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An Inducible Tyrosine Kinase Receptor for Axonal Regeneration

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Neurotrophins have been shown to augment neuronal survival, function, and axon growth in numerous animal models of neuronal degeneration and nervous system injury. The inability to traverse the blood–brain barrier to reach target areas in the CNS and dose-limiting adverse effects from broad exposure of nontargeted structures in the CNS and PNS limit their application in clinic translation. Reaching effective concentrations of neurotrophic factors within a CNS target and the ability to regulate growth factor expression or signaling pathways downstream from growth factor receptors are desirable goals. Thereby, the intrinsic ability of neurons for regeneration might be enhanced. An inducible trkA (ItrkA) system has been shown to regulate embryonic DRG neuronal survival and neurite outgrowth in vitro. The inducible trkA receptor exploits the inherent dimerization-induced activation of receptor tyrosine kinases to trigger trkA signaling in response to the small-molecule ligand AP20187. To further confirm the ability of ItrkA to regulate trkA signaling, biological responses and signaling pathways influencing embryonic and adult DRG neuronal differentiation and neurite outgrowth were investigated using viral and non-viral gene delivery. Based on this，
the main purpose of this study was to establish reproducible conditions for gene delivery and cultivation of adult and embryonic DRGs and to investigate the effects of ItrkA plasmid electroporation (non-viral gene delivery) on survival and neurite growth of adult and embryonic DRG neurons in vitro and to investigate the impact of ItrkA lentiviral transfection on survival and neurite growth of adult and embryonic DRG neurons in vitro.
In a previously described ItrkA plasmid, one adenine (A) was found to be missing 3’ from the myristoylation signal resulting in a frame-shift mutation. A new ItrkA plasmid was therefore established by correcting the sequence of the original plasmid. Based on the presumably different localization of the expressed protein at the membrane, the original plasmid was named ItrkA-cytosol ($ItrkA_{cyto}$) and the new plasmid ItrkA-membrane ($ItrkA_{memb}$). Adult DRGs were dissected from adult Fischer 344 rats (8–14 weeks) and embryonic DRGs were dissected from E16 rat embryos. DRGs were digested to obtain single cell suspensions. For non-viral gene delivery, cells were electroporated with plasmid DNA using the Lonza 4D-Nucleofector X-unit system. Cells were treated with AP20187, vehicle or 50ng/mL NGF. All cells were cultured at 37˚C and 5% CO_2 for 48 hours. For viral gene delivery, adult and eDRGs were plated and immediately transduced with GFP or ItrkA lentivirus at an MOI of 50 and treated with AP20187. After cultivation, adult and eDRGs were immunolabeled for GFP

and beta-III-tubulin. Twelve imaged and automatically stitched non-overlapping montages of 4×5 frames covering more than 90% of the 35mm well surface were used for quantification. Neurite outgrowth assessments were done by manually tracing the longest neurite of each neuron. Cell diameters were also measured and averaged for each well. To understand the mechanisms of ItrkA activation, protein expression after $ItrkA_{memb}$ transfection and trkA downstream signaling were investigated by Western-blotting.

Following adult DRG electroporation, no difference in neurite length between all GFP control groups was detected, and neurite length was within the range of naïve neurons. 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. Following eDRGs electroporation, $ItrkA_{memb}$ transfected eDRG neurons showed increased neurite growth independent of AP20187 whereas increased neurite growth of $ItrkA_{cyto}$ transfected cells was dependent on AP20187 as expected. After lentivirus transduction, AP20187 treatment of $ItrkA_{memb}$ virus-transfected eDRG neurons significantly increased neurite growth compared to untreated $ItrkA_{memb}$ and GFP virus-transfected control neurons. Similarly, adult DRGs infected by $ItrkA_{memb}$ lentivirus also showed increased neurite length in the presence of AP20187. Quantification of the mean diameter of transfected DRGs demonstrated that $ItrkA_{memb}$ 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. 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. The diameter of transfected E16 DRG neurons was also not affected after membrane and cytosolic ItrkA electroporation or ItrkA lentiviral transfection. Western-blotting confirmed $ItrkA_{memb}$ expression in electroporated and virus-transfected cells, but the latter showed a much higher expression signal. Regarding Akt and Erk1/2 phosphorylation, 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. Comparing electroporation and virus transfection, AP20187 treatment appeared to result in higher phosphorylation levels of Erk1/2 and Akt in $ItrkA_{memb}$ transfected DRGs.

In conclusion, the responsiveness of adult/embryonic DRG neurons after ItrkA transfection differs, which is reflected in the change of cell soma size and/or neurite growth, depending on the gene delivery technique, expression level and the localization of ItrkA.