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Research paper

Inhibitor of DNA binding 2 promotes axonal growth through upregulation of Neurogenin2

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ABSTRACT

Manipulation of developmentally regulated genes presents a promising strategy to enhance the intrinsic growth capability of adult neurons. Inhibitor of DNA binding 2 (Id2), a negative regulator of bHLH transcriptional factors, promotes axonal growth after its forced expression in post-mitotic neurons. Neurogenin2 (Ngn2) is a neural specific bHLH factor which controls neuronal fate and drives neuronal differentiation during development. In this study, we investigated the mechanism of Id2 in promoting axonal growth and revealed that Ngn2 contributed to the growth-activating role of Id2 in neurons. Ngn2 expression was upregulated with increased Id2 activity by assessing RNA and protein levels. Forced expression of Id2 or Ngn2 in cortical neurons significantly promoted axonal growth with little effect on dendrites. Furthermore, knockdown of Ngn2 impaired the axonal growth promoting effect of Id2, implying that the effect of Id2 on axonal growth depends on Ngn2. These findings suggest that elevation of neuronal Ngn2 may be a new therapeutic strategy to stimulate axonal regeneration.

1. Introduction

Axons in the mammalian central nervous system (CNS) grow vigorously during early development, and this intrinsic axonal growth ability declines progressively after birth (Goldberg et al., 2002). As neurons mature, they enter a quiescent state of axonal growth and fail to regenerate their axons after CNS injury (Fawcett and Verhaagen, 2018; Mar et al., 2014). The loss of intrinsic axonal growth capability is attributed, at least partly, to the developmental downregulation of genes promoting axonal growth as well as the upregulation of genes suppressing axonal growth (Moore et al., 2011; Moore et al., 2009; Park et al., 2008). Thus, identification and characterization of developmentally regulated genes affecting axonal growth may lead to promising approaches for promoting axonal regeneration. The expression of one such gene, inhibitor of DNA binding 2 (Id2), is high early in development and declines with neuronal maturation (Tzeng and de Vellis, 1998), and overexpression of Id2 increased axonal growth (Ko et al., 2016; Lasorella et al., 2006; Yu et al., 2011), implying a role of Id2 in intrinsic growth regulation.

Id2 belongs to the inhibitor of differentiation/DNA binding (Id) protein family considered to serve as a dominant negative regulator of the basic helix-loop-helix (bHLH) transcription factors (Benezra et al., 1990). Members of the Id protein family share a homologous HLH domain which can dimerize with bHLH transcription factors. Id proteins lack the basic DNA binding region, therefore, the heterodimers of Id proteins with bHLH factors are unable to bind the *E*-box consensus sequences in DNA to activate downstream gene transcription (Wang and Baker, 2015). During neuronal maturation, Id2 protein is targeted for degradation by a complex of E3 ubiquitin ligase anaphase-promoting complex (APC) and its co-activator Cdh1 (Cdh1-APC) (Konishi

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et al., 2004; Lasorella et al., 2006). Degradation of Id2 can be suppressed by mutating the key residues in its D-box motif which is the recognition site of Cdh1, and introducing the D-box mutated Id2 (Id2DBM) promotes axonal growth (Kim and Bonni, 2007; Lasorella et al., 2006). We also found that overexpression of Id2DBM in dorsal root ganglion (DRG) neurons promoted axonal growth in culture, prevented axonal retraction and stimulated a few axons regenerating into the lesion after spinal cord injury (Yu et al., 2011). More recently, Ko et al. (2016) reported phosphorylation of Id2 at serine 14 by Akt augmented protein stability and promoted axonal growth through an interaction with the cytoskeletal protein radixin. Several potent axonal growth inhibitory genes are induced upon Id2 degradation (Lasorella et al., 2006), suggesting a mechanism of Id2 in regulating axonal growth. However, how the elevation of Id2 activates intrinsic axonal growth program is still not well understood.

In this study, we performed RNAseq analysis of gene expression changes in neurons following overexpression of Id2DBM. We identified upregulation of Neurogenin2 (Ngn2), a proneural gene expressed in neural progenitor cells (Sommer et al., 1996). Our results indicate that Id2 promotes axonal growth through elevating Ngn2 expression in neurons.

2. Materials and methods

2.1. Adenovirus, AAV9 and lentivirus generation

Id2DBM, a degradation resistant form of Id2, was cloned as previously reported (Yu et al., 2011). The key residues Arginine (AGG) and Leucine (CTG) in the D-box motif of Id2 were changed to Glycine (GGG) and Valine (GTG), respectively. The Id2DBM adenovirus (Ad-Id2DBM-3 × Flag-EGFP) was generated by inserting the Id2DBM into the GV314 (CMV-MCS-3 × FLAG-SV40-EGFP) vector with a C-terminal 3 × Flag tag and with an unfused EGFP reporter co-expressed. The Id2 overexpressing AAV9 virus (AAV9-Id2–3 × Flag-EGFP) was generated by inserting mouse Id2 into GV410 (CMV bGlobin-MCS-3 × FLAG-2A-EGFP) with a C-terminal 3 × Flag tag and with an unfused EGFP reporter co-expressed. The Ngn2 overexpressing lentivirus (LV-Ngn2–3 × Flag-Cherry) was constructed by inserting mouse Ngn2 into CV186 (Ubi-MCS-3 × FLAG-SV40-Cherry-IRES-puromycin) with a 3 × Flag tag fused to its C-terminus and with an unfused Cherry reporter co-expressed. The Ngn2 shRNA knockdown lentivirus (LV-shNgn2-Cherry) was created by inserting the shRNA sequences into GV298 vector (U6-MCS-Ubiquitin-Cherry-IRES-puromycin) with an unfused Cherry reporter co-expressed. The three sequences of shRNA targeting Ngn2 were 5′-TCGCCAGGGACTGTATCTA-3′; 5′-AGGATGCC AAGCTCACGAA-3′; 5′- ATGCACGAGTGCAAGCGT-3′. The control sequence was 5′-TTCTCCGAACGTGTCACGT-3′. After screening, the sequence (5′- ATGCACGAGTGCAAGCGT-3′) that showed the best knockdown was used for further experiments. The packaging service of adenovirus, AAV9, and lentivirus was provided by GeneChem (Shanghai, China).

2.2. Primary cortical neuron isolation

The cerebral cortex was isolated from brains of E17-E18 mouse embryos and digested with 0.05% trypsin (Gibco, Grand Island, NY) as previously reported (Jin et al., 2018). After dissociation into a single cell suspension, cells were cultured in Neurobasal medium containing B27 (1: 50, *v*/v) supplemented with Glutamax (all from Gibco).

2.3. Neurite outgrowth assay

The capacity of neurite outgrowth was assessed using mouse cortical neurons following functional gain of Id2 and functional gain or loss of Ngn2. For Id2DBM overexpression, dissociated mouse cortical neurons were adjusted to a density of 1×10^4/mL, added to equal amounts of Ad-Id2DBM-3 × Flag-EGFP or Ad-EGFP virus (0.5 μL/well, 2×10^{10} pfu/mL) and then plated onto 24-well plates (1 mL/well) containing coverslips pre-coated with PLL (200 μg/mL). After 3, 5, and 7 days culture *in vitro* (DIV), cells were fixed and stained for imaging. DIV3 and DIV5 neurons were stained with mouse anti-β-tubulin III (1:500, Sigma, Saint Louis, MO) and chicken anti-GFP (1:1000, Abcam, Cambridge, MA). DIV7 neurons were stained with rabbit anti-MAP2 (1:250, Millipore, Temeula, CA), mouse anti-Tau1 (1:250, Millipore), and chicken anti-GFP. To visualize the growth cone morphology, some DIV3 neuron samples were also co-stained with TexRed-Phalloidin (1:50, Life Technologies Corporation, Eugene, Oregon). In another experiment, dissociated mouse cortical neurons were first cultured for 4 days before being infected with Ad-Id2DBM-3 × Flag-EGFP or Ad-EGFP virus. After being cultured for an additional 3 days (DIV7(4 + 3)), cells were fixed and stained with MAP2, β-tubulin III, and GFP antibodies.

For Ngn2 overexpression, cortical neurons were infected with an equal amount of LV-Ngn2–3 × Flag-Cherry or LV-Cherry lentivirus (1 μL/well, 1×10^9 TU/mL) and then plated in 24-well plates (1×10^4 cells/well) containing coverslips pre-coated with PLL. Cells were fixed at DIV3 and stained with β-tubulin III.

For Ngn2 knockdown and Id2DBM overexpression, cortical neurons were first infected with an equal amount of LV-shNgn2-Cherry or LV-shControl-Cherry (1 μL/well, 1×10^9 TU/mL) and plated onto 24-well plates (1×10^4 cells/well) containing PLL-coated coverslips. After being cultured for 2 days, Ad-Id2DBM-3 × Flag-EGFP or Ad-EGFP was then added and cultured for an additional 3 days before fixation. Cells were stained with β-tubulin III and GFP.

Images were obtained using a Zeiss 700 LSM confocal microscope. Neurons exhibiting a polarized morphology with multiple short neurites (dendrites) and one long neurite (axon) are included for analysis. Neurite length and growth cone size were measured using Image J (Schneider et al., 2012). Data were collected from at least 3 independent cultures, and a total of 66–180 neurons were measured for each group.

2.4. RNAseq analysis

Dissociated mouse cortical neurons were infected with Ad-EGFP or Ad-Id2DBM-3 × Flag-EGFP and cultured for 3 days on 3.5 cm dishes. Total RNAs was extracted using Takara MiniBEST Universal RNA Extraction kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instruction. RNA concentrations and quality were assessed using a NanoDrop 2000 (Thermo Fisher). RNA integrity was evaluated by the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA). Sequencing service was provided by BioMarker Technologies (Beijing, China). The sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (New England Biolabs Inc.) and were sequenced on an Illumina HiSeq X Ten platform (Illumina Inc.). The clean reads of high quality were obtained and aligned to mouse reference genome and the Fragments Per Kilobase of transcript per Million fragments mapped (FPKM) values were calculated. Differential expression analysis of two samples was performed using the EBSeq R package. The false discovery rate (FDR) < 0.01 and the absolute value of log2(fold change) > 1 were set as the thresholds for significant differential expression.

2.5. Western blotting

To study changes in endogenous Id2 in cultured neurons during maturation, mouse cortical neurons were harvested at 3, 7, 9, 12, 15, 18, and 22 days. Protein lysates were collected at indicated time points for immunoblotting with Id2 (1:400, Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (1:1000, Sangon Biotech, Shanghai, China) antibodies. Protein lysates of the same batches of dissociated cells from E18 mouse cortical tissues were used as a baseline control.

For assessing the protein level changes of Ngn2 and Tal1 induced by Id2DBM expression, dissociated mouse cortical neurons were freshly harvested or were firstly cultured for 4 days on 3.5 cm dishes, followed by infection with Ad-Id2DBM-3 × Flag-EGFP or Ad-EGFP virus. Three days after infection, protein lysates from the neurons were collected and subjected to immunoblot analysis using anti-Ngn2 (1:600, Cell Signaling Technology, Danvers, MA) and rabbit anti-Tal1 (1:2000, Cell Signaling Technology) antibodies. Immunoblotting with chicken anti-GFP (1:1000, Abcam), mouse anti-Flag (1:1000, Sigma), and rabbit anti- Id2 antibodies (1:400, Santa Cruz) were also performed for confirming the correct expression of Id2 by the recombinant viruses in each experiment. To assess the effects of gain and loss of Ngn2 function, mouse cortical neurons were infected with LV-Ngn2–3 × Flag-Cherry/LV-Cherry lentivirus or with LV-shNgn2-Cherry (#1, #2, #3)/LV-shControl-Cherry on 12-well plates. After being cultured for 3 days, cell lysates were collected and subjected to immunoblot assays with anti-Flag antibody for confirming Ngn2 overexpression, and with anti-Ngn2 (1:600, Cell Signaling Technology) to evaluate Ngn2 knockdown.

2.6. AAV9 injection

Experiments were performed on adult female C57BL/6 mice (20-22 g). All experiments were approved by the Laboratory Animal Ethics Committee of Jinan University and conducted following their guidelines. Under anesthesia with 1.25% avertin administered intraperitoneally at 20 mL/kg body weight, the T13 thoracic vertebra was immobilized using a pair of stainless steel arms attached to a mouse stabilizer (Wu et al., 2018). A T13 laminectomy was conducted, and the dura was opened using iridectomy scissors. Microinjection of 0.5 μl of AAV9-EGFP or AAV9-Id2–3 × Flag-EGFP was performed stereotaxically into the spinal cord using a glass micropipette. The entry points into the spinal cord were 0.4 mm lateral to the midline bilaterally with injections into the ventral spinal cord at a depth of 1.0 mm. The glass micropipette remained *in situ* following the injection for 5 min to prevent leakage. After the micropipette was removed, the muscles and skin were sutured in layers, and mice were allowed to recover on a heating pad. One mL saline was injected subcutaneously to prevent dehydration. Four weeks after the AAV9 injection, mice were killed by transcardial perfusion with PBS (pH 7.4), followed by ice-cold 4% PFA in PBS. Spinal cords were removed for cryosection, followed by immunostaining with GFP and Ngn2 antibodies. Images were obtained using a Zeiss 700 LSM confocal microscope.

2.7. Statistical analysis

Data were presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6 (GraphPad, San Diego, CA) by one-way or two-way ANOVA with Tukey's *post hoc* test to compare differences between individual groups or by a two-tailed Student's *t*-test to compare differences between 2 groups. Statistical significance was defined as $p < .05$.

3. Results

3.1. Id2 expression declined with neuronal maturation and Id2DBM enhanced axonal growth

Id2 was highly expressed in cells freshly dissociated from E18 mouse cortex tissue. However, it was drastically reduced in cortical neurons after 3 days *in vitro* (DIV3); the level of Id2 further decreased with days in culture (DIV3-DIV22) (Fig. 1A and B). Expression of Id2DBM in cortical neurons significantly increased both total neurite length (DIV3, $p < .0001$; DIV5, $p = .0001$) and the length of the longest neurite (DIV3, $p < .0001$; DIV5, $p = .007$) at DIV3 and DIV5 (Fig. 1C and D). The size of the growth cone was also increased in neurons expressing Id2DBM ($p = .0012$) (Fig. 1E and F).

Neurons at DIV7 were stained with MAP-2 and Tau-1 to quantify dendritic and axonal growth. We found that Id2DBM mainly promoted axonal growth ($p = .0234$) with a minimal effect on dendrite growth ($p = .6468$) (Fig. 2A and B). The promotional effect on axonal growth ($p < .0001$) was still observed when Id2DBM was introduced at a later stage of development (DIV4 and fixed at DIV7), while dendritic growth remained unaltered ($p = .4639$) (Fig. 2C and D).

3.2. Elevation of Id2 induced Ngn2 expression in cultured cortical neurons in vitro and in adult spinal cord neurons in vivo

To reveal the mechanism or possible downstream pathways of Id2, RNAseq analysis was performed to screen for gene expression changes in neurons following expression of Id2DBM. The differentially expressed genes with FDR < 0.01 and the absolute value of log2(fold change) > 1 are shown in Table S1. The neural-specific bHLH transcription factor Ngn2 was one of the genes that showed significant upregulation with Id2DBM overexpression. We confirmed the upregulation of Ngn2 protein by Western blot analysis ($p = .0011$) (Fig. 3A and B) which was consistent with its RNAseq result. Overexpressing Id2DBM at a later stage of neuronal differentiation (DIV4 and fixed at DIV7) also resulted in elevation of Ngn2 protein expression ($p = .0002$) (Fig. 3C and D). Another bHLH factor, TAL1, was significantly downregulated on RNAseq analysis and was excluded from further study as its protein level was unchanged by Western blot analysis (Fig. 3A-D).

We then evaluated whether Id2 could upregulate Ngn2 *in vivo* by expressing Id2 or EGFP in the adult mouse spinal cord. We found a low level of Ngn2 expression in adult spinal cord neurons in the EGFP-expressing animals. In contrast, significant Ngn2 elevation was observed in the neurons overexpressing Id2 (Fig. 3E). Ngn2 as a transcription factor was thought to be mainly localized in the nucleus of neurons. However, the re-expression of Ngn2 in adult spinal cord neurons induced by Id2 was both localized in the nucleus and distributed extensively in the neuronal cytoplasm (Fig. 3E).

3.3. Overexpression of Ngn2 in cortical neurons promoted neurite outgrowth

A lentiviral vector system was used to increase expression of Ngn2 in freshly dissociated cultured cortical neurons, which were fixed 3 days later. At DIV3, the average total neurite length and longest neurite of the neurons expressing Ngn2 were significantly longer than that of the control ($p = .002$ and $p = .003$, respectively) (Fig. 4). This recombinant Ngn2 contains a C-terminally fused 3 × Flag tag, we therefore checked the distribution of Ngn2-Flag fusion protein by immunostaining with a Flag antibody, and found that Ngn2-Flag protein was predominantly distributed in the cytoplasm rather than in neuronal nucleus (Fig. 4C). A considerable amount of Flag signal was also found along the neurite and at the growth cones (Fig. 4C). This indicated that, in addition to work as a transcription factor, Ngn2 may function in the neuronal cytoplasm or growth cone to regulate neurite outgrowth.

3.4. Knockdown of Ngn2 level blocked the effect of Id2DBM on neurite outgrowth

To further determine the role of Ngn2 on Id2 induced axonal growth, shRNA targeting Ngn2 was introduced prior to Id2DBM overexpression. Three different shRNAs were screened, and shRNA #3 was shown to have the best knockdown effect (Fig. 5A). Both the total neurite length and the longest neurite length of the neurons expressing shControl + Id2DBM were significantly increased as compared to those expressing the shControl + EGFP ($p < .0001$). The neurite length promotion effect of Id2DBM was markedly reduced with Ngn2 knockdown. The total neurite length ($p < .0001$) and the longest neurite length ($p = .0014$) of the shNgn2 + Id2DBM treated neurons became significantly shorter than those of the shControl + Id2DBM treated neurons

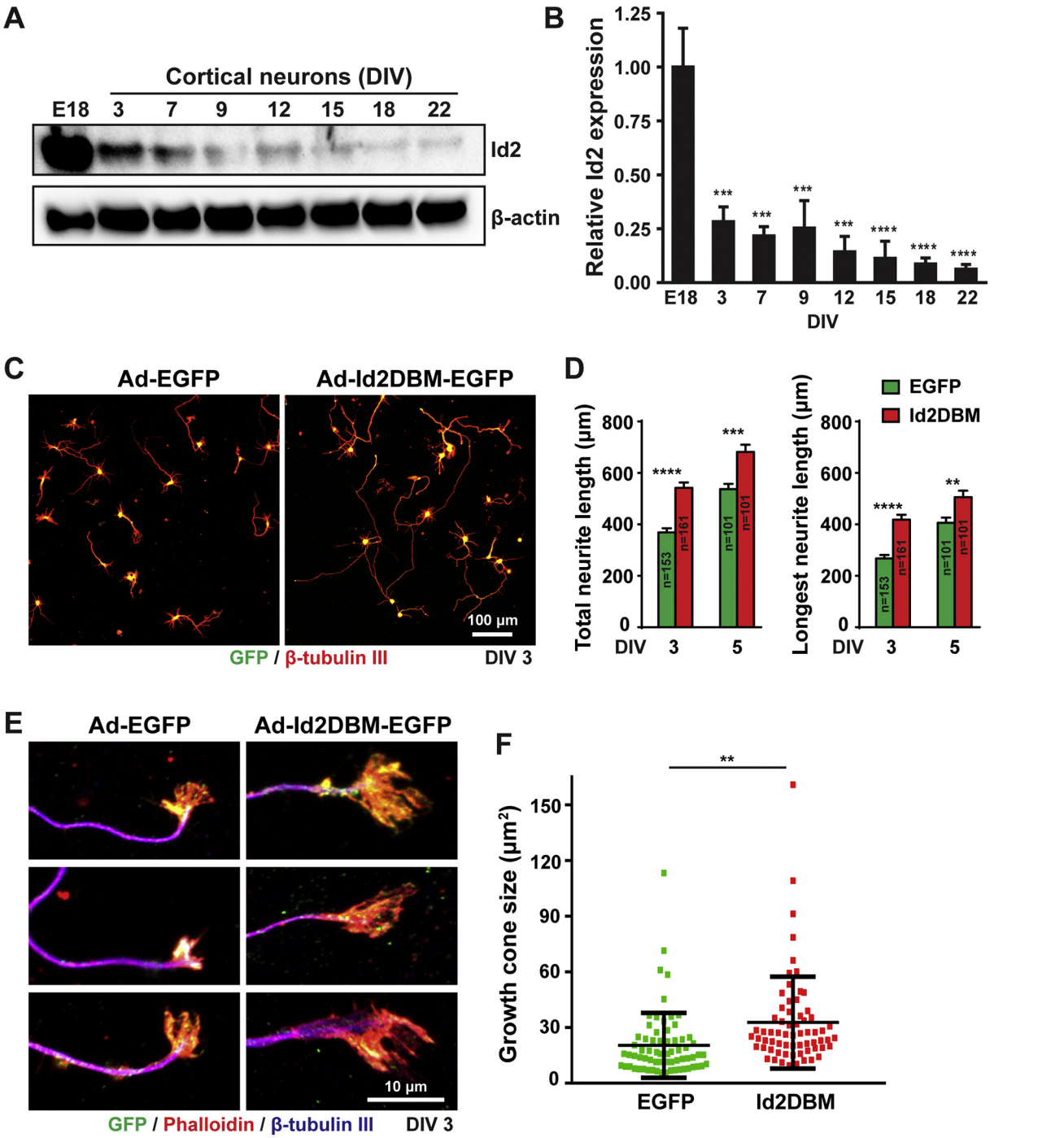


Fig. 1. Id2DBM promoted neurite outgrowth and increased growth cone size of cortical neurons. (A) Representative immunoblot of endogenous Id2 protein levels in cultured cortical neurons from DIV3 to DIV22. Protein lysates of dissociated cells from E18 mouse cortical tissues were used as a baseline control. β-actin was used as an internal control. (B) Quantitative analysis showing that Id2 expression declined with time in culture. The intensity of Id2 bands were calibrated with β-actin levels and then normalized to the Id2 expression level in E18. Data were presented as mean ± SEM; ***, $p < .001$; ****, $p < .0001$; compared to the E18 sample; by one-way ANOVA with a Tukey *post hoc* test. (C) Representative images of DIV3 cortical neurons infected with Id2DBM or a control EGFP adenovirus. Neurons were stained with β-tubulin III (red) and GFP (green) antibodies for visualization and measurements of neurite length. Scale bar: 100 μm. (D) Quantitative analysis showing Id2DBM promoted neurite outgrowth of cortical neurons at DIV3 and DIV5. Data were presented as mean ± SEM. **, $p < .01$; ***, $p < .001$; ****, $p < .0001$; by two-way ANOVA with a Tukey's *post hoc* test. (E) Representative growth cone images of DIV3 cortical neurons infected with Id2DBM or control EGFP adenovirus. TexRed-Phalloidin was used to label growth cones. Scale bar: 10 μm. (F) Quantitative analysis showing Id2DBM expressing neurons had increased growth cone size. **, $p < .01$; by two-tailed Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

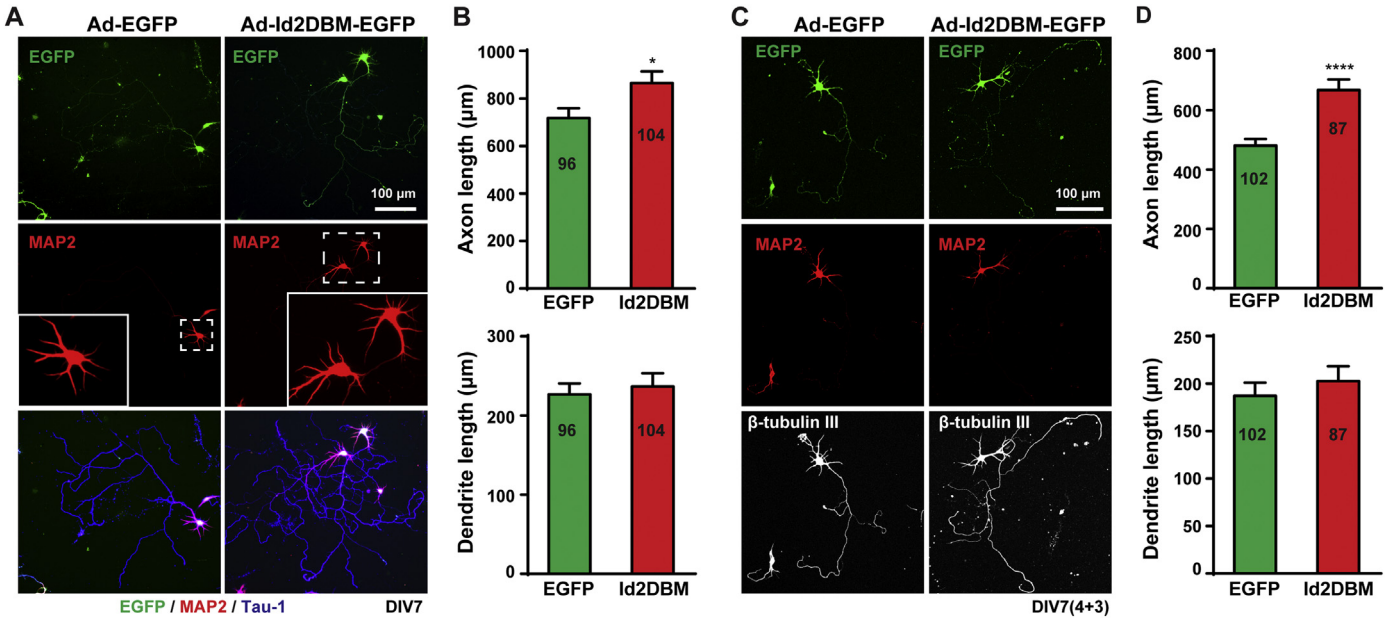


Fig. 2. Expression of Id2DBM at early or a later stage of neuronal development promoted axonal growth. (A) Representative images of DIV7 cortical neurons infected with Id2DBM or control EGFP adenovirus. Neurons were stained with MAP2 (dendrites, red), Tau-1 (axons, blue), and GFP (green) antibodies for visualization and measurement of axon and dendrite lengths. Scale bar: 100 μm. (B) Quantitative analysis of axon and dendrite lengths showing Id2DBM increased axonal length but not dendrite length of cortical neurons. Data were presented as mean ± SEM. *, $p < .05$; by two-tailed Student's t-test. (C) Representative images of cortical neurons infected with Id2DBM or EGFP adenovirus at DIV4 and cultured for an additional 3 days before fixation (DIV7(4 + 3)). Neurons were stained with MAP2 (red), β-tubulin III (white), and GFP (green) antibodies. The MAP2 labels dendrites, while the β-tubulin III labels all neurites. Scale bar: 100 μm. (D) Quantitative analysis of axon and dendrite lengths showed that expression of Id2DBM in cortical neurons at DIV4 increased axon lengths when analyzed at DIV7. ****, $p < .0001$; by two-tailed Student's t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 5B and C). Thus, knockdown of Ngn2 curtailed the effect of Id2DBM on promoting neurite outgrowth.

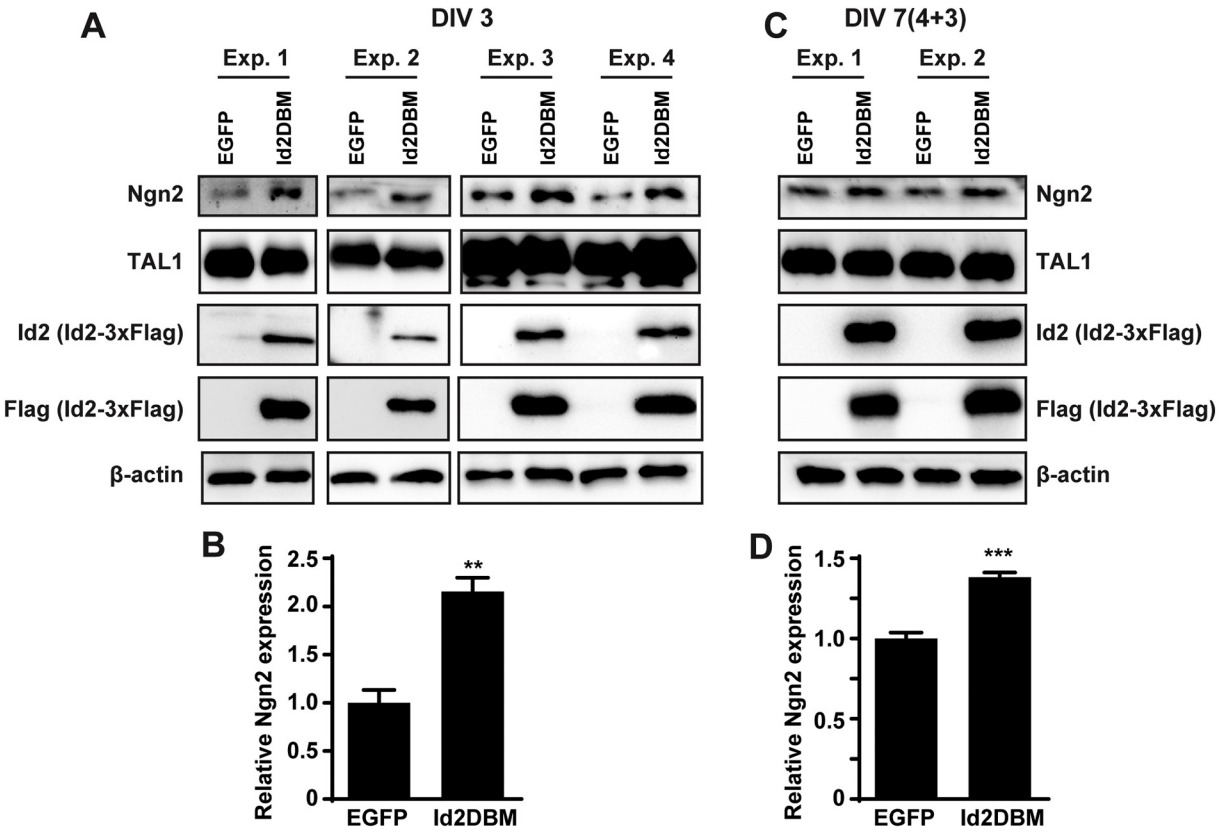
4. Discussion

Id2 and its family members have been widely studied and well recognized for their role in promoting cell proliferation while inhibiting cell differentiation (Ling et al., 2014). Several studies have demonstrated an additional role for Id2 in promoting axonal growth (Ko et al., 2016; Lasorella et al., 2006; Yu et al., 2011), but the mechanism of action of Id2 remains obscure. Our study demonstrated that overexpression of Id2 primarily promoted axonal growth rather than that of dendrites in both stage 1 (early) and stage 4 (later) neurons, based on the reported stages that classify the maturation of hippocampal neurons in culture (Dotti et al., 1988). This suggests that forced expression of Id2 in neurons can maintain the intrinsic axonal growth capacity that declines with neuronal maturation. Furthermore, our study found that Id2 elevated or induced the re-expression of Ngn2 in neurons, which promoted axonal growth.

Ngn2 is a proneural gene essential for neuronal fate commitment (Lacomme et al., 2012; Mizuguchi et al., 2001; Sommer et al., 1996). It is transiently expressed during neurogenesis and then disappears with neuronal maturation, which plays important roles in neuronal differentiation, migration, axonal projection, and proper formation of cortical circuitry during CNS development (Hand et al., 2005; Hand and Polleux, 2011; Heng et al., 2008). Postnatally, Ngn2 is restricted to the neurogenic regions including the subventricular zone and dentate gyrus and markedly decreased with age (Ozen et al., 2007). Ngn2 has also been used to convert or reprogram non-neuronal cells into neurons. Forced expression of Ngn2 induced astrocytes into glutamatergic neurons (Heinrich et al., 2010) and converted embryonic stem cells, induced pluripotent cells, or neural progenitor cells into excitatory neurons (Zhang et al., 2013). In combination with other compounds or factors, Ngn2 is able to directly transform fibroblasts or human umbilical cord mesenchymal stem cells into induced neurons without passing through a proliferative neural progenitor state (Araujo et al., 2018; Liu et al., 2013; Smith et al., 2016). However, few studies have focused on its role in post-mitotic neurons, especially mature neurons. In this study we reveal that maintaining Ngn2 levels in immature neurons has the potential to boost their intrinsic growth capacity. It also implies that re-introduction of Ngn2 in mature neurons may rejuvenate them into an intrinsically active state beneficial for axonal regrowth, although the role of Ngn2 in axonal regeneration after CNS injury still needs further investigation.

Id2 is a negative regulator of the bHLH factor, while Ngn2 is a neural tissue specific bHLH transcription factor. Id2 and Ngn2 seem to have opposite effects on neurogenesis: Id2 promotes proliferation of neural progenitor cells and Ngn2 drives neuronal differentiation. It has been shown that overexpression of Id2 in neural progenitor cells blocks the expression of neuron-specific genes and led to apoptosis. In contrast, overexpression of Id2 in post-mitotic neurons has no effect either on survival or on neuronal-specific gene expression (Toma et al., 2000), and instead could promote axonal growth (Ko et al., 2016; Lasorella et al., 2006; Yu et al., 2011). These indicate that different pathways may be triggered in neurons *versus* neural progenitors by Id2. Ngn2 promotes neuronal differentiation through activating neuronal specific gene transcription. Whether elevation of Ngn2 level in neurons enhances axonal growth by influencing the same or a different set of genes is unknown.

We observed re-expression of Ngn2 protein in adult spinal cord neurons by Id2 *in vivo*, and notably Ngn2 protein distribution was not restricted to the nucleus but was also localized in the cytoplasm. In addition, in the overexpression experiments, we also observed distribution of Ngn2-flag fusion protein in the cytoplasm as well as growth cone of neurons. This imply possible roles of Ngn2 in other signaling activities to promote axonal growth that do not require DNA binding. Id2, which contains the homologous HLH domain but lacks the basic DNA binding domain, was previously found to be enriched in the



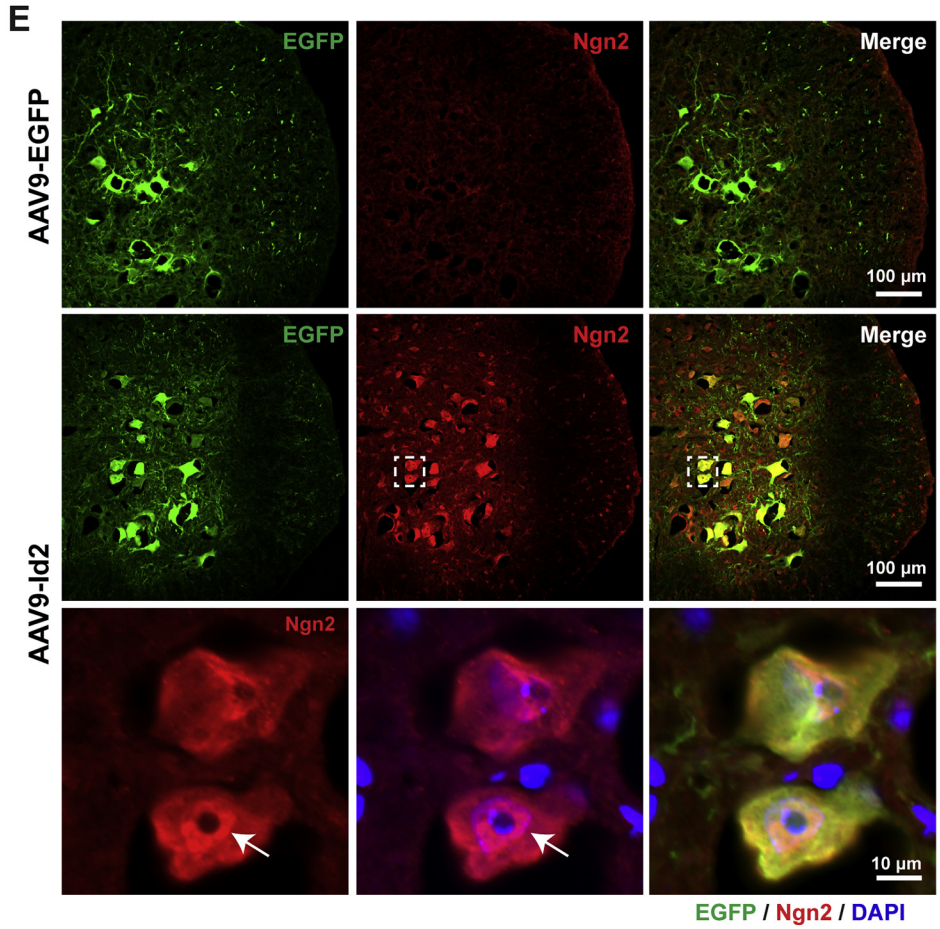


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Fig. 3. Id2 induced Ngn2 expression. (A) Immunoblot results of DIV3 cortical neurons isolated from 4 independent cultures. Cortical neurons were infected with Id2DBM or EGFP adenovirus at the time of plating. Cell lysates collected at DIV3 were subjected to immunoblot with the indicated antibodies. (B) Quantification of Ngn2 expression of DIV3 neurons (**, $p < .01$; by two-tailed Student's t-test). The intensity of Ngn2 bands was calibrated with β-actin levels and then normalized to the Ngn2 expression level in EGFP control group. (C) Representative immunoblot results of DIV7 (4 + 3) cortical neurons. Cortical neurons were cultured for 4 days before being infected with Id2DBM or EGFP adenovirus. Neurons were cultured for additional 3 days before cell lysates were collected and subjected to immunoblot with the indicated antibodies. (D) Quantification of Ngn2 expression of DIV7 (4 + 3) neurons (***, $p < .001$; by two-tailed Student's t-test). The intensity of Ngn2 bands were calibrated with β-actin levels and then normalized to the Ngn2 expression level in EGFP control group. (E) Representative immunostaining images of Ngn2 (red) in adult mouse spinal cord infected with AAV9-EGFP or AAV9-Id2. Images in the bottom row are high magnification images of the dotted lined boxes of the middle row. Id2 induced the expression of Ngn2, which was distributed not only in the nucleus (arrows) but also in the cytoplasm of neurons. Scale bars: 100 μm in the top and middle rows, 10 μm in the bottom row. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

growth cone that regulates cytoskeletal organization (Ko et al., 2016). The bHLH protein Ngn2 may similarly interact with the cytoskeleton system in neurons.

It has been previously reported that forced expression of Ngn2 resulted in a marked upregulation of Id2 expression rather than inhibition (Dubreuil et al., 2002). Our results on the other hand showed that the forced expression of Id2 caused a marked upregulation of Ngn2. Whether Id2 induces Ngn2 expression through a direct transcriptional regulation or through other indirect mechanisms remains to be elucidated. Nevertheless, this study disclosed a mechanism for Id2 to promote axonal growth.

In summary, we demonstrated the role of Id2 in promoting axonal growth by elevating the expression of Ngn2, a neural progenitor cell expressing gene, in post-mitotic neurons. Understanding the role of Ngn2 in neurons may provide valuable insight into the intrinsic mechanisms of axonal growth.

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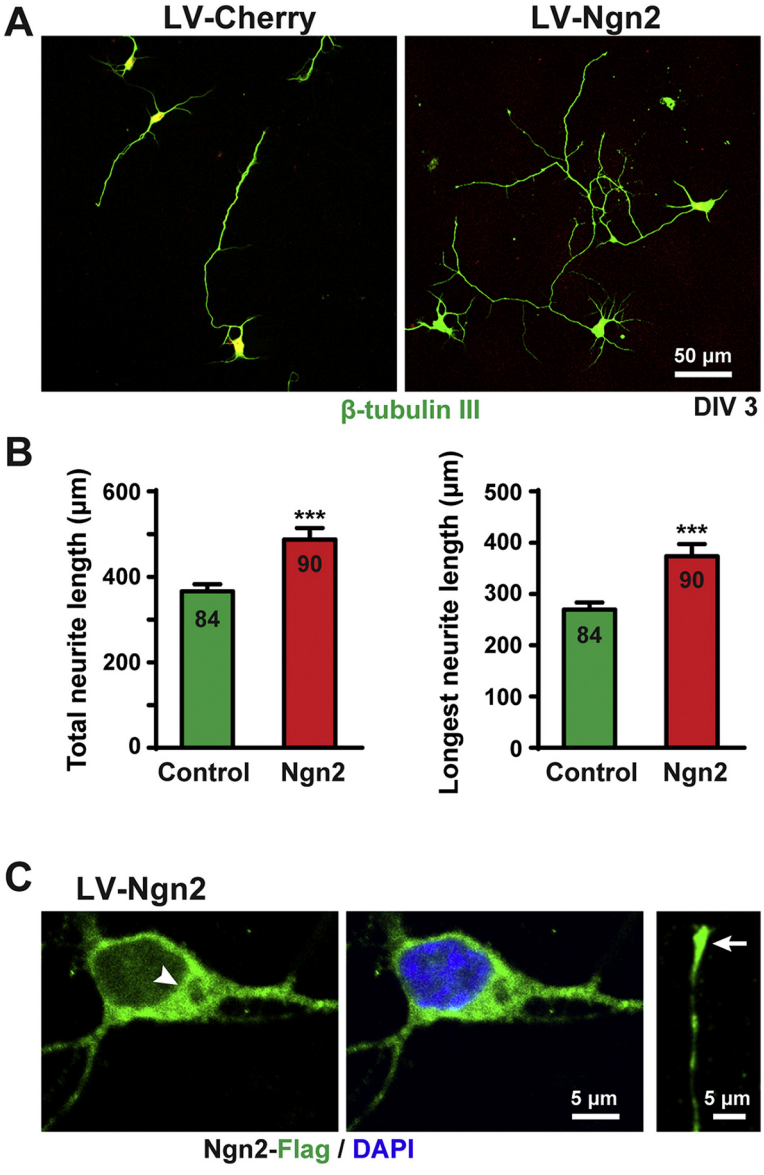

Fig. 4. Overexpression of Ngn2-Flag in cortical neurons promoted neurite outgrowth. (A) Representative images of DIV3 cortical neurons infected with Ngn2 or control lentivirus. Neurons were stained with β-tubulin III (green) antibody for visualization and measurements of neurite length. Scale bar: 50 μm. (B) Quantitative analysis showing Ngn2 increased the total neurite length as well as the longest neurite length of cortical neurons. Data were presented as mean ± SEM. ***, $p < .001$; by two-tailed Student's t-test. (C) Distribution of Ngn2-Flag (green) fusion protein stained by Flag antibody, showing protein localization in neuronal cytoplasm (arrowhead) and growth cone (arrow). Scale bar: 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

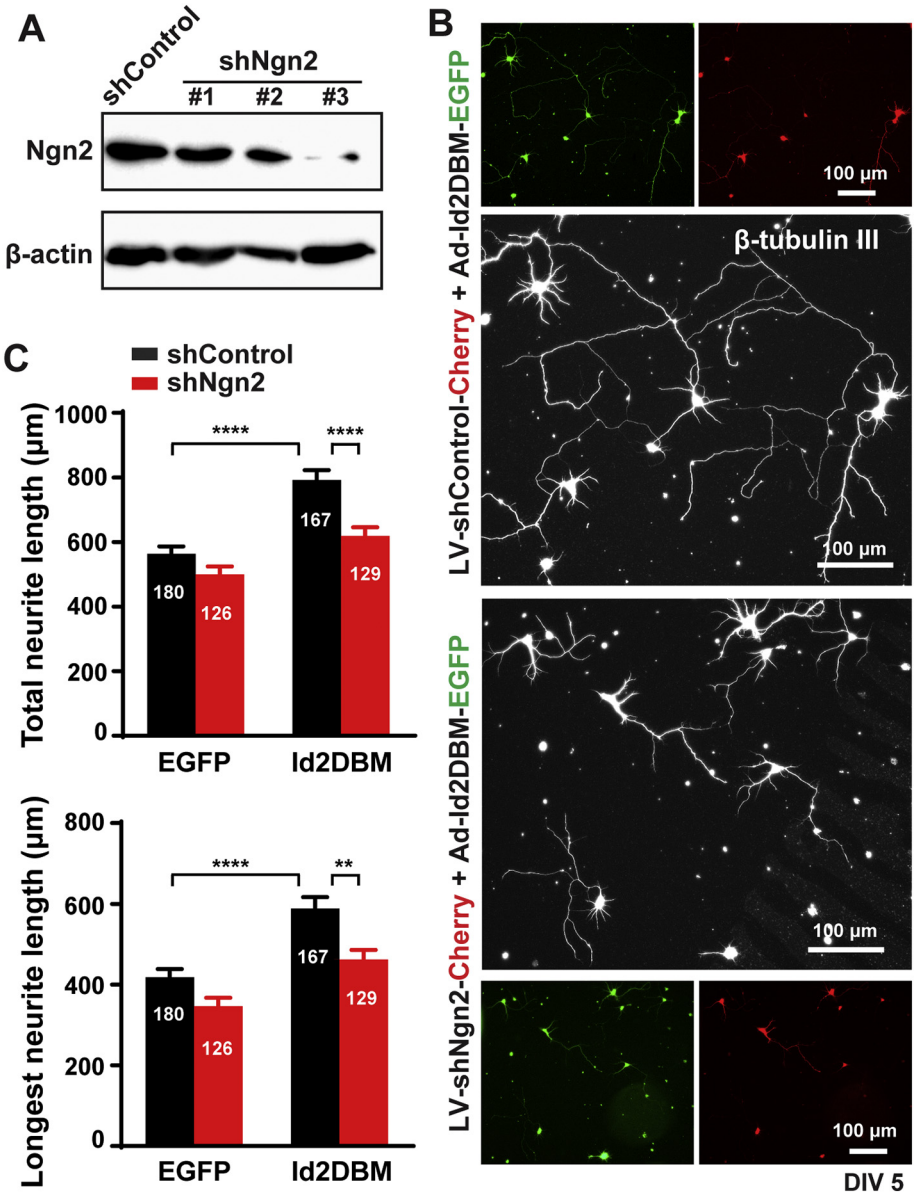

Fig. 5. Knockdown Ngn2 using shRNA blocked the axonal growth promoting effect of Id2DBM. (A) Western blot results confirmed the reduced Ngn2 level by Ngn2 shRNA #3. (B) Representative images of DIV5 cortical neurons infected with lentivirus (LV) encoding control shRNA (shControl) or Ngn2 shRNA (shNgn2) at the time of plating, followed by the addition of adenovirus (Ad) encoding Id2DBM (Ad-Id2DBM) at DIV2, neurons were fixed at DIV5 and were stained with β-tubulin III (white) antibody for visualization and measurement of neurite length. LV-shRNA also encodes a fluorescent reporter Cherry (red), and the adenovirus contains an unfused EGFP (green) reporter. Scale bar: 100 μm. (C) Quantitative analysis of the total neurite length and the longest neurite length showing that the growth promoting effect of Id2DBM was disrupted by Ngn2 shRNA. Data were presented as mean ± SEM. **, $p < .01$; ****, $p < .0001$; by two-way ANOVA with Tukey's *post hoc* test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conflicts of interests

None declared.

Author contributions

PY and BZ: Conceptualization, resources, supervision, methodology, paper writing and editing; ZH, JL, JJ, and QC: Data acquisition and analysis; LS, YZ, CS, and LZ: Resources, supervision, methodology, and paper editing.

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