recent study shows that synphilin-1 can act through its c-terminal proteolytic fragments to inhibit precaspase-3 hydrolysis and to reduce the levels of active caspase-3 resulting in protection against taurosporine and 6-

hydroxydopamine toxicity (Giaime et al., 2006). Taken together, these findings suggest that synphilin-1 plays a neuroprotective role in PD pathogenesis.

In summary, synphilin-1 promoted N1E-115 cell proliferation in 10 % FBS DMEM culture condition. Synphilin-1 increased ERK 1/2 activation resulting in neurite outgrowth of N1E-115 cells under differentiation culture condition. Synphilin-1 protected against Rotenone-induced cell death via reduction of Ronone-induced caspase-3 activation and PARP cleavage. The results indicate that synphilin-1 displays trophic and protective effects *in vitro,* suggesting that synphilin-1 may play a protective role in PD pathogenesis.

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Abbreviations:

DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
LB	Lewy body
PARP	poly (ADP-ribose) polymerase
PD	Parkinson's disease.

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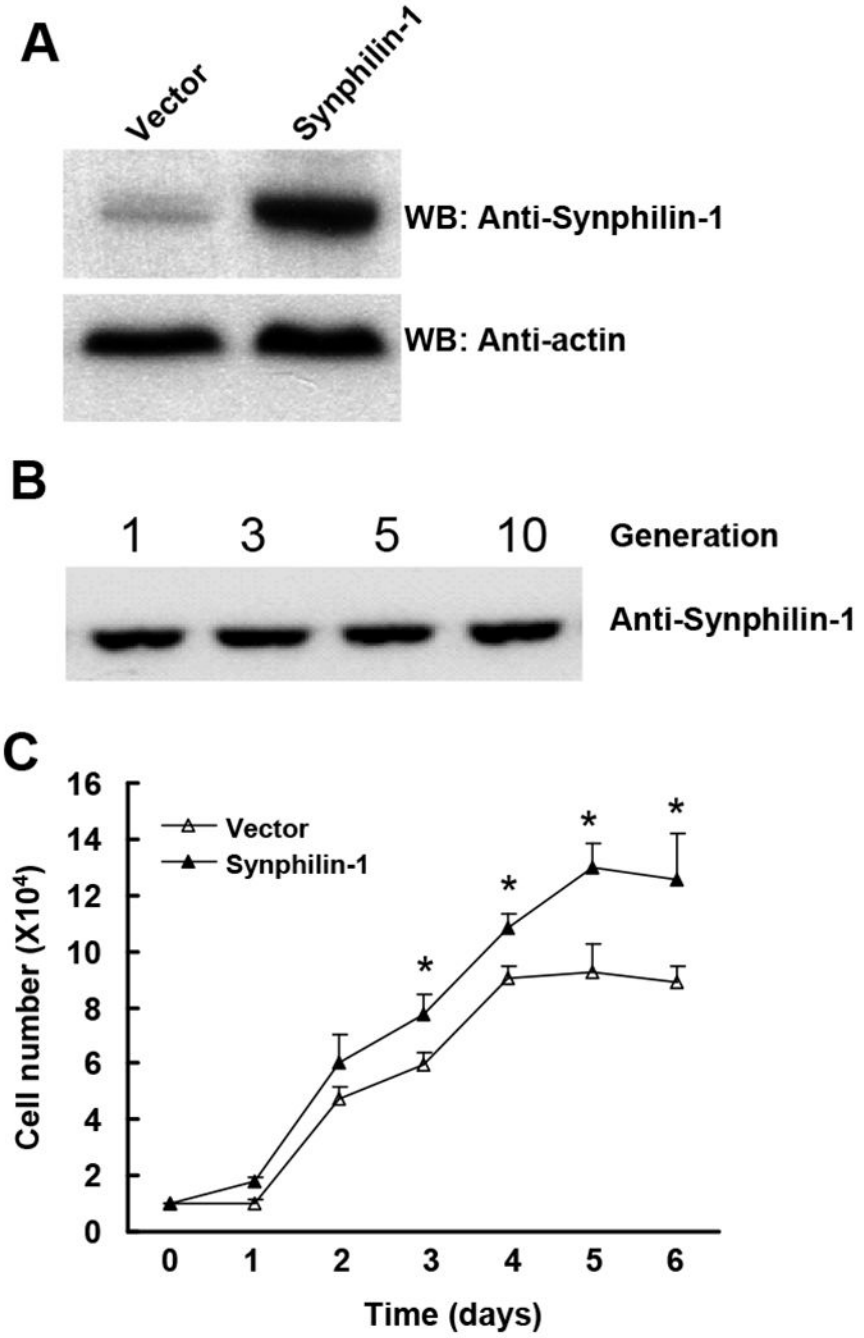

Fig. 1. Synphilin-1 promotes N1E-115 cells proliferation.

A. Western blot analysis of N1E-115 cells stably expressing human synphilin-1 using an anti-synphilin-1 antibody. **B.** Western blot analysis of N1E-115 cells stably expressing human synphilin-1 at indicated passage generations. **C.** The growth curve of N1E −115 cells expressing human synphilin-1. Cell viability was measured by Trypan blue exclusion method. Data are mean ± SE for three separate experiments. *p<0.05 versus cells expressing empty vector.

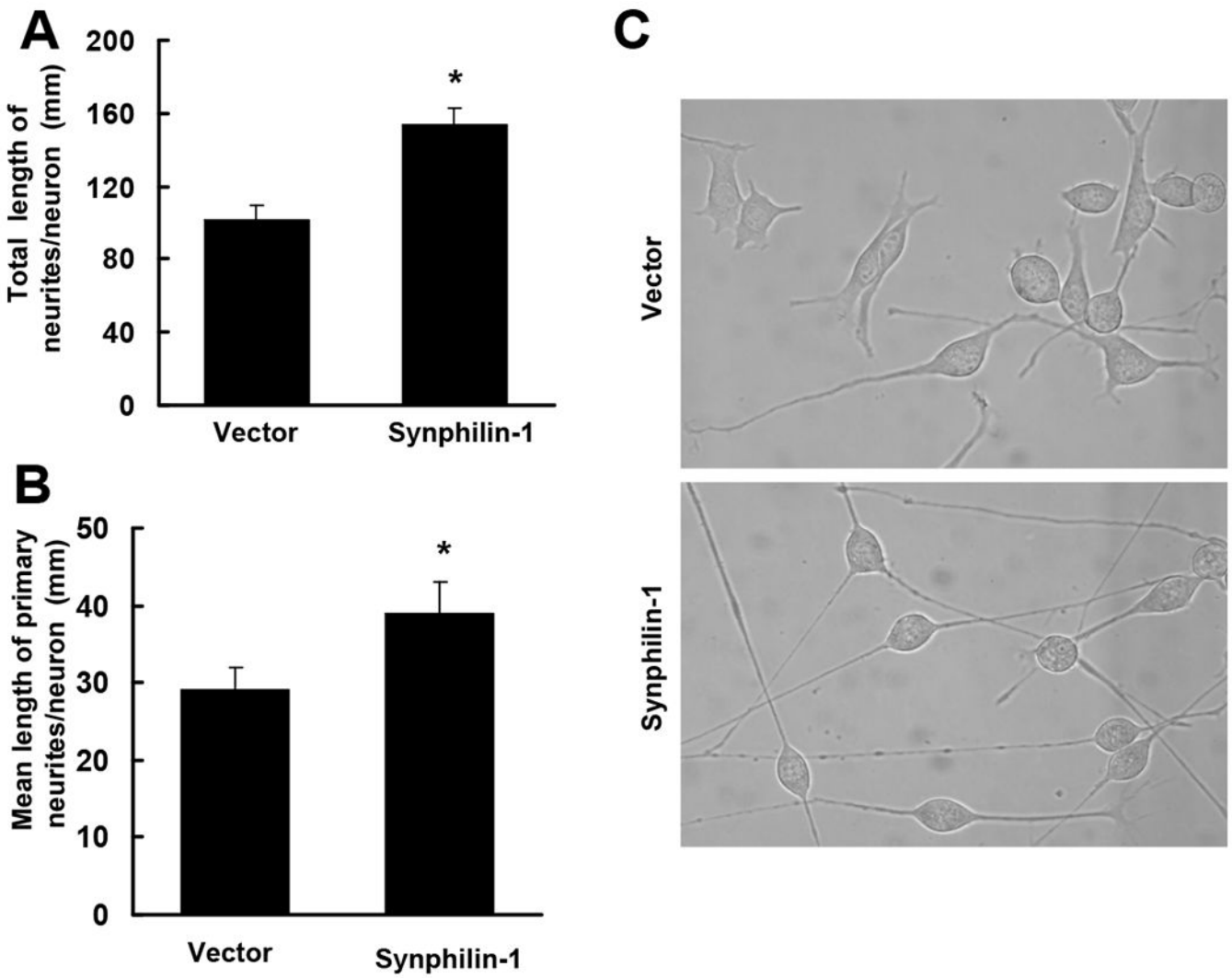

Fig. 2. Synphilin-1 enhances neurite outgrowth of N1E-115 cells.

Cells expressing empty vector or human synphilin-1 were differentiated in DMEM media with 0.5 % FBS and 1.5 % DMSO for 72 h. The images of cells were captured and neurite length was quantified. **A.** The total length of neurites per neuron. **B.** The mean length of primary neurites per neuron. Data are mean ± SE for three separate experiments. *p<0.05 versus cells expressing empty vector. **C.** Representative images of cells in each experimental group.

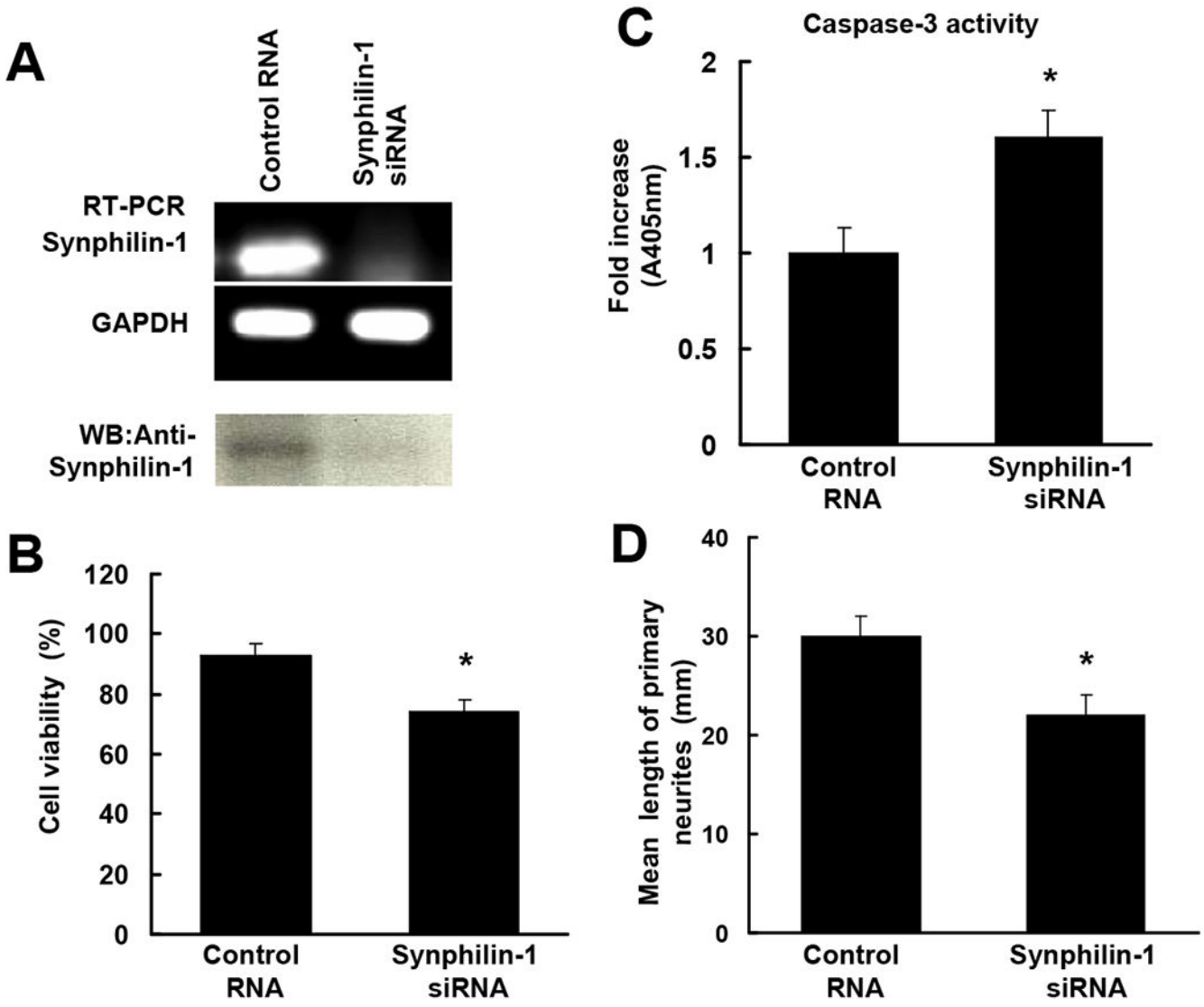

Fig. 3. Knockdown of endogenous synphilin-1 causes neuronal toxicity and reduced neurite outgrowth.

A. Cells were transfected with siRNA targeting mouse synphilin-1 for 72 h. Cell lysates were harvested and subjected to RT-PCR and western blot analysis using anti-synphilin-1 antibodies. Representative images of RT-PCR product (top) and western blot (bottom). **B** and **C.** N1E-115 cells were transfected with either synphilin-1 siRNA or scrambled control RNA for 72 h. **B.** Cell viability was measured by Trypan blue exclusion. **C.** Caspase-3 activity was measured using DEVD-p-nitroanilide as a substrate. **D.** N1E-115 cells were transfected with either synphilin-1 siRNA or scrambled control RNA. After 4 h transfection, media were removed and changed with differentiation media. After 3-day transfection and differentiation, the cell images were captured and neurite outgrowth was quantified. Data are mean ± SE for three separate experiments. *p<0.05 versus cells transfected with control RNA.

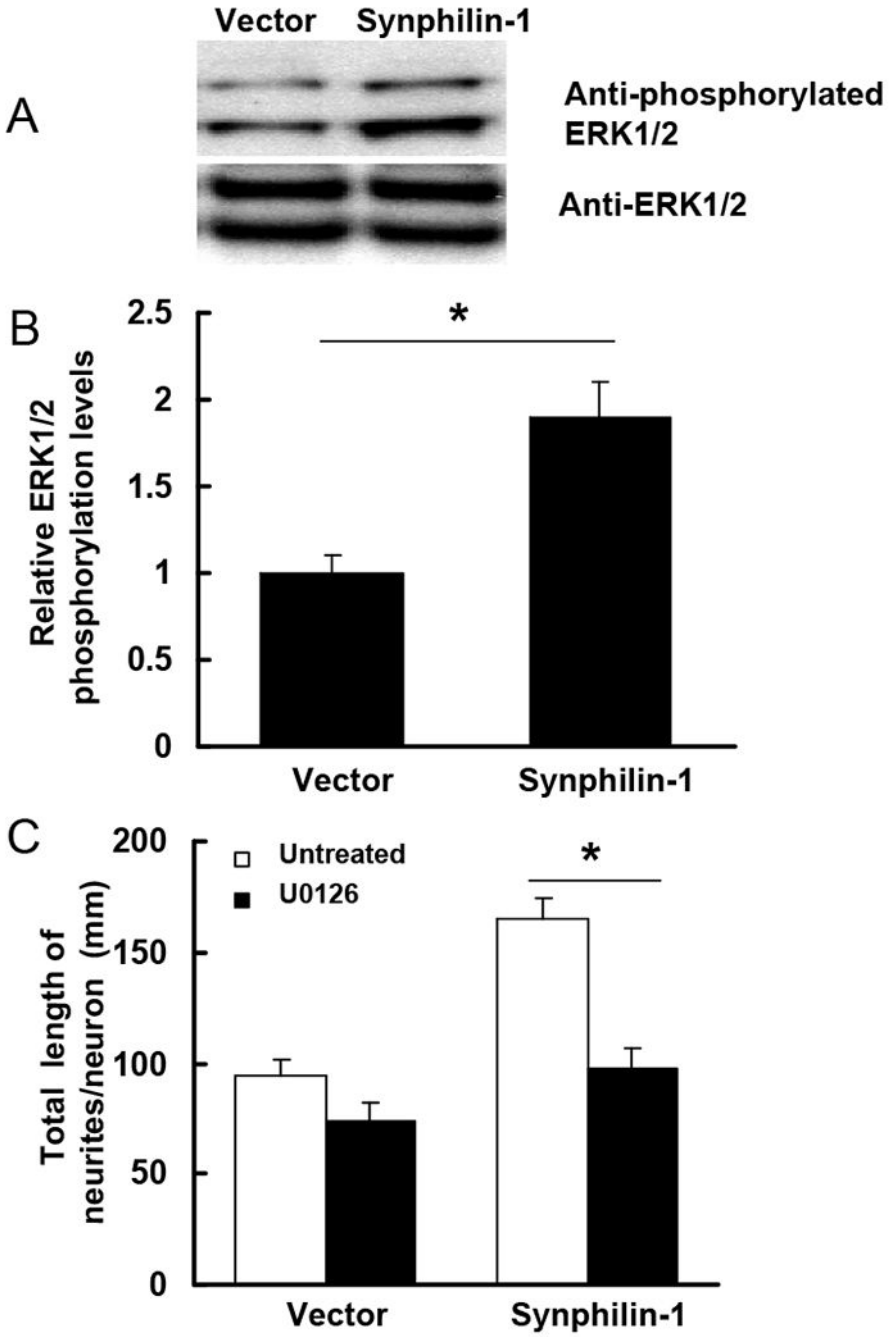

Fig. 4. Synphilin-1 increased ERK1/2 phosphorylation.

A. Cells expressing empty vector or human synphilin-1 were differentiated in DMEM media with 0.5 % FBS and 1.5 % DMSO for 72 h. Cells were harvested and cell lysates were subjected to western blot analysis using anti-phopshorylated ERK1/2 and anti-ERK1/2 antibodies. Shown are the representative blots three separate experiments. **B.** Quantification data of A. **C.** Cells expressing empty vector or human synphilin-1 were differentiated in DMEM media with 0.5 % FBS and 1.5 % DMSO for 24 h and then cells were left untreated or treated with 4 μM U0126 for 48 hours. The images of cells were captured and neurite

length was quantified. Data are mean ± SE for three separate experiments. *p<0.05 by ANOVA.

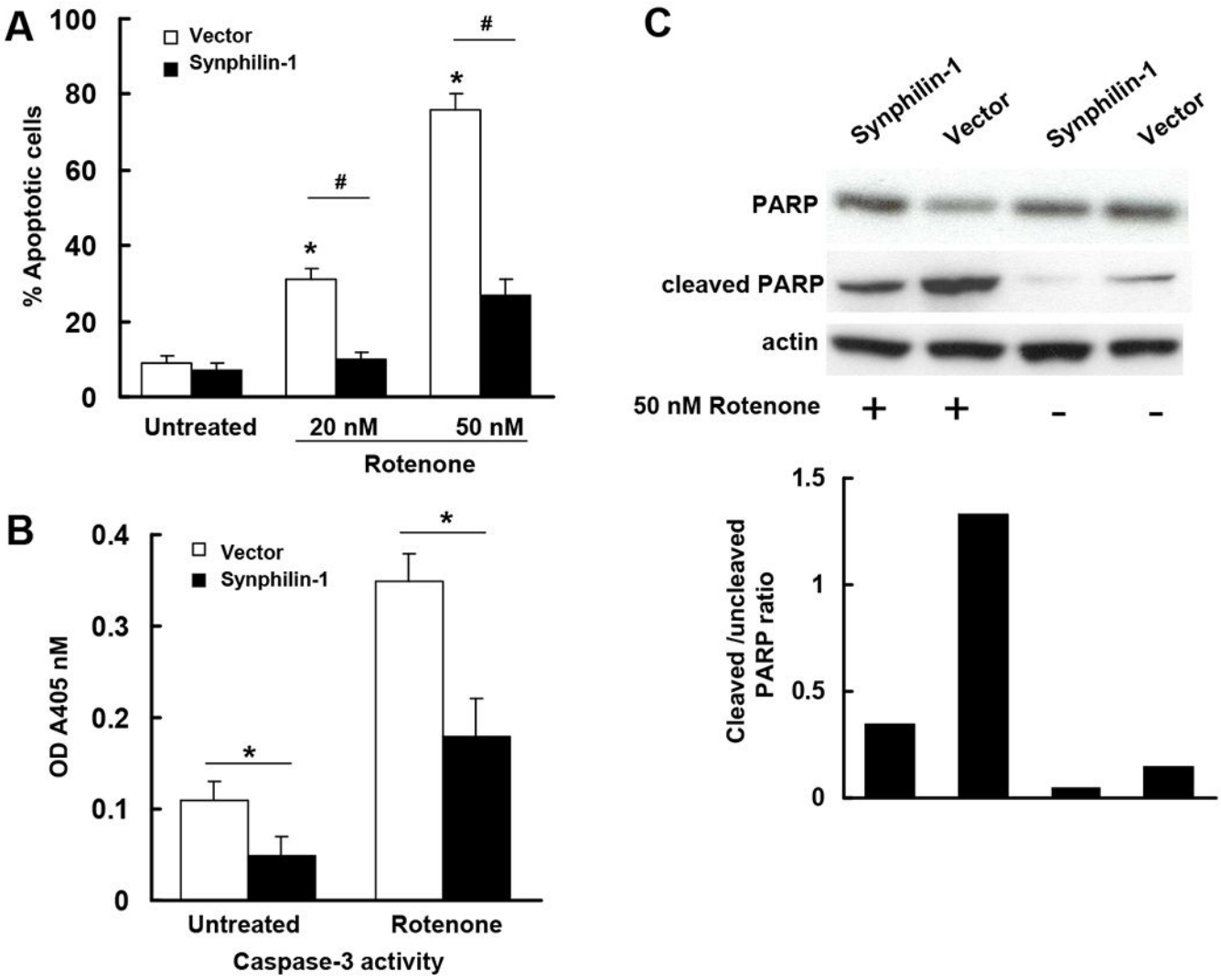

Fig. 5. Synphilin-1 protects against Rotenone-induced toxicity.

A. Cells expressing empty vector or human synphilin-1 were treated with Rotenone at concentrations of 0, 20, 50 nM for 24 hours. **A.** Apoptotic cells were measured by counting apoptotic nuclei using Hoechst/PI labeling. **B.** Cells expressing empty vector or human synphilin-1 were left untreated or treated with Rotenone at 50 nM concentration for 24 hours. Caspase-3 activity was measured using DEVD-p-nitroanilide as a substrate. Data are mean ± SE for three separate experiments. *p<0.05 versus untreated cells expressing empty vector. # p<0.05 versus Rotenone treated vector cells. **C.** Cells expressing empty vector or human synphilin-1 were left untreated or treated with Rotenone at 50 nM concentration for 24 hours. Cell lysates were harvested and subjected to western blot analysis using anti-PARP, anti-cleaved PARP and anti-actin antibodies. Top, representative western blot of three separate experiments. Bottom, quantification data of uncleaved and cleaved PARP ratio.