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Original Article

An inducible tyrosine kinase receptor for axonal regeneration

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Abstract: The prevention or reduction of neuronal degeneration remains a challenge in neurotrophins therapy. An inducible trkA (ItrkA) system has been shown to regulate embryonic dorsal root ganglion (DRG) neuronal survival and neurite outgrowth in vitro. A new ItrkA plasmid ItrkA-membrane ($ItrkA_{memb}$) with one adenine at 3' terminal was established by correcting the sequence of the original plasmid ItrkA-cytosol ($ItrkA_{cyto}$). Adult DRGs were dissected from adult Fischer 344 rats (8-14 weeks) for the treatment with AP20187 (membrane-permeable small-molecule ligand), vehicle or NGF (Nerve Growth Factor). Neurite outgrowth assessments were done by manually tracing the longest neurite of each neuron. Cell diameters were also measured and averaged for each well. Protein expression after $ItrkA_{memb}$ transfection and trkA downstream signaling were investigated by Western-blotting. Neurite length of $ItrkA_{memb}$ transfected DRGs was not influenced by AP20187 or NGF but cells displayed shorter neurites compared to GFP control groups. While $ItrkA_{cyto}$ transfected DRGs cultured with AP20187 had the longest neurite growth compared to $ItrkA_{memb}$ transfected neurons and $ItrkA_{cyto}$ transfected cells treated with vehicle or NGF, no significant difference to GFP controls was detected. Quantification of the mean diameter of transfected DRGs demonstrated that $ItrkA_{memb}$ electroporation significantly increased cell diameter, while the diameter of $ItrkA_{cyto}$ transfected neurons and GFP controls were almost the same as naïve neurons. In contrast to electroporated adult DRG neurons, $ItrkA_{memb}$ virus transfection did not affect the diameter of infected adult DRG Neurons. No obvious difference was observed between the $ItrkA_{memb}$ and GFP electroporated cells, and only cells transduced with $ItrkA_{memb}$ treated with AP20187 seemed to show higher phosphorylation both of Akt and Erk1/2. The effect of adult DRG neurons after ItrkA transfection differs, which depends on the change of cell soma size and/or neurite growth, gene delivery technique, expression level and the localization of ItrkA.

Keywords: Inducible tyrosine kinase receptor a, dorsal root ganglion, nerve growth factor, AP20187, Erk1/2, Akt

Introduction

Neurotrophins have been shown to augment neuronal survival, function, and axon growth in numerous animal models of neuronal degeneration and nervous system injury [1]. Several clinical trials delivering neurotrophic factors by ex vivo and in vivo gene therapy in neurodegenerative disorders were done such as Alzheimer's disease and Parkinson's disease [2-4]. Despite promising results from these trials, regulation of neurotrophin expression or regulated neurotrophic signaling pathways remains highly desirable to optimize the dose of neurotrophin delivered and to increase the safety of growth factor gene therapy. Neurotrophin therapy has been hampered in clinical translation by the inability of the proteins, which play roles in traversing the blood-brain barrier (BBB) to reach target areas of the central nervous system (CNS). It therefore remains a challenge to achieve the desired concentration of neurotrophic factors within a target area undergoing neuroregeneration. Recently, in vivo studies on gene transfer focus on the regulation of growth factor expression as well as signaling pathways downstream from growth factor receptors.

NGF (Nerve Growth Factor), belongs to neurotrophic factors, expressed within discrete areas in the CNS and is secreted by tissues targeted by sympathetic and some sensory neurons. It

plays a role in neuroprotection through the trkA (Tyrosine Kinase Receptor A) receptor and triggers apoptosis in some cells through the p75 receptor [5-7]. The binding of NGF to trkA receptor facilitates receptor dimerization and tyrosine residue phosphorylation of the cytoplasmic tail by adjacent trk receptors [8]. Previous studies have shown that blocking PI3K or Akt activity resulted in death of sympathetic neurons in culture, although NGF was present [9]. Besides, NGF-induced cell signaling via trkA for cell survival mainly depend on MAPK/Erk1/2 [6, 8, 10]. Binding of a ligand to trkA can trigger the survival signaling pathway through phosphatylinositol 3-kinase (PI3K), and active RAS-MAPK signaling pathway leading to survival and differentiation [11].

The inducible trkA receptor (ItrkA) exploits the inherent dimerization-induced activation of receptor tyrosine kinases to trigger trkA signaling in response to the membrane-permeable small-molecule ligand, AP20187. The activation of ItrkA can efficiently regulate eDRG neuronal differentiation and neurite outgrowth in vitro [12]. DRG (Dorsal Root Ganglion) ganglia contain a diverse group of sensory neurons that convey different sensory stimuli, such as pain, temperature, touch and body posture, to the brain. Each DRG neuron possesses one axon stemming from the cell body, which branches into two axons: a peripheral axon branch innervating peripheral targets and an ascending central branch projecting into the dorsal horn of the spinal cord. In the last decade, it has proven to be one of the most versatile non-viral techniques for studying adult neurons, even if efficient used for DRGs transfection for genetic dissection of axon regeneration in vivo [13]. In this paper, we utilized an inducible trkA (ItrkA) system to investigate the effect of activated signaling pathways downstream from neurotrophin receptors on neurons regeneration.

Materials and methods

Cloning and viral vector construction

The original plasmid was from the University of California, San Diego 18. Briefly, the cDNA of rat trkA intracellular domain (1410-2458; accession No. NM_021589) was amplified by PCR with the following primers: 5'-ACTTCTAGAAATTTGGGATCAACCGCCCTG-3' and 5'-CTGTCTAGAGCCCAGAACGTCCAGGTAACT-3'. The 1,048-base-pair PCR product was then subcloned into the XbaI site of plasmid $pC_4M\text{-}F_V2E$ to generate an inducible trkA chimeric protein (ItrkA) consisting of the rat trkA intracellular domain fused with an amino-terminal myristoylation signal, two tandem FK506 binding domains ($FKBP_{36v}$), and a c-terminal hemagglutinin tag (HA). The $FKBP_{36V}$ domain is based on the human protein FKBP12, engineered to bind only a synthetic small ligand for dimerization (AP20187), and not endogenous FKBP ligand. A 2,418-bp BamHI/NheI ItrkA fragment was then excised after the insertion of an EcoRI/NheI/AgeI linker (5'-AATTGGATCCGCTAGCG-3' and 5'-AATTCGCTAGCGGATCC-3') and cloned 3' region of a CAG promoter into a self-inactivating lentivirus vector based on pRRL. An internal ribosomal entry site (IRES) for green fluorescent protein (GFP) reporter gene expression was also included to construct the plasmid p156 sinRRL-ItrkA-IRES-GFP (p225, the original plasmid). The same lentiviral plasmid only expressing GFP (p155) was used as control. Because p225 was found to contain a frame-shifting mutation, a new plasmid (p645) was established after correcting the sequence of original plasmid (specific difference shown in result). Plasmid DNA was completely dissolved and mixed well in TE buffer (Thermo Fisher Scientific, USA).

Production of lentivirus

Highly purified plasmid DNA (1 mg/mL) was prepared for the lentivector transfer vector and helper plasmid with an endotoxin-free plasmid kit. 293T cells (ATCC, US) were cultivated in DMEM high glucose with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P.S) and 1% L-glutamine 37. For the virus production, 293T cells were transfected using calcium chloride DNA precipitates and HEPES buffer (Thermo Fisher Scientific, USA) before confluent. 293T cells were plated on 10 × 150 mm tissue culture dish plates, at a density of 1×10^6 cells per plate, in 15 ml cell culture medium. These were incubated at 37°C, 10% CO_2 over the weekend. The cells were re-plated after 3 days into approximately 12 plates (about 1×10^7 293T cells per 150 mm tissue culture dish in 15 ml culture medium) and incubated at 37°C, 10% CO_2 overnight for transfection. Gritty precipitate around the cells was checked after about 3 hours.

Until 70-90% of the cells were transfected through GFP fluorescence, the culture medium was removed and replaced with 15 ml serum-free fresh media at 37°C, 10% CO_2 overnight. The medium was harvested again and 15 ml fresh serum-free cell culture medium was added to each dish. The harvested supernatants were immediately filtered through a 250 ml filter unit with a 0.45 μm pore size. Filtered medium was centrifuged in 30 ml polyallomer Beckman conical tubes in a Beckman SW28 rotor at 69,000 g (19,400 rpm) for 2 h at 4°C. After the centrifugation, the supernatants were carefully removed and pellets were pooled in 3 ml HBSS (w/Ca and Mg) and transferred to a 3 ml polyallomer Ultracone centrifuge tube (Seton Scientific Ultracone #03922) and centrifuged at 24,000 rpm in a Sorvall AH-650 rotor for 2 h at 4°C. The pellet was suspended in 50-100 μl HBSS without any bubbles. Virus was spun at 13000 rpm for 15-30 sec to pellet non-resuspendable debris, and 10 μl aliquots were stored at -80°C.

Determination of lentiviral titers was based on the expression of a reporter gene (GFP) in a cell based assay (infectious units assay) 37. For the measurement of infectious units, 50,000 of 293T cells were seeded per well in 1 ml cell culture medium into a 24-well plate. For Serial virus dilutions of the concentrated virus were prepared: 1 μl virus prep was added to 1000 μl medium (10^{-3} dilution) and serial dilutions were made (up to 10^{-7}) by adding 100 μl of the previous dilution to 900 μl medium. The cells were incubated at 37°C, 10% CO_2 for 2 days and the number of colonies were counted under a fluorescence microscope, then the virus titers were calculated.

Dorsal root ganglion culture

For adult DRG culture, six-well polystyrene plates were first coated with 1.5 ml poly-D-lysine (Sigma; 16.67 μg/ml in sterile water) per 35 mm well for 1 h at room temperature. The plates were washed twice with equal volumes of water. Diluted laminin (Sigma; 0.5 μg/ml in D'PBS) was then added to the plates (1.5 ml/well) and incubated at room temperature for 3 h. After two washing steps with equal volumes of D'PBS, the wells were filled with 2 ml of complete culture medium 18 and plates were placed at 37°C until cell plating 25.

Adult Fischer 344 rats (8-14 weeks) were sacrificed by an anesthesia mixture (125 mg/kg ketamine, 6.35 mg/kg xylazine, 1.25 mg/kg acepromazine), according to the guidelines on animal care of State and Institutional Animal Care and Use Committee and Society for Neuroscience. Following decapitation, the spinal column, including the muscles surrounding the vertebrae was removed. The caudal end was cut so as to barely expose the opening of the vertebral canal. A 10 ml syringe filled with cold Hank's Balanced Salt Solution (HBSS) (Thermo Fisher Scientific, USA) with an 18-gauge needle was inserted into the caudal opening of the vertebral canal, and the cord was quickly extruded through the rostral opening. The column was cut longitudinally in half along the dorsal and ventral axes. When necessary, the remaining dura mater was removed. At this point, all DRGs were exposed, lying in the intervertebral foramina along the vertebral canal. For each DRG, the dorsal root was grasped with microforceps to pull the ganglion gently away from the foramen, and the nerve distal to the DRG was cut with microscissors. Then DRG was carefully trimmed of nerve roots, and cut half way into the center of the ganglion, and placed in Hibernate A medium on ice until all DRGs from each rat were collected.

The DRGs were washed once in cold Ca/Mg-free HBSS and incubated in a 1:1 mixture of Collagenase XI (2.5 mg/ml final concentration; = 1200 collagenase digestive units/mg) and Dispase I (Worthington; 5 mg/ml final concentration; = 4 units/mg) at 37°C for 30 min, shifting every 10 min to equally distribute the DRGs in the tube. The enzyme solution was removed, and the cells were briefly washed once with warm complete medium with 10% FBS to stop the digestion and once with warm serum-free complete medium to remove any remaining serum. Mechanical trituration was carried out using a sterile fire-polished glass Pasteur pipette as following: the ganglia were placed in 1 ml warm serum-free complete medium and triturated approximately 15 times, avoiding air bubbles. Large remaining pieces of tissue debris were allowed to sink to the bottom of the tube (approximately 1-2 min), and the supernatant containing the single-cell suspension was transferred to a new tube. Cells were counted under bright-field light microscopy using a hemocytometer. From one rat, between 1.25

and 1.75 × 10^6 cells including neurons and glia were typically obtained after dissociation. Naïve cells were plated at a density of 15,000 cells per well in 2 ml complete medium. DRGs were cultured in incubator at 37°C and 5% CO_2.

Transfection

An optimized electroporation protocol was used to transfection DRGs with plasmid 25 [14]. Cells were centrifuged at room temperature for 5 min at 800 rpm after dissociation. Cell pellet was resuspended in D'PBS, and centrifuged again at 800 rpm for 5 minutes. Then cell pellet was resuspended in P3 buffer (Qiagen, Germany) (as recommended by Lonza for neurons) at a concentration of 3 × 10^5 cells per 20 µl. 20 µl of cell suspension was transferred to a new tube and combined with 2.2 µg plasmid DNA per reaction, composing up to 10% of the reaction mixture volume (2.2 µl of 1 mg/ml DNA per 20 µl cell suspension). The DNA/cell mixture was then transferred to the 20 µl reaction strip. Electroporation was carried out using the Lonza 4D-Nucleofector X-unit system (Code: DR 114) (Lonza, Switzerland). After plating for 3 hours, the medium was replaced and cells were immediately transduced with GFP or ItrkA lentivirus at an MOI of 50 and treated with AP20187 at a concentration of 125 nM (1:100,000 dilution in PBS from a 12.5 mM stock solution in 100% ethanol). Controls were treated with 0.001% ethanol in PBS. The cells were cultured at 37°C and 5% CO_2 for 72 hours.

Immunocytochemistry

Adult and embryonic DRGs were fixed with 4% ice-cold paraformaldehyde (PFA) in 0.1 M phosphate buffer and washed 4 × 5 minutes with 0.1 M Tris-buffered saline (TBS). Cells were blocked for 1 h at room temperature in TBS with 0.1% Triton X 100 and 5% donkey serum and incubated with primary antibodies in TBS with 0.1% Triton X 100 and 1% donkey serum overnight at 4°C. Cells were washed 4 × 5 min with TBS and incubated with secondary antibodies diluted in TBS for 2.5 h at room temperature in the dark. Cells were washed 4 × 5 min with TBS. In the third wash, 4-6-Diamidino-2-phenylindole (DAPI) (Sigma, USA) was added at a concentration of 1:1000 (stock concentrations: 0.1 mg/ml) for naïve cells. Antibodies used for fluorescent immunocytochemistry were the following: rabbit anti-GFP (1:1000; Life Technologies, USA), mouse anti-beta-III-tubulin (1:1000; Promega, USA), donkey anti-rabbit Alexa 488 (1:1000; Molecular Probes), and donkey anti-mouse Alexa 594 (1:1000; Molecular Probes, USA).

Imaging

Imaging of plates was automated using an Olympus I X 81 inverted microscope equipped with a motorized stage and controlled by Olympus Cell-P Software using a 4 × objective (Olympus, Japan). Image files were opened in NIH image J (Image science, Germany), and neurons were identified by beta-III-tubulin labeling and transfected neurons by GFP double labeling. Neurite outgrowth assessments were done by manually tracing the longest neurite of each neuron double labeled for GFP and beta-III-tubulin, starting at the base of the neurite at the edge of the cell soma. For neurons without neurites, a small line was traced along the edge of the soma, amounting to no more than 10 pixels (16 µm). Cell counts and neurite lengths were averaged for each well.

Western blot

Two wells of cells in each group were washed twice with ice-cold D'PBS, then cells were scraped off the plate in D'PBS and collected in a 15 mL conical tube and centrifuged to get cell pellets and placed on ice. One hundred microliter of 1 × loading buffer (2% SDS, 15% glycerol, 50 mM Tris/ HCl, 0.1% bromophenol blue, 0.1 M DTT) was added per tube, which included 8 µl complete protease inhibitor cocktail (one tablet dissolved in 2 mL H_2O used for 25 mL extraction solution) and 10 µl phosphatase inhibitor cocktail (one tablet dissolved in 1 mL water used for 10 mL extraction solution) (Roche, Germany), and the samples were heated in 90°C water bath for 3-5 minutes and extracts were pipetted up and down several times. Ten microliters of protein extract/well was separated on 4-12% Bis-Tris gels (Invitrogen, USA) and transferred to polyvinylidene difluoride (PDF) membranes for immunoblotting. Membranes were blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline Tween-20 (TBST, 0.1% Tween 20 in TBS) for 1 hour, and washed 3 × 10 min with TBST, then incubated with the following primary antibodies overnight at 4°C: Rabbit anti-HA (1:1000;

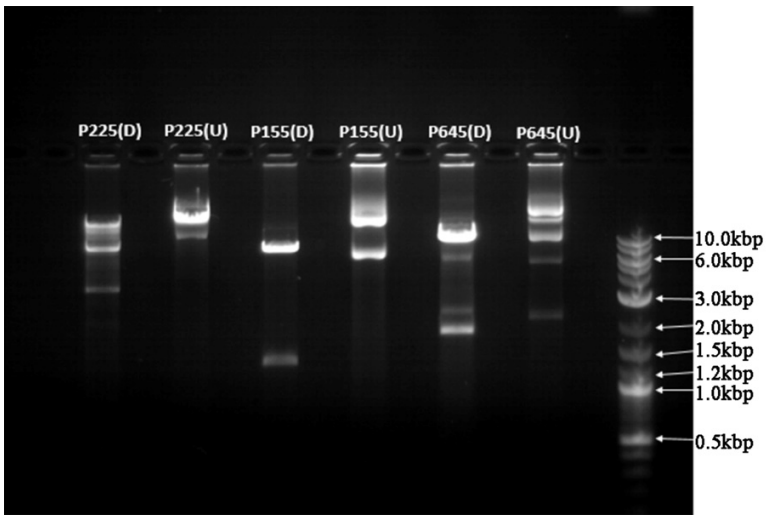

Figure 1. PCR detection of three digested plasmids. After digestion (AgeI/BamHI for p645, StuI for p225 and EcoRI for p155), digested plasmid DNA fragments are visible using a 2-log ladder as marker. The size of expected bands is as followed: p645 (1815 bp and 9129 bp), p225 (3323 bp and 7620 bp), p155 (1358 bp and 7196). U: undigested DNA; D: digested DNA.

Sigma-Aldrich, USA), Erk1/2 (1:1,000; Cell Signaling, USA), Phospho-p44/42 MAPK (pErk1/2) (1:2,000; Cell Signaling), and Akt (1:1,000; Cell Signaling, USA), and pAkt (1:1,000; Cell Signaling, USA). Membranes were washed 3 × 10 min with TBST, and incubated with HRP-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibodies (made in goat, PerkinElmer, USA) used at 1:10,000 dilution for 1 hour. After washing 3 × 10 min with TBST and 1 × 10 min with TBS, bands were visualized using an ECL (PerkinElmer, USA) as chemiluminescent substrate and imaged by fusion detection system (PeQlab, Germany).

Statistics analysis

Data are presented as means and standard errors of the mean. Group means were compared by unpaired two-tailed Student's t-test and one-way ANOVA followed by Tukey's multiple comparisons test to assess significant differences. A significance criterion of $P < 0.05$ was used in all statistical tests.

Results

Plasmid vectors for expression of ItrkA

This study focused on the role of ItrkA effects using the new corrected plasmid (p645) in adult or embryonic DRG neurons in response to AP20187. The same lentiviral plasmid expressing only GFP (p155) and the original lentivector p225 were used as control. According to the different localization and function of the vectors, they were named: p155: GFP control; p225: ItrkA-cytosol ($ItrkA_{cyto}$); p645: ItrkA-membrane ($ItrkA_{memb}$). To examine the sequence of the obtained plasmid DNA, the program MacVector was used to select restriction enzyme(s) to digest plasmid DNA. After digestion with restriction enzymes (AgeI/BamHI for p645, StuI for p225 and EcoRI for p155), plasmid DNA was separated in a 1% agarose gel, and the size of expected bands were as followed: p645 (1815 bp and 9129 bp), p225 (3323 bp and 7620 bp), p155 (1358 bp and 7196). In addition to the expected bands, p645 and p225 also had additional bands, which are likely derived from undigested plasmid DNA with different supercoiling (**Figure 1**).

Cultures of adult dorsal root ganglion neurons

Naïve adult DRGs were cultivated to determine the suitable culture condition. DRGs were isolated from adult rats and cultivated for 48 h. Networks had formed between neurons and glia, and irregularly shaped growth cones could be seen at the end of neurites. The soma of neurons was generally circular, oval, polygonal, or triangular, with a strong halo in light microscopy, larger than glia, and extending neurites (**Figures 2**, **3**). Upon fixation with cold PFA and immunocytochemical labeling with anti-beta-III-tubulin antibody, cell soma and neurites of DRG neurons were easily distinguished from other cells (**Figures 2**, **3**).

Freshly isolated, dissociated DRGs were transduced with plasmids of ItrkA or GFP control via electroporation (using the Lonza 4D-Nucleofector X-unit System) or via viral transfection. The transfected DRG neurons and glia expressed the transgene strongly or moderately as visualized by GFP fluorescence. In immunocytochemical labeling assay, beta-III-tubulin

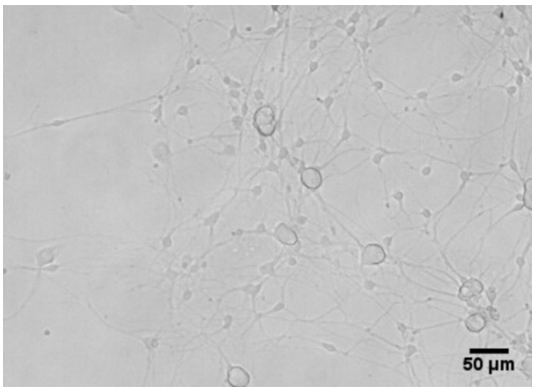

Figure 2. Image of cultured adult DRGs in vitro without staining. DRG neurons form networks after 2 days in vitro. Neuronal somata are generally circular, oval or polygonal, are much larger than other cell somata, and extend long neurites. Scale bar = 50 µm.

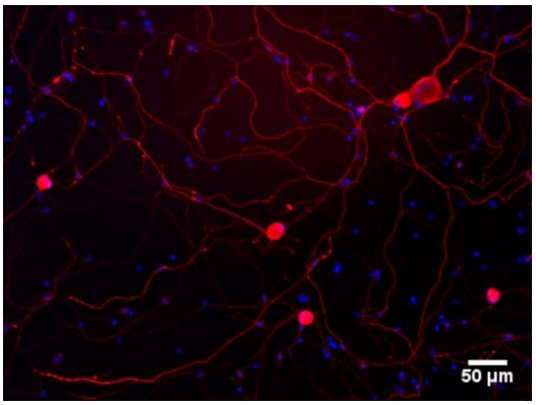

Figure 3. Immunocytochemical staining of DRGs. Under epifluorescent illumination, DRG neurons cultured for 48 h in vitro were labeled against beta-III-tubulin (red) and nuclei stained with DAPI (blue) presenting round, oval or polygonal DRG somata that extend neurites. Each well of culture plates contains hundreds to thousands of DRG neurons and numerous DAPI-stained glial nuclei around DRGs. Scale bar = 50 µm.

labeling (red) indicated neurons, while GFP labeling (green) identified transfected cells. Neurons were evenly distributed throughout each well, and the low plating density allowed for neurite tracing and quantification. Neurite outgrowth assessments of transfected DRGs were done by manually tracing the longest neurite of each double labeled neuron (**Figures 4**, **5**).

Effects of ItrkA expression in electroporated adult DRG neurons

The effect of electroporation of adult DRGs with ItrkA plasmids and GFP control plasmids on the neurite growth of transfected DRGs in response to AP20187 was determined. In the three GFP control groups, no difference in neurite length was observed (Tukey's multiple comparisons test, $P > 0.05$), and neurite length was within the same range as naïve untransfected neurons. Similar to the GFP controls, neurites length of $ItrkA_{memb}$ transfected DRGs did not differ and no influence of AP20187 or NGF could be detected (Tukey's multiple comparisons test, $P > 0.05$). However, all $ItrkA_{memb}$ transfected neurons displayed shorter neurites compared to GFP control groups (ANOVA $P < 0.001$, Tukey's multiple comparisons test; $^{***}P < 0.001$), indicating that $ItrkA_{memb}$ transfection reduced neurite outgrowth. The $ItrkA_{cyto}$ transfected DRGs cultured with AP20187 had the longest neurite growth, significantly higher than the $ItrkA_{memb}$ groups and $ItrkA_{cyto}$ transfected cells that were untreated or NGF-treated. (ANOVA $P < 0.001$, Tukey's multiple comparisons test; $^{***}P < 0.001$; $^{****}P < 0.0001$). Despite this enhanced growth, no significant difference to GFP control was detected (ANOVA, $P > 0.05$) (**Figure 6**).

Neurite outgrowth in lentivirus transducted DRGs

Lentiviral transduction of adult DRGs with $ItrkA_{memb}$ increased neurite length in the presence of AP20187 (ANOVA, $P < 0.001$, Tukey's multiple comparisons test, $^{***}P < 0.001$). The $ItrkA_{memb}$ transfected DRGs treated with ethanol and GFP controls demonstrated neurite length within the expected range of naïve adult DRG neurons (**Figure 7**), indicating that viral transfection did not have negative effect on neurite outgrowth of adult DRG neurons.

ItrkA influences cell soma size

When the neurites of transfected adult DRG neurons were traced, cell somata of $ItrkA_{memb}$ transfected DRG neurons appeared to have a larger size. The diameter of electroporated DRGs was measured, to determine whether transfection may have influenced cell soma size instead of neurite growth, Quantification of mean diameter of transfected DRGs after immunocytochemical cell labeling demonstrated $ItrkA_{memb}$ electroporation significantly increased cell diameter. Although NGF treatment of $ItrkA_{memb}$ electroporated DRGs decreased this effect, the cell diameter was still larger than $ItrkA_{cyto}$ and GFP-electroporated control groups

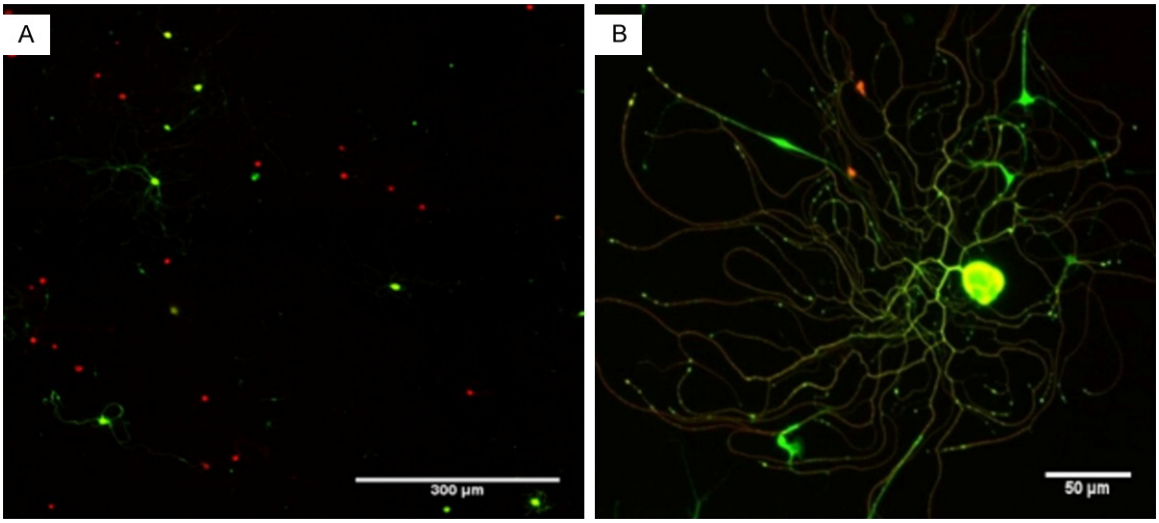

Figure 4. Images of DRGs after electroporation with plasmid or viral transfection. (A) Beta-III-tubulin labeling (red) indicates neurons, and GFP labeling (green) identifies transfected cells. (B) Higher magnification shows one transfected neuron with strong transgene expression, with GFP detected in the cell soma and neurites. Scale bar = 300 μm in (A) and 50 μm in (B).

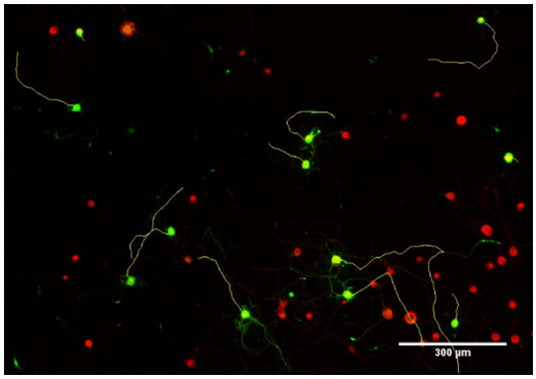

Figure 5. Tracing the longest neurite of transfected DRG neurons. Neurite outgrowth assessments of transfected DRGs were done by manually tracing the longest neurite of each neuron double labeled for GFP and beta-III-tubulin, starting at the base of the neurite at the cell soma. For neurons without neurites, a short line was traced along the edge of the soma, amounting to no more than 10 pixels (16 μm). Scale bar = 300 μm.

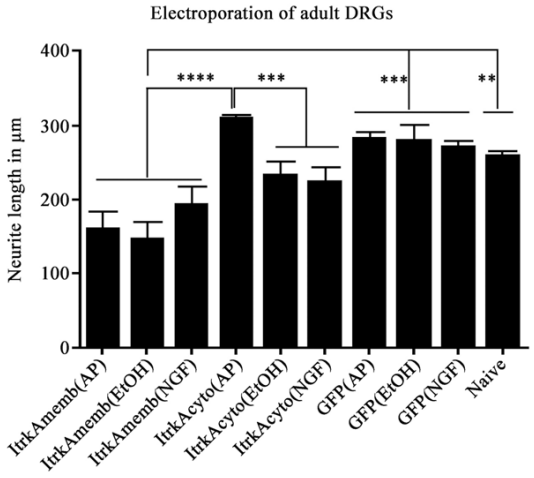

Figure 6. Neurite length of transfected adult DRG neurons after electroporation. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

(ANOVA, $P < 0.0001$; Tukey's multiple comparisons test, $^{****}P < 0.0001$). The diameter of $ItrkA_{cyto}$ transfected neurons and GFP controls were almost the same as naïve neurons (ANOVA, $P > 0.05$), indicating that $ItrkA_{cyto}$ and GFP electroporation had no effect on the change of cell somata (**Figure 8**). In contrast to electroporated adult DRG neurons, the diameter of $ItrkA_{memb}$ lentivirus-transfected adult DRG Neurons was not significantly different between the groups, showing that $ItrkA_{memb}$ viral transfection did not increase cell soma size (ANOVA, $P > 0.05$) (**Figure 9**).

Transgene expression and downstream signaling

The proteins level in cells electroporated with plasmid $ItrkA_{memb}$ and GFP control and in $ItrkA_{memb}$ and GFP lentivirus transfected adult DRGs were investigated. As expected, HA-tagged protein was only detectable in

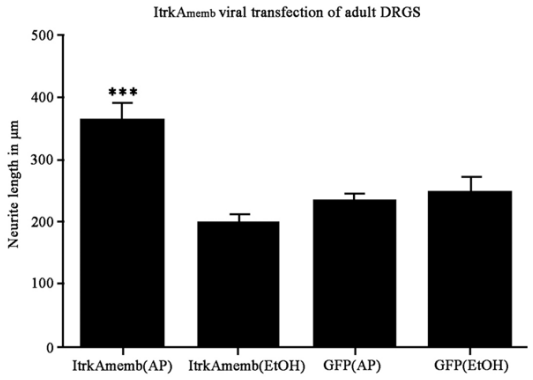

Figure 7. Inducible neurite outgrowth of adult DRG neurons after lentiviral transfection. ***P < 0.001. AP, AP20187; EtOH, ethanol.

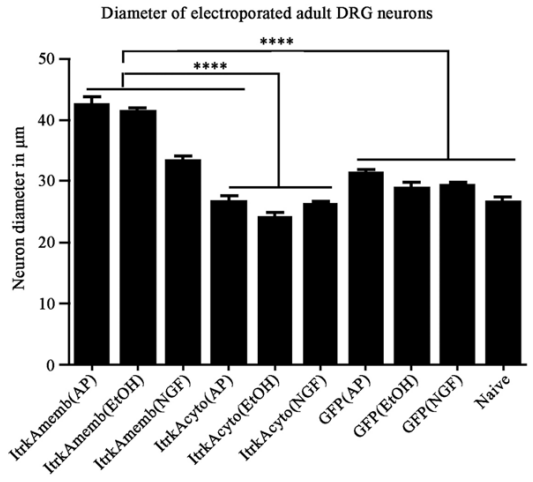

Figure 8. Mean diameter of electroporated adult DRG neurons. ****P < 0.0001. AP, AP20187; EtOH, ethanol, NGF, neuron growth factor.

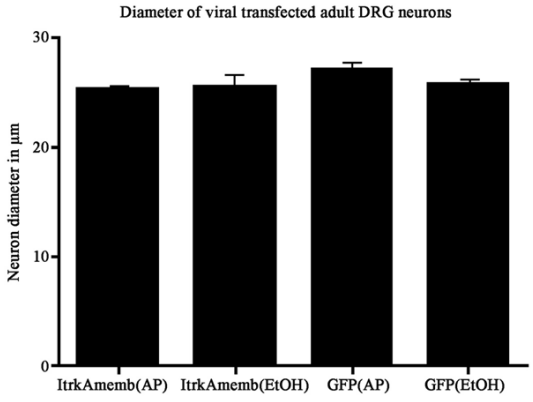

Figure 9. Mean soma diameter of virus-transfected adult DRG neurons. AP, AP20187; EtOH, ethanol.

$ItrkA_{memb}$ transfected groups and not in GFP controls, demonstrating successful transfection. Regarding Akt and Erk1/2 phosphorylation, no obvious difference was observed between the groups, although pErk1/2 signal was slightly higher in cells electroporated with $ItrkA_{memb}$ treated with AP20187 (**Figure 10**). Likewise, only $ItrkA_{memb}$-virus transfected cells demonstrated HA protein expression indicating successful virus transfection. Regarding Akt and Erk1/2 phosphorylation, however, only cells transduced with $ItrkA_{memb}$ and treated with AP20187 seemed to show higher phosphorylation both of Akt and pErk1/2 (**Figure 11**). As both of them express the HA transgene, all transfected DRGs showed a signal for HA, but the viral transfections showed a much stronger signal for HA than plasmid electroporation. In both, electroporation and virus transfection, AP20187 treatment appeared to result in a higher phosphorylation level of Erk1/2 and Akt in $ItrkA_{memb}$ transfected DRGs (**Figure 12**).

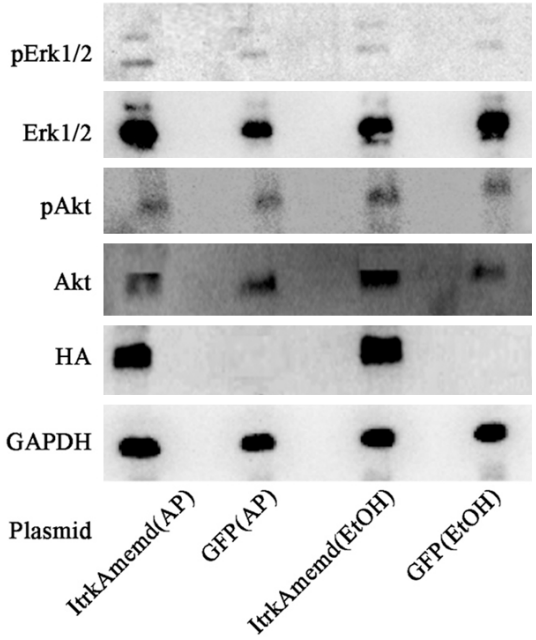

Figure 10. Western blot of ItrkA downstream signaling in electroporated adult DRGs. AP, AP20187; EtOH, ethanol.

Discussion

In this study, we aim to illustrate the mechanisms of ItrkA activity to trigger trkA signaling pathway for neurite growth. We firstly constructed p645 ($ItrkA_{memb}$), after one more adenine was added into the sequence of the original plasmid, in order to investigate the mechanism of trkA signal transduction and biological

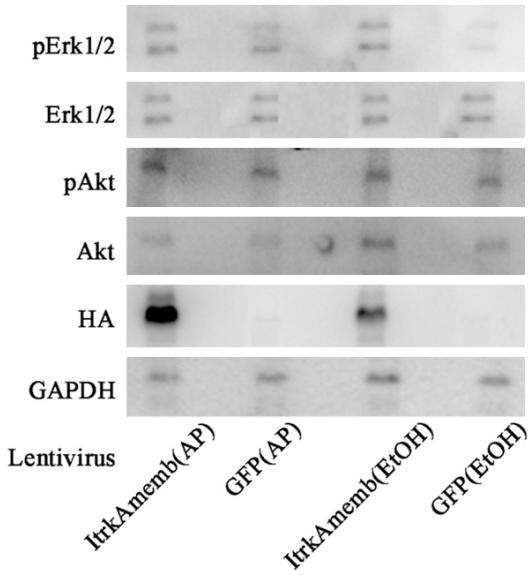

Figure 11. Western blot of ItrkA downstream signaling in $ItrkA_{memb}$ and GFP viral transduced adult DRGs. AP, AP20187; EtOH, ethanol.

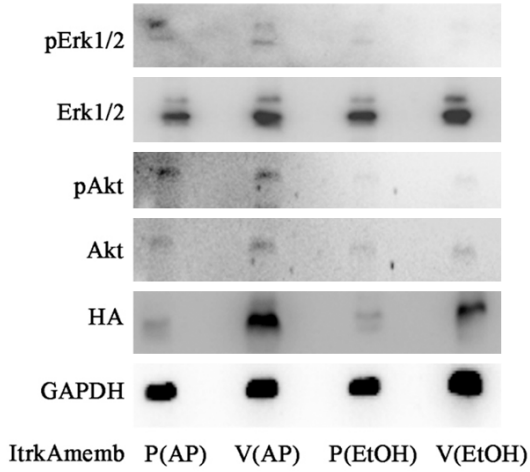

Figure 12. Western blot of ItrkA downstream signaling in $ItrkA_{memb}$ plasmid electroporation and its lentiviral transfection. P, plasmid; V, virus; AP, AP20187; EtOH, ethanol.

responses that is correlated with the regulation of a chimeric fusion protein containing only the membrane-anchored intracellular domain of trkA fused to two domains for small ligand-induced dimerization. With the expression of a membrane-anchored $ItrkA_{memb}$ transgene, electroporated adult DRG neurons showed larger cell-soma and reduced neurite growth, while neurite growth of virus-transfected adult DRG neurons was promoted in the presence of AP20187.

$ItrkA_{cyto}$-electroporated neurons treated with ethanol or NGF did not differ from naïve neurons also indicating that electroporation with $ItrkA_{cyto}$ plasmid had no effect on the neurite growth similar to GFP controls. Because of the virtually identical plasmid size, the electroporation itself should not affect $ItrkA_{memb}$ transfected neurons either. Thus, we propose that other reason(s) for the reduction of neurite growth of $ItrkA_{memb}$ transfected neurons must exist. Besides, after transduction of adult DRGs with $ItrkA_{memb}$ lentivirus, which exhibits less damages to cells [15-17], increased neurite length in the presence of AP20187 was observed as previously reported for $ItrkA_{cyto}$ lentiviral transduced eDRGs. The neurite length of $ItrkA_{memb}$-transfected DRGs treated with ethanol and GFP controls was within the range of naïve adult DRG neurons, indicating that viral transfection did not have a negative effect on neurite growth of adult DRG neurons. Electroporation and lentiviral transfection themselves don't have effects on neurite growth of adult DRG neurons but have a negative effect on neurite growth of embryonic DRG neurons.

In addition, NGF-treated neurons within each plasmid group were not different from the ethanol group in each electroporation, indicating that NGF has no effect on the neurite growth of electroporated adult DRGs. Naïve DRGs treated with NGF were not investigated here, but previous evidence showed that treatment with 50 ng/mL NGF increased neurite length using different culture condition and methods to measure neurite length [18-21]. Interestingly, only a little NGF effect can be detected when treating $ItrkA_{memb}$ transfected neurons. Though not different from the other two groups, the neurite length of $ItrkA_{memb}$ with NGF is longest in the three $ItrkA_{memb}$ groups, and is similar to the group of $ItrkA_{cyto}$ not treated with AP20187. This indicates that the effect of NGF can partly neutralize the effect of reducing neurite growth after electroporation with $ItrkA_{memb}$ plasmid. It has been demonstrated that ItrkA expression didn't interfere with normal NGF signal transduction in PC12 cells [12]. No observation of NGF effect might be due to the reason that all DRG neurons were dissected, transfected and traced. The roles of NGF are expected to be limited to the small diameter and some larger diameter neurons that express trkA, the receptor for NGF. Medium sized and large diameter

neurons expressing trkB or trkC and unresponsive to NGF are also in the cultures and quantified [22-24]. Previous receptor-binding studies on adult DRG neurons, transport of iodinated NGF and immunocytochemistry indicated that only about 40-45% of adult DRG neurons normally express trkA receptor [25]. Our study confirmed the previous study that the ability of ItrkA to regulate trkA signaling and biological responses, in the absence of NGF [12].

It is known that the peripheral nervous system has an intrinsic ability to regenerate after injury. Regeneration remains a significant clinical problem, especially in cases when the trauma results in complete transection of the nerve, though autologous nerve grafting techniques has been skillfully used for bridging nerve gaps [7]. Adult DRG neurons are among a few adult neurons known to regenerate robustly after peripheral injury. Injuries of axons of DRG neurons have been widely used as an excellent model system to study the cell-intrinsic mechanisms that regulate axonal regeneration. The peripheral and central branches of DRG neurons differ in their capacity to regenerate. The peripheral branches of the DRG neurons regenerate readily after peripheral nerve injury, whereas the central branches do not regrow after spinal cord injury. However, if peripheral axotomy occurs before the dorsal column injury (a process called conditioning lesion), central branches regain some ability to grow inside the spinal cord [26, 27]. The mechanisms underlying the conditioning effect remain incompletely understood, but transcriptional programs are found necessary for enhanced axonal growth after conditioning lesions [28]. Many potential candidate genes were identified that can be tested for growth-promoting effects [27, 29]. While an in vivo research would require much time and isolate neuron-specific effects, transfection of DRG neurons in vitro with a brief reliable culture, immediate growth assessment would be most appropriate and reliable to narrow the list of candidate genes for further testing in vivo.

Our data presented here to illustrate the role of ItrkA on regulating survival and neurite growth of adult or embryonic DRG neurons in response to AP20187. With sufficient concentrations of AP20187, it is likely to readily cross the blood-brain barrier [30], and has effect on CNS for further investigation in vivo. Several previous studies have shown that neurite length was reduced post-transfection in postnatal DRG neurons and adult retinal ganglion cells [31, 32]. Out data found that, similar to GFP-transfected controls, neurite length of $ItrkA_{memb}$ transfected DRG neurons was not influenced by AP20187 or NGF. Intriguingly, all $ItrkA_{memb}$ transfected neurons extended shorter neurites compared to GFP controls, indicating that $ItrkA_{memb}$ transfection reduced neurite outgrowth. Although the number of transfected cells was higher after electroporation than after viral transfection, viral transfections showed a much stronger signal for HA than plasmid electroporation, contrary to what was speculated. This may indicate that only with a high level of $ItrkA_{memb}$ in viral transfected DRG neurons, AP20187 can promote neurite growth, and that little $ItrkA_{memb}$ not localized close to each other on the membrane of electroporated DRG neurons is insufficient to be dimerized by AP20187.

Downstream signaling of trkA via Erk1/2 and PI3K/AKT phosphorylation was then investigated in $ItrkA_{memb}$-transfected DRGs with or without AP20187. Regarding Akt and Erk1/2 phosphorylation, no obvious difference was observed between $ItrkA_{memb}$ and GFP electroporated cells, although the pErk1/2 signal was slightly higher in $ItrkA_{memb}$ electroporated cells treated with AP20187. This explains why neurite growth of $ItrkA_{memb}$ transfected neurons that were treated with AP20187 was hardly different from $ItrkA_{memb}$ transfected, vehicle treated neurons. In contrast, in lentivirus transduced cells, only cells transduced with $ItrkA_{memb}$ treated with AP20187 seemed to show higher phosphorylation both of Akt and pErk1/2, explaining the reason that AP20187 treatment can promote neurite growth of $ItrkA_{memb}$ lentiviral transfected DRG neurons. $ItrkA_{memb}$ viral transduced neurons treated with vehicle have a normal length of neurite length but also seemed to show higher phosphorylation of pErk1/2, which might be mainly utilized for cell survival.

In both, electroporation of $ItrkA_{memb}$ and $ItrkA_{memb}$ viral transfection, AP20187 treated cells appear to show a higher phosphorylation level of Erk1/2 and Akt than cells treated with vehicle. It suggests that AP20187 treatment

can increase the phosphorylation of Erk1/2 and Akt in cells expressing $ItrkA_{memb}$. This result just indicates that trkA downstream signals can be activated following the dimerization of $ItrkA_{memb}$ by AP20187 as expected. With $ItrkA_{cyto}$ transgene and AP20187 treatment, electroporated adult DRG neurons showed only a slightly increased neurite growth compared to naïve, while electroporated embryonic DRG neurons showed increased neurite growth compared to naïve. With $ItrkA_{memb}$ transgene, electroporated embryonic DRG neurons showed increased neurite growth independent of AP20187. The $ItrkA_{memb}$ protein was expressed stronger in virus-transfected cells and less in electroporated cells. The trkA downstream signaling pathway can be regulated by dimerization of $ItrkA_{memb}$ with AP20187.

The survival and neurite growth of adult/embryonic DRG neurons in response to ItrkA can be further understood, once transfection of adult DRG neurons with $ItrkA_{cyto}$ lentivirus has not been done. Our study was not designed to distinguish between localized axonal and somal ItrkA signaling and retrograde transport of activated ItrkA signaling complexes, but the results suggest that both survival and differentiation signals can be regulated by membrane anchored ItrkA dimerization in the absence of NGF. In the future, using compartmentalized neuronal cultures in vitro or localized application of the small-molecule ligand AP20187 to axons distant from the cell body in vivo will allow for the investigation of potential retrograde ItrkA signaling and its effects on neuronal survival and neurite growth.

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Disclosure of conflict of interest

None.

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