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MiR-126 Regulates Growth Factor Activities and Vulnerability to Toxic Insult in Neurons

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Abstract

Dysfunction of growth factor (GF) activities contributes to the decline and death of neurons during aging and in neurodegenerative diseases. In addition, neurons become more resistant to GF signaling with age. Micro (mi)RNAs are posttranscriptional regulators of gene expression that may be crucial to age- and disease-related changes in GF functions. miR-126 is involved in regulating Insulin/IGF-1/PI3K/AKT and ERK signaling and we recently demonstrated a functional role of miR-126 in dopamine neuronal cell survival in models of Parkinson's disease (PD)-associated toxicity. Here, we show that elevated levels of miR-126 increase neuronal vulnerability to ubiquitous toxicity mediated by staurosporine (STS) or Alzheimer's disease (AD)-associated amyloid beta 1-42 peptides ($A\beta_{1-42}$). The neuroprotective factors IGF-1, NGF, BDNF, and soluble amyloid precursor protein α (sAPPα) could diminish but not abrogate the toxic effects of miR-126. In miR-126 overexpressing neurons derived from Tg6799 familial AD model mice, we observed an increase in $A\beta_{1-42}$ toxicity but, surprisingly, both $A\beta_{1-42}$ and miR-126 promoted neurite sprouting. Pathway analysis revealed that miR-126 overexpression downregulated elements in the GF/PI3K/AKT and ERK signaling cascades, including AKT, GSK-3β, ERK, their phosphorylation, and the miR-126 targets IRS-1 and PIK3R2. Finally, inhibition of miR-126 was neuroprotective against both STS and $A\beta_{1-42}$ toxicity. Our data provide evidence for a novel mechanism of regulating GF/PI3K signaling in neurons by miR-126 and suggest that miR-126 may be an important mechanistic link between metabolic dysfunction and neurotoxicity in general, during aging, and in the pathogenesis of specific neurological disorders, including PD and AD.

Keywords

miR-126; Growth factors; Neurotoxicity; Neuroprotection; PI3K/AKT signaling; Aβ

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Conflict of Interest

The authors have no conflicts of interest.

Supplementary Material

Additional Material is available at *Molecular Neurobiology* online.

Introduction

Growth factor (GF) signaling pathways are essential for the function and survival of neurons, and their dysfunction has been implicated in the decline or death of neurons during aging and in neurodegeneration. In particular, Insulin/IGF-1 signaling pathways have been associated with age-related neuronal dysfunction and neurodegenerative diseases, such as Parkinson's (PD) and Alzheimer's disease (AD) [1–8]. The detailed mechanisms of GF dysfunctions in aging and neurodegeneration, however, are still not well understood. One current hypothesis is that neurons become more resistant to GF actions over time [4–6,8,9].

Micro (mi)RNAs regulate gene expression at the posttranscriptional level [10]. They are involved in all aspects of cell functions, including key signaling pathways that are important in the maintenance of cellular homeostasis and response to stress. There is evidence that miRNAs are involved in neuronal aging and in the pathogenesis of neurodegenerative disorders, but their precise relationships with GF activities are poorly understood [11–18]. In non-neuronal cells, IGF-1/PI3K and ERK signaling is, in part, modulated by miR-126 (reviewed in [14,19]), and in hepatocytes, upregulation of this miRNA has been associated with insulin resistance [20]. miR-126 has also been described in the neuronal context [21–27], and we have recently shown that miR-126 was upregulated in dopamine (DA) neurons in postmortem PD patients' brains and in pyramidal cortical neurons from Schizophrenia patients [14,28,29]. Moreover, our recent data showed that elevated levels of miR-126 in the DA neuronal context is neurotoxic to 6-OHDA by impairing IGF/PI3K/AKT and ERK signaling, while its inhibition is neuroprotective [28].

Because of the critical roles of GF-activated PI3K/AKT and ERK signaling in neuronal function and age- or disease-associated dysfunction, we hypothesized that miR-126 might play a general role in regulating or deregulating the effects of a variety of GFs in neurons, including nerve growth factor (NGF) whose diminished trophic effects on cholinergic neurons has been linked to cognitive decline in aging and AD [30–33]; soluble amyloid precursor protein α (sAPPα), one of the cleaved products of amyloid precursor protein (APP) that has neurotrophic and neuroprotective properties [34,35], acts synergistically with NGF and IGF-1, and can reverse the toxic effects of amyloid beta (Aβ) [36,34], another product of APP cleavage which may be the primary toxic agent in AD pathogenesis [37,35]; and BDNF which is involved in neuroplasticity and protection and has been associated with aging and a variety of neuropsychiatric and neurodegenerative disorders [38,39].

Here, we show that elevated levels of miR-126 in cortical and hippocampal neurons are neurotoxic and enhance the effects of ubiquitous toxicity mediated by staurosporine (STS), a general kinase inhibitor [40,41], and cell-specific toxicity due to $A\beta_{1-42}$ peptides, which directly interact with GF/PI3K signaling pathways [8,34,42,43]. Neurotoxicity could be diminished, but not abrogated, by IGF-1, NGF, BDNF, and sAPPα, and inhibition of miR-126 was neuroprotective. In neurons derived from Tg6799 mice, which is a model of familial AD (FAD) [44], we observed an increase in miR-126 expression and $A\beta_{1-42}$ toxicity, but surprisingly, and in contrast to littermate controls, both $A\beta_{1-42}$ and miR-126 promoted neurite sprouting. On the mechanistic level, overexpression of miR-126 caused a downregulation of factors in the PI3K and ERK signaling cascades. Our data provide

evidence for a novel mechanism of regulating GF/PI3K signaling in neurons by miR-126, and suggest a functional role of this miRNA, broadly, in aging neurons, and the pathogenesis of neurodegenerative diseases.

Material and Methods

Lentivirus vectors and cell transduction

The third generation lentivirus system was kindly provided by Drs. D. Trono and R. Zufferey, University of Geneva, Switzerland [45,46]. For neuron-specific expression of rno-miR-126, the Synapsin promotor was subcloned from pHIV7/Syn-EGFP (kindly provided by Dr. Atsushi Miyanohara (UCSD)) and inserted together with approx. 270 bp upstream and downstream sequences of the rat miR-126 pre-miRNA [28], and an IRES-GFP cassette downstream of the miRNA gene into the pRRL.cPPT.WPRE.Sin-18 backbone. The following primers were used to amplify the miRNA sequences from genomic DNA by PCR (given without flanking sequences for restriction sites): rno-miR-126 5′: GCACTATGCTGAGGGCTGATTC; rno-miR-126 3′: TTCTACACCTCCTCTCTCACC. The human sAPPα cDNA was cloned according to the strategy by Turner et al. [47] and inserted into the pRRL.PGK.cPPT.WPRE.Sin-18 backbone. The lentivirus construct CAG.NGF.GFP that expresses NGF from the chicken beta actin promoter with a CMV enhancer element was kindly provided by Dr. I. Verma, Salk Inst. La Jolla, CA [48,49]. For downregulating trkB expression, a set of 4 trkB siRNA-expressing lentivirus vectors was used (TRCN0000023416, TRCN0000023699, TRCN0000023701, TRCN0000023703; The RNAi Consortium (http://www.broadinstitute.org/rnai/public/); Thermo Scientific/ Dharmacon (http://dharmacon.gelifesciences.com/openbiosystems)).

All cloning experiments were based on standard molecular biology techniques. Virus production, concentration by ultracentrifugation, qRT-PCR- or p24 Elisa- (Clontech Laboratories, Mountain View, CA) based titer determination were be performed according to published protocols [50–52]. Average virus titers were 10^6–10^7 transducing units per μl.

Cell transductions were performed with multiplicity of infections (MOI) of 10–20 in the presence of 5–7 μg/ml hexadimethrine bromide (Polybrene, Sigma Aldrich, St. Louis, MO). Cells were incubated with virus and polybrene for 5–6 hrs before changing to fresh media. Expression of virus vectors were determined by GFP fluorescence and qRT-PCR using the miR-126 TaqMan® MicroRNA Assay from Life Technologies Corporation (Cat. # 4427975) and rat snoRNA (Cat. # U64702), or The Exiqon mmu-mir-126-5p and RNU5G control (Exiqon, Woburn, MA).

Animals and primary cell culture

All procedures involving animals were approved by the IACUC committees at McLean Hospital or Hanyang University. Primary cortical or hippocampal neurons were obtained from embryonic day 18 (E18) rat embryos (Sprague-Dawley, Charles River, MA), Tg6799 transgenic (MT) mice (Jackson Laboratory, Bar Harbor, ME), or littermate controls (LM) at postnatal day 1 as described [53,28]. Briefly, dissected cortex and hippocampus brain tissues were incubated in Accutase (Invitrogen) for 10 min at 37 °C, and then mechanically

triturated using fire-polished Pasteur pipettes. The cell suspension was plated onto glass coverslips in 24-well plates pre-coated with 37.5 μg/ml poly-D-lysin or poly-L-ornithine (Sigma), 2.5 μg/ml fibronectin (Sigma) or laminin (Sigma) at a density of 3.6×10^4 cells/cm^2 in neurobasal media (neurobasal media, 1% heat-inactivated FBS, penicillin, streptomycin, B27 supplement, glutamax, 2 mg/ml glutamic acid, and additional 1% horse serum for cortical cells (Invitrogen)) or DMEM/F-12 supplemented with 10% fetal bovine serum (GenDEPOT, San Diego, CA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO), 2 mM L-glutamin (Gibco, Carlsbad, CA), 5% B-27 supplement (Gibco, Carlsbad, CA), and 10 ng/ml bFGF (Invitrogen, Carlsbad, CA). Neurobasal media was half-changed with neural differentiation media (neurobasal media without serum and glutamic acid) 4 days after plating to induce cholinergic differentiation, and changed once a week. Cells were transduced with lentiviruses 6 days after plating, and media was half-changed with insulin-free media 5 days after transduction. Dox-inducible miR-126 expressing PC12 cell lines were used for miR-126 inhibition assays by transfection with 50–100 nM scrambled controls or miR-126 targeting Locked Nucleic Acids (LNA™, Exiqon, Woburn, MA) using lipofectamine (Life Technologies, Rockville, MD) as previously described [28].

Drug treatment and measurement of cell viability

Neuronal cultures were maintained in insulin-free media for 6 days before treatment with STS (Cayman Chemical Company, Ann Arbor, MI), $A\beta_{1\text{-}42}$ (AnaSpec Inc., Fremont, CA), IGF-1 (Peprotech, Rocky Hill, NJ), BDNF (Peprotech), or the IGF-1R tyrosine kinase inhibitor AG1024 (Millipore, Tenecula, CA). STS was dissolved in DMSO (100 μM stock solution) and applied for 24 hrs at a final concentration of 0, 25, 50, 100, or 300 nM in the primary cultures or PC12 cells, respectively. Lyophilized $A\beta_{1\text{-}42}$ peptides were dissolved in 1% NH_4OHM and then immediately diluted with 1X Dulbecco's Phosphate Buffered Saline (PBS) without $MgCL_2$ and $CaCl_2$ (Gibco/Life Technologies #14200-075), as a 150 μM final stock solution for stabilization. The $A\beta_{1\text{-}42}$ stock solution was incubated at 37 °C for 72 hrs to produce oligomers. For titration, cells were treated with 0, 0.1, 1, 2 or 10 μM $A\beta_{1\text{-}42}$ oligomers for 48–72 hrs. 1 μM of final concentration was applied in primary cultures and 2 μM in PC12 cells. Formation of toxic $A\beta_{1\text{-}42}$ oligomers were confirmed in Western blots and toxicity titration assays. IGF-1 (20 ng/ml) was added 30 min before addition of STS or $A\beta_{1\text{-}42}$. AG1024 was prepared as 2 mM stock solution in DMSO and 0.5 μM of the final concentration was added 30 min prior to IGF-1 treatment.

Cell viability was determined using the activity of lactate dehydrogenase (LDH) in collected cell culture medium, according to the manufacturer's instructions (Roche, Indianapolis, IN), and absorbance measured at 490 nm.

Protein sample preparation and Western blot

Protein samples were purified from harvested cells in lysis buffer (100 mM Tris-HCl (pH 7. 5), 10 mM EDTA, 10 mM EGTA, 1% SDS, 20 mM NaCl (Sigma, St. Louis, MO)) containing 1 mM PMSF, Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc., Waltham, MA). Lysates were centrifuged at 14,000 rpm for 30 min at 4 °C and supernatants collected and stored at −80 °C before use. Equal amounts of

protein sample were used for Western blots as previously described [50,28]. Western blots were performed with the following primary antibodies: PI3-kinase p85β (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,250); IRS-1, AKT, phospho-AKT, ERK, phospho-ERK, GSK-3β and phosphoGSK-3β (Cell Signaling; 1:1,250), 6E10 (Covance, Princeton, NJ, 1:1,000), 22C11 (Millipore, 1:1,000), and β-actin (Covance, 1:10,000). Alkaline phosphatase (AP)-conjugated anti-mouse or -rabbit secondary antibodies (Invitrogen, 1:2,500) and Immun-Star™ AP Substrate (Bio-Rad, Hercules, CA) were used for protein detection. sAPPα in the media from PGK.sAPPα transduced PC12 cells was measured in Western blots using the 22C11 or 6E10 antibody. Quantification of immunoreactive bands was performed using Image J (NIH, http://rsb.info.nih.gov/ij/). Experiments were performed at least in triplicate for the same samples.

Immunocytochemistry

Cultured neurons were fixed in 4% paraformaldehyde (Fisher Scientific, Waltham, MA) and rinsed with phosphate-buffered saline (PBS). Cells were then incubated with blocking buffer (10% normal goat serum and 0.1% Triton X-100) for 30 min at room temperature. Immunostaining was performed using primary antibodies against choline acetyltransferase (ChAT; Millipore, Tenecula, CA, 1:500), beta-III tubulin (Tuj1; Covance, 1:1,000), or *Tau* (Tau46, Cell Signaling, 1:500), followed by incubation in Alexa Fluor 568 or Alexa Fluor 488 conjugated anti-mouse or -rabbit secondary antibodies (Invitrogen; 1:1,000), or alkaline phosphate substrate solution (Vector Lab, Burlingham, CA). After counterstaining with 1 μg/ml Hoechst 33342 (Sigma) for 2 min, cover glasses were mounted onto glass slides using Gel-Mount anti-fade media (Electron Microscopy Sciences, Hatfield, PA).

Cell counting and neurite length measurement

Neurons were counted from images taken with an inverted Zeiss Axiovision microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) connected to a fluorescence light source and digital camera (Zeiss AxioCam HRc). In each condition, 30 sections per coverslip were quantified and a total of 300–1400 cells were analyzed. Two investigators, blinded to the treatment groups, independently performed counting and duplicate analyses. Neurite lengths of *Tau* positive cells were counted from 16 microscopic images per condition and analyzed using Image J (NIH, http://rsb.info.nih.gov/ij/) by two independent assessors blinded to the conditions. The length of neurites were measured using the freehand line tool by drawing lines starting from the basal line of the cell surface to the end of the neurite projection on the image. Each protruding tip from the basal line of the soma was counted as an individual neurite (83–114 cells and 300 neurites per group). The lengths and counts of neurites were presented as a relative value compared to untreated LM control group.

Statistical analysis

Microsoft Excel software (Microsoft Corp., Redmond, WA) was used for statistical analyses. Data were compared between different experimental groups or within a group using unpaired two-tailed Student's t-test. Differences of comparison were considered statistically significant when p values were less than 0.05 ($p < 0.05$).

Results

Overexpression of miR-126 increases STS toxicity and decreases the neuroprotective effects of IGF-1

To express GFP or miR-126 together with GFP specifically in neurons, we used lentivirus vectors that contain the Synapsin promoter (Syn.GFP and Syn.miR-126, respectively) (Fig. 1a, b). Virus-transduced cortical or hippocampal primary cultures were tested in STS or $A\beta_{1\text{-}42}$ toxicity assays (Supplementary Material, Fig. S1a–c) in combination with trophic factors expressed from viral vectors (CAG.NGF [48,49] and PGK.sAPPα (Supplementary Material, Fig. S1d)), or were supplemented to the cultures (IGF-1 and BDNF).

We first tested the effects of miR-126 on toxicity to STS, which causes a general inhibition of protein kinase activities and whose effects can be ameliorated by IGF-1 [40,41]. Overexpression of miR-126 increased STS toxicity and reduced the protective effects of IGF-1 when compared to naïve and virus GFP controls (Fig. 1c). The effect of IGF-1 was inhibited in the presence of the IGF-1 receptor (IGF-1R) inhibitor AG1024 in both control and miR-126 transduced cells, confirming that miR-126 acts on IGF-1 signaling pathways [54,20,55,28] (Fig. 1d). To confirm apoptotic neuronal cell death measured by LDH, we immune-stained the cultures with βIII-tubulin and the nuclear marker Hoechst 33342. Apoptotic neurons were characterized by swollen cytoplasm and condensed or fragmented nuclei (Supplementary Material, Fig. S2). The percent of apoptotic neurons over all apoptotic cells was increased in Syn.miR-126 transduced cultures, demonstrating neuron-specificity of the miR-126 effects. When we examined miR-126 expression levels, we found that the endogenous miRNA was increased (1.5–2 fold) in STS and IGF-1-treated cells (Fig. 1e), while in the Syn.miR-126 transduced cells, STS caused a decrease and IGF-1 a slight increase in miRNA levels, which could have been associated with different Synapsin promoter regulation as a consequence of cell treatment.

Overexpression of miR-126 is neurotoxic, increases $A\beta_{1\text{-}42}$ toxicity, and modulates neuroprotection by IGF-1, NGF, BDNF, and sAPPα

We next focused on other factors that are associated with PI3K or ERK signaling and which are involved in the neuronal aging process or disease-specific pathogenesis. $A\beta_{1\text{-}42}$, which is thought to be the primary toxic agent in AD pathogenesis [37,35] acts on the Insulin/IGF-1 or NGF receptor and, thus, competes with Insulin/IGF-1 or NGF on PI3K signal activation [34]. In contrast, sAPPα can act synergistically with NGF and IGF-1 to reverse amyloid Aβ toxicity [36,34]. We, therefore, tested the effects of overexpressed miR-126 on toxic $A\beta_{1\text{-}42}$ peptides, and neuroprotection by IGF-1, NGF, and sAPPα in $A\beta_{1\text{-}42}$ vulnerable cell types, including cortical and hippocampal neurons (Figs. 2 and S3). No differences between naïve and control Syn.GFP transduced cells were observed regarding $A\beta_{1\text{-}42}$ toxicity and the effects of trophic factors (Supplementary Material, Fig. S3a, b). In the miR-126 overexpressing neurons, the miRNA alone was cell toxic and exaggerated $A\beta_{1\text{-}42}$ toxicity (Fig. 2a, Supplementary Material, Fig. S3c). IGF-1, NGF, and sAPPα reduced $A\beta_{1\text{-}42}$ toxicity in both virus controls and miR-126 overexpressing cells, and sAPPα acted synergistically with IGF-1 and NGF, and the effects of IGF-1 in Syn.GFP control, Syn.miR-126, and Syn.miR-126/PGK.sAPPα transduced cells could be inhibited in presence

of AG1024 (Fig. 2b). Assessment of miRNA expression levels revealed no marked changes in the virus-transduced cells and factor-treated cells (Fig. 2c, d).

We also tested the effects of miR-126 overexpression in BDNF treated cortical neurons, because recent data have shown that BDNF protects cortical neurons from Aβ toxicity [56]. BDNF had a neuroprotective effect towards $A\beta_{1-42}$ toxicity in both Syn.GFP controls and Syn.miR-126 transduced cells, but could not fully abrogate the increased neurotoxicity caused by miR-126 (Fig. 3a). In both conditions, the neuroprotective effects of BDNF were diminished when the expression of its receptor trkB was inhibited by trkB-siRNAs, demonstrating that miR-126 affects the BDNF/trkB signaling cascade (Fig. 3a, b).

miR-126 increases $A\beta_{1-42}$ toxicity in Tg6799 neurons and modulates neurite sprouting

We next evaluated the effects of miR-126 in primary cortical cultures from Tg6799 mutant mice, because these animals exhibit a massive accumulation of $A\beta_{1-42}$ in the brain [44]. Overexpression of miR-126 increased $A\beta_{1-42}$ toxicity in both littermate controls (LM) and Tg6799 mutant (MT) cells and to a greater extent in the latter cell population (Fig. 4a). As shown in the rat primary cultures (Fig. 2), endogenous miR-126 expression was not significantly upregulated in LM neurons after $A\beta_{1-42}$ treatment, but increased in Tg6799 cells (Fig. 4b).

In addition to neurotoxicity we also evaluated the effects of miR-126 on neurite sprouting in *Tau*-immune-stained LM control and Tg6799 MT neurons. In the LM cells, $A\beta_{1-42}$ slightly decreased the length of neurites per cell and this effect was significantly exaggerated when miR-126 was overexpressed (Fig. 4c). Untreated Tg6799 neurons had a reduction in neurite lengths when compared to LM controls and both $A\beta_{1-42}$ treatment and miR-126 overexpression significantly increased neurite lengths to levels seen in the untreated LM controls, however, to a lesser extent in $A\beta_{1-42}$ treated miR-126 overexpressing cells (Fig. 4c). These data indicate that in normal neurons elevated levels of miR-126 exaggerate an inhibitory effect of $A\beta_{1-42}$ on neurite sprouting, while in Tg6799 neurons, miR-126 promotes neurite sprouting to a similar extent as seen with $A\beta_{1-42}$, but has no synergistic effect.

Increased levels of miR-126 affect the expression of factors in IGF-1/PI3K/GSK-3β and ERK signaling

To evaluate the effects of increased miR-126 on cellular signaling events in STS or $A\beta_{1-42}$ toxicity, we measured the expression of factors in the IGF-1/PI3K/AKT and ERK pathways (Figs. 5 and 6, Supplementary Material, Fig. S4), including GSK-3β, which is regulated by pAKT and has been linked to *Tau* phosphorylation, amyloid production, and neuronal death [57].

In STS toxicity, IRS-1 the adapter molecule of IGF-1R and a validated target of miR-126 [20,58] was upregulated in controls when exposed to IGF-1, but its expression was reduced in the miR-126 overexpressing cells (Fig. 5a and b). In IGF-1 untreated controls, STS-treatment did not change the expression levels of AKT, ERK, and GSK-3β, but caused a downregulation of pAKT, pERK, and pGSK-3β, and altered their respective ratios (Fig. 5a

and c). Addition of IGF-1 increased AKT, pAKT, pERK, and pGSK-3β, but not ERK and GSK-3β. In contrast, overexpression of miR-126 in STS and IGF-1 treated neurons caused downregulation of AKT, ERK, GSK-3β, pAKT, pERK, and pGSK-3β, an upregulation of the AKT/pAKT and ERK/pERK ratios, while the pGSK-3β/GSK-3β ratio in the IGF-1 condition was decreased.

In $A\beta_{1-42}$ toxicity, IGF-1, NGF, and sAPPα caused upregulation of IRS-1 in control neurons, but downregulation when miR-126 was overexpressed (Fig. 6a and b, Supplementary Material, Fig. S4a). The expression levels of p85β, a component of the PI3K complex and another validated target of miR-126 [55,59,54], were also increased in the controls, but unchanged or only slightly increased in the miR-126 transduced cells (Fig. 6a and b, Supplementary Material, Fig. S4a). In controls, $A\beta_{1-42}$ and GF treatment were associated with upregulation of AKT, pAKT, ERK, pERK, and GSK-3β, and to lesser extent pGSK-3β (Fig. 6a and c, Supplementary Material, Fig. S4b). In contrast, except for an upregulation of pERK and pGSK-3β, these molecules were downregulated in miR-126 overexpressing neurons. Moreover, in $A\beta_{1-42}$ and factor treated controls the pAKT/AKT ratios were largely unchanged, while the pERK/ERK and pGSK-3β/GSK-3β ratios were reduced. In contrast, miR-126 overexpression caused a slight decrease of the pAKT/AKT ratios and a striking increase in the pERK/ERK and pGSK-3β/GSK-3β ratios. Altogether, overexpression of miR-126 in neurons had profound impacts on the activation status of signaling pathways related to IGF-1, NGF, and sAPPα in STS and $A\beta_{1-42}$ toxicity.

Inhibition of miR-126 is neuroprotective

Finally, we evaluated whether inhibition of miR-126 would be neuroprotective to STS and $A\beta_{1-42}$ toxicity. For this, we used a toxicity assay based on virus-transduced PC12 cells as previously published in [28]. Consistent with our data on 6-OHDA, inhibition of miR-126 reduced STS toxicity and enhanced the neuroprotective effects of IGF-1 (Fig. 7a), and also diminished the toxic effects of $A\beta_{1-42}$ (Fig. 7b).

Discussion

Neuronal functions depend on a balance between neurotoxicity and neuroprotection, with the latter mediated in part by GF/PI3K signaling pathways. In aging and age-related neurological diseases slow progressive neuronal dysfunction is a consequence of an imbalance of many mechanisms, which may be general or disease-specific. Because of its implication in aging and neurodegenerative diseases, Insulin/IGF-1 signaling is one of the pathways of great interest. For example, there is evidence that AD may be a metabolic disorder with an impairment of glucose utilization and energy production, as a consequence of insulin deficiency and resistance to Insulin/IGF-1/PI3K signaling in the brain (sometimes called “brain-diabetes” or “type-3-diabetes”) [2–8,60–62]. Dysfunctional Insulin/IGF-1 signaling contributes to all aspects of AD-type neurodegeneration, including dysregulated Aβ, *Tau* hyperphosphylation, and oxidative stress [8,63–67,35]. The PI3K signaling pathway is also used by other GFs, including BDNF and NGF, and there is evidence that resistance to NGF signaling contributes to the loss of cholinergic neurons and cognitive decline seen both in aging and dementia [30–33]. The molecular mechanisms underlying

these disturbances are largely unknown, and our data provide evidence that miR-126 may play a role in these processes.

miR-126 is involved in regulating IGF-1/PI3K/AKT, p38 MAPK, or ERK signaling in a multitude of non-neuronal cells [14,54,19,68–70]. In the neuronal context, it is expressed in rodent or human cortical, hippocampal, cerebellum, ventral mesencephalon, and motor neurons [21,22,26,23,27], and the miRNA is differentially expressed in cortex, hippocampus, and cerebellum during early human development [27]. Recently, we found an upregulation of miR-126 in postmortem DA neurons from PD and in pyramidal cortical neurons from Schizophrenia patients [14,28,29], and in DA cell systems elevated levels of miR-126 increased neurotoxic to 6-OHDA by downregulating IGF-1/PI3K and ERK signaling [28]. Our new finding that miR-126 also increases STS and $A\beta_{1-42}$ toxicity in cortical and hippocampal neurons and diminishes the protective effects of a variety of growth factors suggests that miR-126 could be involved in the general survival mechanisms of neurons and that its deregulation could contribute to neuronal dysfunction in aging and in combination with cell- and disease-specific events to neurodegeneration. Thus, miR-126 may be an important mechanistic link between metabolic dysfunction and neurotoxicity.

In AD pathogenesis, there is evidence that Insulin/IGF-1 affects Aβ metabolism and function, e.g., stimulating its trafficking from the Golgi apparatus, its extracellular secretion, and increasing transcription of Aβ degrading proteins [63–67,35]. In turn, Aβ acts on the Insulin/IGF-1 or NGF receptor and, thus, competes with Insulin/IGF-1 or NGF on PI3K signal activation [34] (Fig. 8). Aβ appears to alter Insulin/IGF-1 signaling by inappropriately increasing the activation of PI3K/AKT/mTOR and JNK signaling and feedback inhibition of normal activation, thereby, reducing normal Insulin/IGF-1 functions, including normal on/off switching and the protective effects of FOXO activation and mTOR inactivation [43]. Also, intracellular Aβ appears to directly interfere with PIK3 activation of AKT and subsequent GSK-3β phosphorylation which is partly responsible for *Tau* hyperphosphorylation and the regulation of *Tau* gene expression [71–73,57,74,34,42]. In contrast, as shown in our study and previously demonstrated by Luo et al. [36] and Jimenez et al. [34], sAPPα acts synergistically with NGF or IGF-1 and reverses amyloid Aβ toxicity. Similarly, a neuroprotective role of BDNF against Aβ toxicity in cortical neurons [56], was also observed in our study. The finding that elevated levels of miR-126 exaggerate Aβ toxicity in both GF untreated and protective conditions suggests that dysfunctional miR-126 may be a central factor in its pathogenesis via specifically deregulating PI3K/AKT signaling cascades (Fig. 8). This notion is corroborated by the observation that cortical neurons from Tg6799 mice had elevated miR-126 and an increase in Aβ toxicity. Both, miR-126 or Aβ alone, or in combination, negatively affected neurite sprouting in littermate control neurons, but increased sprouting in the cells with FAD-associated mutations. FAD mutations seem to have inhibiting or promoting effects on neurite growth and plasticity, and the latter has been associated with increased GSK3β activity and $A\beta_{1-24}$ induced phosphorylation of *Tau* [75,76]. However, the exact role that miR-126 plays in these functions needs further investigation.

A series of miR-126 targets have been described (summarized in [14]), including factors in ERK signaling, which was also downregulated in the miR-126 overexpressing neurons. One

of these targets is SPRED1 [54,28], which inhibits MAPK/ERK signaling and this pathway is involved in neuronal cell function, aging, and degeneration, including *Tau* regulation [77]. Recently, delta-like 1 homolog (Dlk1), an epidermal growth factor-like homeotic protein that can control extracellular IGF-1 levels by binding IGF-binding protein 1 (IGFBP1)/IGF-1 complexes [78], was identified as a novel target of miR-126 [79]. Dlk1 activates the MEK/ERK pathway [80], plays a functional role in motor neurons [81], and has been identified as a novel target of the orphan nuclear receptor Nurr1 in meso-diencephalon DA neurons [82]. miR-126 may also not exclusively regulate PI3K signaling. For example, in the hippocampus of aged Ames dwarf and growth hormone receptor knock out mice, miR-470, -669b, and -681 were identified as potential suppressors of IGF-1R and AKT [17]; in glioblastoma cells miR-7 inhibits IGF-1/AKT signaling by targeting IRS-1 [83]; and in transfected N2A cells that stably express APP, overexpression of miR-98 downregulates its target IGF-1 and indirectly increases Aβ production and *Tau* phosphorylation [84]. In addition, miR-320, which is expressed in neurons, including pigmented neurons in the substantia nigra [22], influences IGF-1 signaling through regulation of IGF-1/2, IGF-1R, phosphoinositide-3-kinase regulatory subunit 1 (p85α; PIK3R1), and the glucose transporter 4 (SLC2A4) [85,86].

Although levels of miR-126 appear to be low in neurons, small increases seem to have striking effects on cell function. Our observation that neurotoxicity is a consequence of 4-fold upregulated miR-126 is consistent with findings in hepatocytes, in which Rotenone-induced dysfunctional mitochondria caused insulin resistance that was associated with a 3- to 4-fold increase of miR-126, a 75% decrease of IRS-1, an insulin-induced reduction of Glycogen, and downregulation of the pAKT/AKT, and pGSK-3β/GSK3-3β ratios [20]. Together, these data indicate that small changes of miR-126 levels could have profound effects on cell function pointing to a potential potent role of this miRNA in fine-tuning and balancing (or dysbalancing) GF/PI3K signaling in neurons. In fact, inhibition of miR-126 is neuroprotective and increases the neuroprotective effects of GFs without seemingly compromising normal neuronal cell function. Given its low expression levels, this could indicate that miR-126 may be dispensable for normal cellular homeostasis, while being detrimental when upregulated in context of neuronal insult. This notion is supported from studies on miR-126 k.o. mice [87,79]. While about 40% of mice die embryonically or perinatally with severe vascular abnormalities, surviving animals appear to be normal with no reported vascular defects or brain damage, supporting the hypothesis that miR-126 may be dispensable in adult brain cells. Therefore, diminishing or eliminating miR-126's function may be a strategy to promote GF activities. The single locus of miR-126 in intron 7 of the EGFL7 gene makes it an optimal target for gene editing, since its targeted deletion does not alter the expression of EGFL7 in homozygous transgenic mice [87].

In summary, our results provide evidence for a novel mechanism of regulating GF/PI3K and ERK signaling by miR-126 in neurons, and suggest that GF/pathway deregulation by dysfunctional miR-126 may be a contributing mechanism in the resistance of neurons to GF signaling events during aging and in the pathogenesis of neurodegenerative diseases, such as PD and AD. While small increases in miR-126 increase neuronal vulnerability to toxic insult in both normal cells and seemingly augmented in cells with disease-associated mutations,

such as in FAD, inhibiting miR-126 confers neuroprotection without compromising normal neuronal cell functions, suggesting that non-functional or dispensable miR-126 in neurons may have therapeutic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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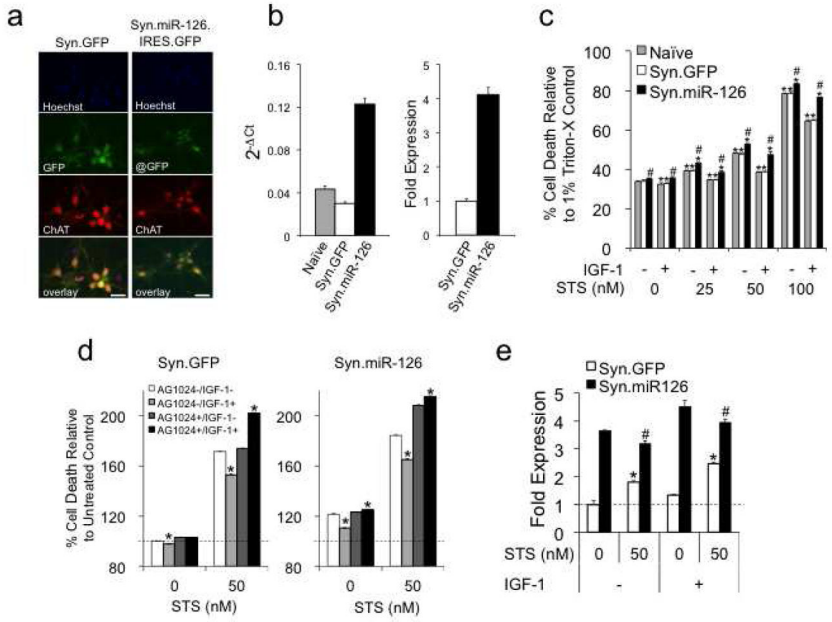

Fig. 1.

Overexpression of miR-126 increases STS toxicity and impairs a protective effect of IGF-1. (**a**, **b**) Transduction of cortical neurons with Syn.GFP control or Syn.miR-126.IRES.GFP (Syn.miR-126) revealed expression of GFP (**a**) and 4-fold upregulation of miR-126 (**b**). Neurons were immunostained for ChAT (red), and miR-126 expression was measured by qRT-PCR. Size bars = 20 μm. (**c**) LDH assays demonstrate an increase of STS toxicity and a reduction of neuroprotection by IGF-1 in miR-126 transduced cortical neurons. Data are plotted as relative cell death to Triton X (1%) induced maximum cell death. *: $p < 0.05$ comparing treated to untreated condition. #: $p < 0.05$ comparing miR-126 to Syn.GFP control. (**d**) The effects of IGF-1 can be inhibited by AG1024 (0.5 μM). Data are plotted as percent cell death relative to untreated Syn.GFP control. *: $p < 0.05$ comparing IGF-1 treated to IGF-1 untreated condition. (**e**) Expression levels of endogenous and virus-expressed miR-126 in STS and IGF-1 treated neurons. *: $p < 0.05$ comparing Syn.GFP to untreated condition. #: $p < 0.05$ comparing Syn.miR-126 to untreated condition.

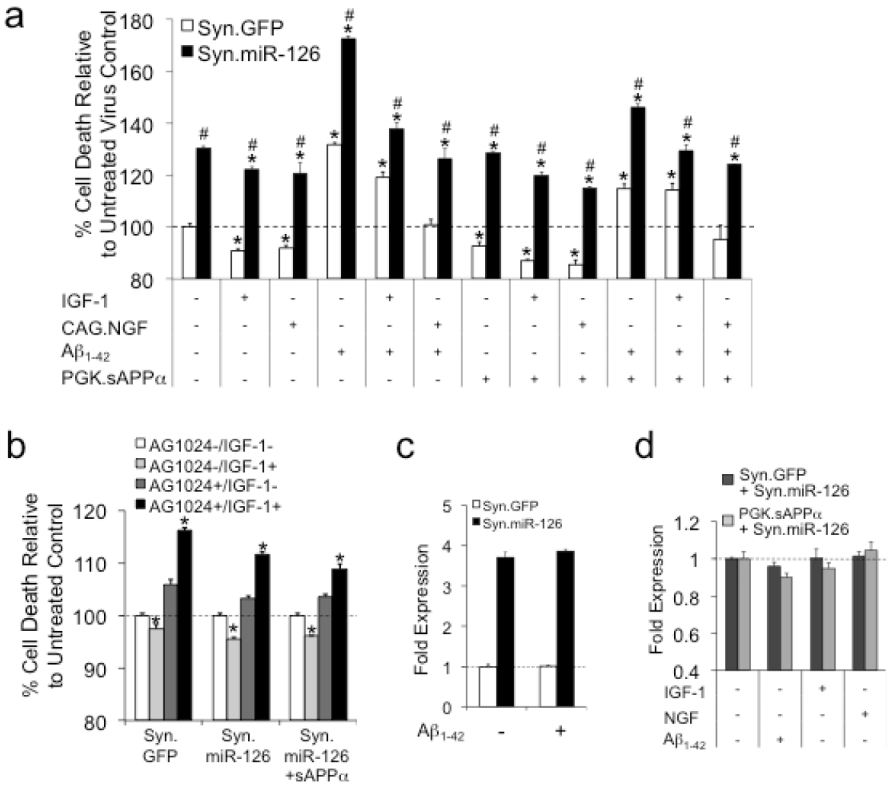

Fig. 2.

Overexpression of miR-126 modulates $A\beta_{1\text{-}42}$ toxicity and the neuroprotective effects of IGF-1, NGF, and sAPPα. (**a**) LDH assays show that overexpression of miR-126 is neurotoxic and increases the toxic effects of $A\beta_{1\text{-}42}$. IGF-1, NGF, and sAPPα protect cortical neurons against $A\beta_{1\text{-}42}$ toxicity in both control and miR-126 virus-transduced neurons with a synergistic effect of sAPPα in combination with IGF-1 and NGF. Data are plotted relative to untreated Syn.GFP control. *: $p < 0.05$ comparing treated to untreated condition. #: $p < 0.05$ comparing Syn.miR-126 to Syn.GFP controls. (**b**) The neuroprotective effects of IGF-1 can be abrogated by AG1024. Data are plotted as percent cell death relative to untreated control. *: $p < 0.05$ comparing IGF-1 treated to IGF-1 untreated condition. (**c**, **d**) $A\beta_{1\text{-}42}$, and IGF-1 or NGF do not significantly change miR-126 levels in virus controls or miR-126 overexpressing cells (**c**), or in control/miR-126 or sAPPα/miR-126 transduced neurons (**d**).

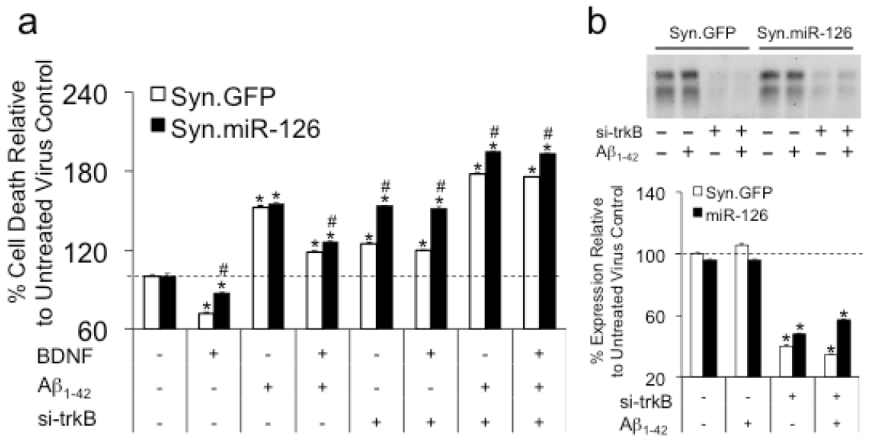

Fig. 3.

Overexpression of miR-126 modulates $A\beta_{1\text{-}42}$ toxicity and the neuroprotective effects of BDNF in cortical neurons. (**a**) LDH assays show that overexpression of miR-126 increases the toxic effects of $A\beta_{1\text{-}42}$ and that BDNF (10 ng/ml) protects against $A\beta_{1\text{-}42}$ toxicity in both virus control and miR-126 transduced neurons, and that the neuroprotective effects of BDNF are inhibited in neurons that express trkB siRNAs. *: $p < 0.05$ comparing treated to untreated condition. #: $p < 0.05$ comparing Syn.miR-126 to Syn.GFP controls. (**b**) Western blots demonstrating >75% downregulation of trkB in trkB-siRNA expressing neurons. *: $p < 0.05$ comparing si-trkB+ to si-trkB− condition.

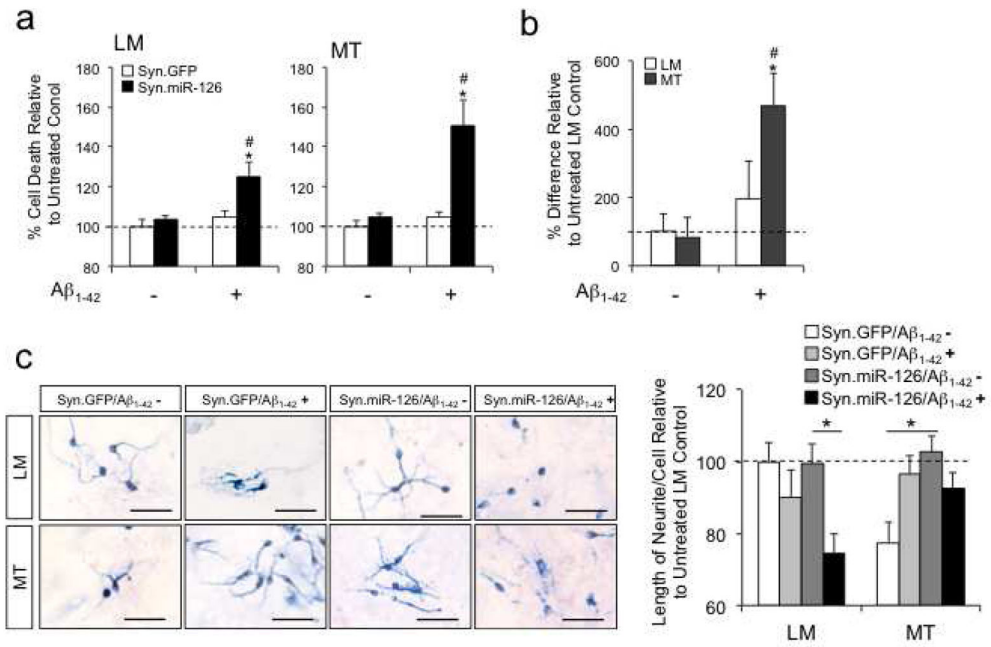

Fig. 4.

Overexpression of miR-126 increases $A\beta_{1\text{-}42}$ toxicity in Tg6799 neurons and modulates neurite sprouting. (**a**) LDH assays show that overexpression of miR-126 increases the toxic effects of $A\beta_{1\text{-}42}$ in littermate (LM) controls and Tg6799 mutant (MT) cortical neurons. *: $p < 0.05$ comparing treated to untreated control. #: $p < 0.05$ comparing Syn.miR-126 to Syn.GFP controls. (**b**) The endogenous miR-126 levels are significantly increased in $A\beta_{1\text{-}42}$ treated Tg6799 MT neurons, but not in LM controls. *: $p < 0.05$ comparing treated to untreated control. #: $p < 0.05$ comparing MT to LM controls. (**C**) Assessment of neurite lengths in *Tau*-immunostained neurons shows that $A\beta_{1\text{-}42}$ treatment significantly decreases neurite sprouting in miR-126 overexpressing LM controls. Tg6799 MT neurons exhibit less neurite sprouting than LM controls, and both miR-126 and $A\beta_{1\text{-}42}$ significantly increase sprouting, which was partly abrogated by $A\beta_{1\text{-}42}$ in the miR-126 overexpressing cells. *: $p < 0.05$.

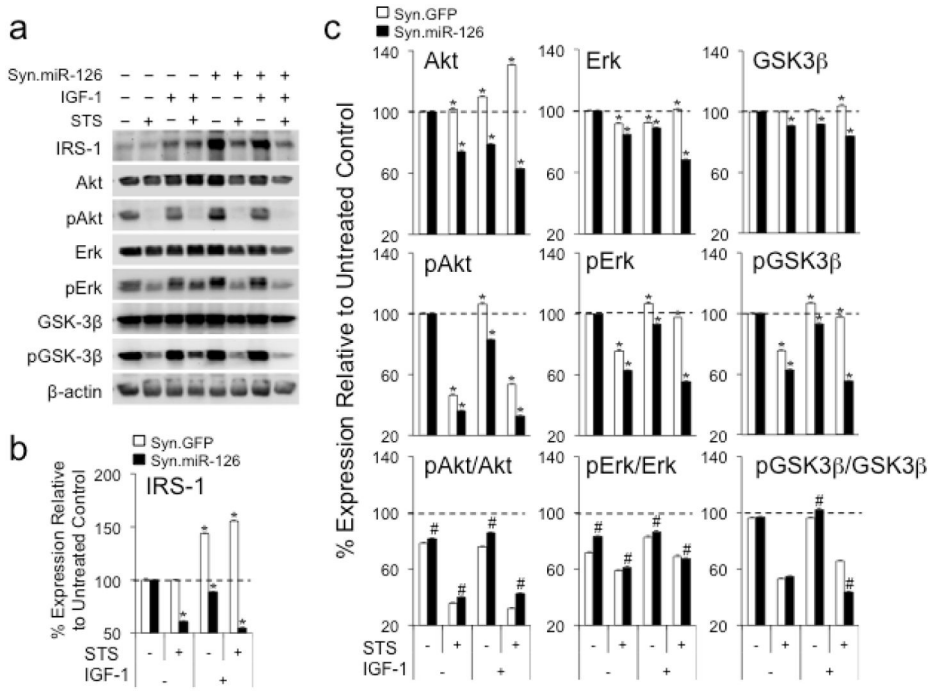

Fig. 5.

Overexpression of miR-126 modulates IGF-1/AKT/GSK-3β and ERK signaling in STS and IGF-1 treated neurons. Quantification of Western blots (**a**) shows that IRS-1 (**b**), AKT, pAKT, ERK, pERK, GSK-3β, and pGSK-3β (**c**) are downregulated in miR-126 overexpressing cells when compared to virus-treated controls. In addition, the pAKT/AKT, pERK/ERK and pGSK-3β/GSK-3β ratios are increased, and the pGSK-3β/GSK-3β ratios decreased in STS/IGF-1 treated and miR-126 transduced cells. Data are plotted as relative percent expression to untreated controls. *: $p < 0.05$ comparing treated to untreated condition. #: $p < 0.05$ comparing ratios for Syn.miR-126 to ratios of Syn.GFP controls.

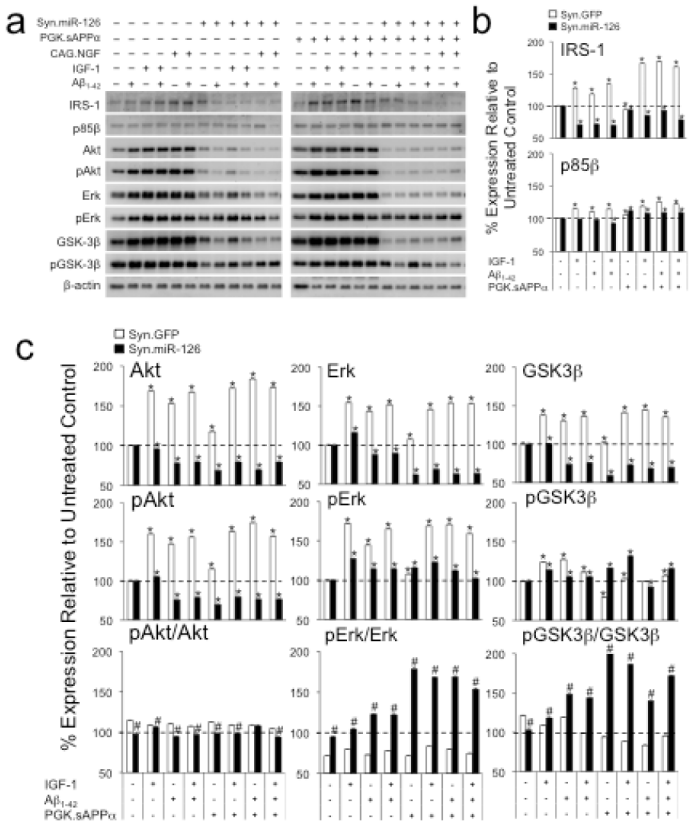

Fig. 6.

Overexpression of miR-126 modulates AKT/GSK-3β and ERK signaling in $A\beta_{1\text{-}42}$, IGF-1 and sAPPα treated neurons. Quantification of Western blots (**a**) shows that IRS-1 is upregulated in $A\beta_{1\text{-}42}$ and IGF-1 or sAPPα treated neurons, but downregulated when miR-126 is overexpressed (**b**). Expression levels of p85β are also increased in controls, but to lesser extent in miR-126 transduced cells. (**c**) $A\beta_{1\text{-}42}$ and IGF-1 or sAPPα treatment cause upregulation of AKT, pAKT, ERK, pERK, GSK-3β, and to a lesser extent pGSK-3β in control cells. In contrast, except for pERK and pGSK-3β, miR-126 overexpressing neurons show downregulation of these molecules. While the pAKT/AKT ratios are unchanged and the pERK/ERK and pGSK-3β/GSK-3β ratios are reduced in the controls, miR-126 overexpressing cells have reduced pAKT/AKT and markedly increased pERK/ERK and pGSK-3β/GSK-3β ratios. In the miR-126 overexpressing cells sAPPα alone or in combination with IGF-1 increases the pERK/ERK and pGSK-3β/GSK-3β ratios. *: $p < 0.05$ comparing treated to untreated condition. #: $p < 0.05$ comparing ratios for Syn.miR-126 to ratios of Syn.GFP controls.

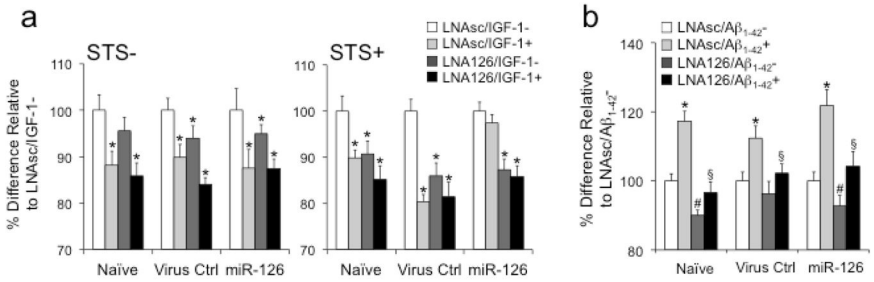

Fig. 7.

Inhibition of miR-126 is neuroprotective. (**a**, **b**) Naive PC12 cells, or transduced cell lines that express a virus control or doxycycline (Dox)-inducible miR126 [28] were transfected with 70–100 nM scrambled (LNAsc) or miR-126 targeting LNAs (LNA126) and treated with 300 nM STS and 20 ng/ml IGF-1 (**a**), or 2 μM $A\beta_{1\text{-}42}$ (**b**). LDH assays show that both IGF-1 and LNA126 improve cell survival in STS-untreated cells and are neuroprotective in STS-treated conditions (*: $p < 0.05$ comparing cell death relative to untreated LNAsc controls). Similarly, LNA126 improve survival of $A\beta_{1\text{-}42}$-untreated cells and are neuroprotective in $A\beta_{1\text{-}42}$-treated conditions. (*: $p < 0.05$ comparing LNAsc/$A\beta_{1\text{-}42}$ + to LNAsc/$A\beta_{1\text{-}42}$ −; #: $p < 0.05$ comparing LNA126/$A\beta_{1\text{-}42}$− to LNAsc/$A\beta_{1\text{-}42}$ −; §: $p < 0.05$ comparing LNA126/$A\beta_{1\text{-}42}$+ to LNAsc/$A\beta_{1\text{-}42}$+).

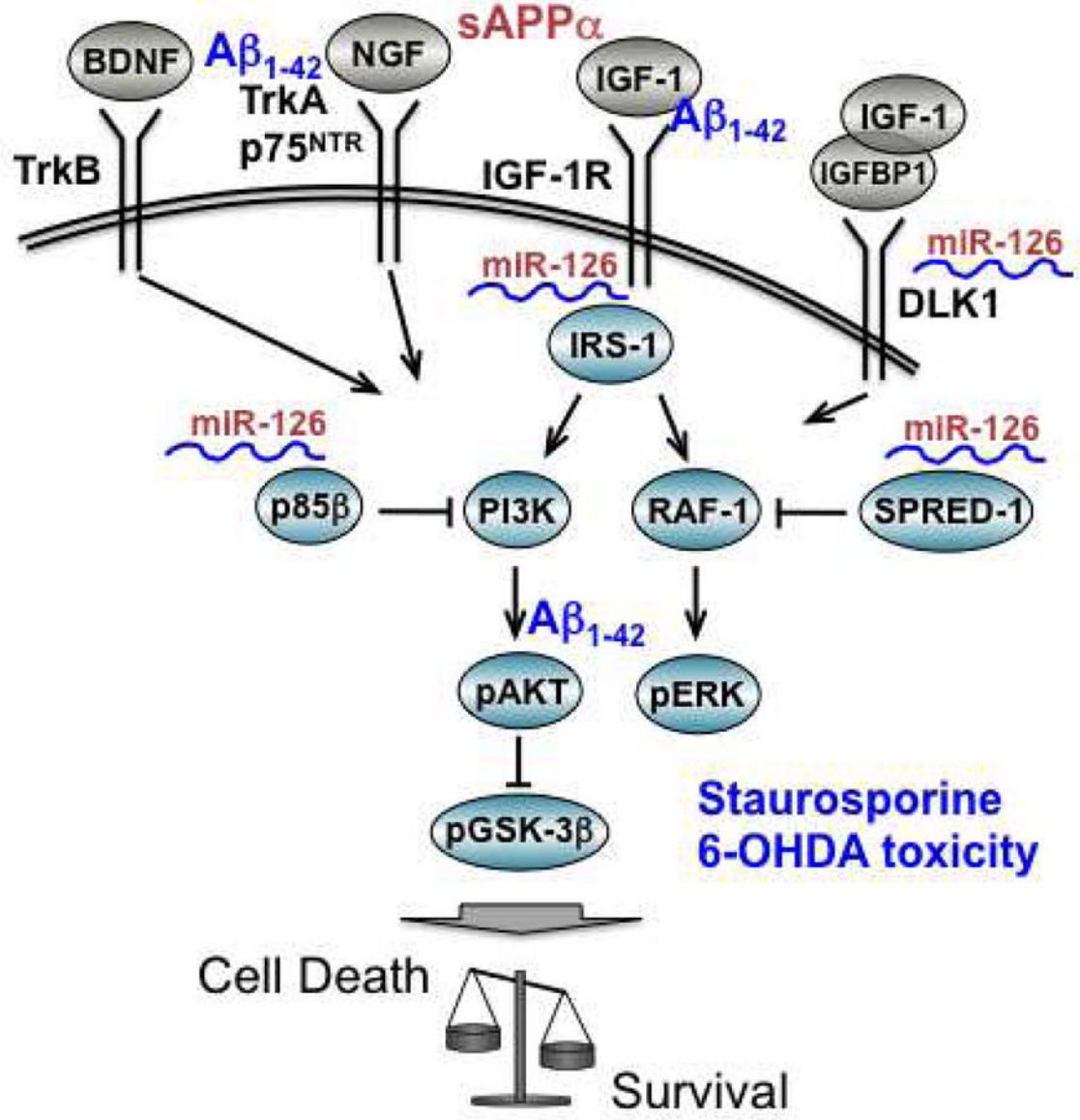

Fig. 8.

Schematic summary of the effects of miR-126 in neurotoxicity and GF protection. miR-126 targets key factors in PI3K/AKT/GSK3β and ERK signaling, including IRS-1, p85β, SPRED1, and DLK-1, and increases the effects of pan-neuronal (STS) or disease-associated (6-OHDA, $A\beta_{1-42}$) toxicity. $A\beta_{1-42}$ competes with IGF-1 and NGF on IR/IGF-1R or TrkA/$p75^{NTR}$ receptor binding and appears to directly interfere with PI3K activation of AKT. sAPPα acts synergistically with NGF or IGF-1 and reverses $A\beta_{1-42}$ toxicity. The recently identified miR-126 target DLK1 binds IGFBP1/IGF-1 complexes and can indirectly or directly influence IGF-1/PI3K and ERK signaling.