Experimental Neurology 279 (2016) 197–211

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Large-scale reconstitution of a retina-to-brain pathway in adult rats using gene therapy and bridging grafts: An anatomical and behavioral analysis☆

Si-Wei You [d,1], Mats Hellström [e,1], Margaret A. Pollett [a], Chrisna LeVaillant [a], Colette Moses [a], Paul J. Rigby [c], Marissa Penrose [b], Jennifer Rodger [b,f], Alan R. Harvey [a,f,*]

[a] School of Anatomy, Physiology and Human Biology, The University of Western Australia, Australia
[b] School of Animal Biology, The University of Western Australia, Australia
[c] Centre for Microscopy, Characterisation and Analysis, The University of Western Australia, Australia
[d] Department of Ophthalmology, Xijing Hospital, The Fourth Military Medical University, Xi'an, China
[e] Laboratory for Transplantation and Regenerative Medicine, Dept. of Obstetrics and Gynecology, Sahlgrenska Academy, University of Gothenburg, Sweden
[f] Western Australian Neuroscience Research Institute, Australia

ARTICLE INFO

Article history:
Received 5 February 2016
Received in revised form 1 March 2016
Accepted 2 March 2016
Available online 10 March 2016

Keywords:
Gene therapy
Peripheral nerve transplantation
Adult rat visual system
Axonal regeneration
Superior colliculus
Neurotrophic factors
Retinal ganglion cells

ABSTRACT

Peripheral nerve (PN) grafts can be used to bridge tissue defects in the CNS. Using a PN-to-optic nerve (ON) graft model, we combined gene therapy with pharmacotherapy to promote the long-distance regeneration of injured adult retinal ganglion cells (RGCs). Autologous sciatic nerve was sutured onto the transected ON and the distal end immediately inserted into *contralateral* superior colliculus (SC). Control rats received intraocular injections of saline or adeno-associated virus (AAV) encoding GFP. In experimental groups, three bi-*cis*tronic AAV vectors encoding ciliary neurotrophic factor (CNTF) were injected into different regions of the grafted eye. Each vector encoded a different fluorescent reporter to assess retinotopic order in the regenerate projection. To encourage sprouting/synaptogenesis, after 6 weeks some AAV–CNTF injected rats received an intravitreal injection of recombinant brain-derived neurotrophic factor (rBDNF) or AAV–BDNF. Four months after surgery, cholera toxin B was used to visualize regenerate RGC axons. RGC viability and axonal regrowth into SC were significantly greater in AAV–CNTF groups. In some cases, near the insertion site, regenerate axonal density resembled retinal terminal densities seen in *normal* SC. Complex arbors were seen in superficial but not deep SC layers and many terminals were immunopositive for presynaptic proteins vGlut2 and SV2. There was improvement in visual function via the grafted eye with significantly greater pupillary constriction in both AAV–CNTF + BDNF groups. In both control and AAV–CNTF + rBDNF groups the extent of light avoidance correlated with the maximal distance of axonal penetration into superficial SC. Despite the robust regrowth of RGC axons back into the SC, axons originating from different parts of the retina were intermixed at the PN graft/host SC interface, indicating that there remained a lack of order in this extensive regenerate projection.

1. Introduction

Injury to the adult mammalian central nervous system (CNS) results in long-lasting functional deficits, a consequence of intrinsic and extrinsic factors that together impact on plasticity and regenerative ability. In the rodent primary visual system, often used as an experimental model of CNS repair, regrowth of retinal ganglion cell (RGC) axons after ON crush can be enhanced using a variety of therapeutic manipulations (Harvey et al., 2006; Berry et al., 2008; Watanabe, 2010; Fischer and Leibinger, 2012). In mice this is most effective after conditional retinal deletion of phosphatase and tensin homologue (PTEN) and in some cases additional deletion of suppressor of cytokine signalling 3 (SOCS3) (Sun et al., 2011; de Lima et al., 2012; Luo et al., 2013). At present this latter type of approach, while important and instructive, is species-specific and dependent on the generation of floxed mouse lines and the use of adeno-associated viral (AAV) vectors to express cre to delete relevant gene(s). It is difficult to translate to different species and to other CNS injury models.

Abbreviations: AAV, adeno-associated virus; AChE, acetyl cholinesterase; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CTB, cholera toxin B subunit; GFP, green fluorescent protein; ON, optic nerve; PN, peripheral nerve; RGC, retinal ganglion cell; SC, superior colliculus; SGS, stratum griseum superficiale.

☆ Funded by the WA Neurotrauma Research Programme, an NHMRC Senior Research Fellowship APP1002258 to JR, and the National Natural Science Foundation of China (no. 81470631) awarded to S-W Y.

* Corresponding author at: School of Anatomy, Physiology and Human Biology, The University of Western Australia, M309, 35 Stirling Highway, Perth, WA 6009, Australia.
E-mail address: alan.harvey@uwa.edu.au (A.R. Harvey).

[1] Co-first authors.

http://dx.doi.org/10.1016/j.expneurol.2016.03.006
0014-4886/

Another way of enhancing mammalian RGC regeneration is to graft segments of peripheral nerve (PN) on to the cut ON. Regeneration of adult RGC axons through these grafts has been achieved in wild-type rats (Vidal-Sanz et al., 1987; Villegas-Perez et al., 1988; Thanos, 1992; Avilés-Trigueros et al., 2000), hamsters (Carter et al., 1989, 1994; Sasaki et al., 1999), mice (Cui and Harvey, 2000), ferrets (Quan et al., 1999) and cats (Watanabe and Fukuda, 2002). When the distal end of PN grafts is inserted into visual centers in the rat or hamster brain, RGC axons re-establish synaptic contacts with central target neurons and some non-patterned visual behaviors have been restored (Vidal-Sanz et al., 1987; Keirstead et al., 1989; Carter et al., 1989, 1994; Sauvé et al., 1995; Sasaki et al., 1999; Heiduschka and Thanos, 2000; Vidal-Sanz et al., 2002), however there is little evidence of restoration of higher level patterned visual function or restitution of visual topography (Sauvé et al., 2001).

In these earlier rodent studies, the PN graft approach was rarely combined with other growth-enhancing therapeutic strategies. Usually less than 20% of the RGC population remained alive, and perhaps 10–20% of these surviving neurons regenerated an axon (about 2000 RGC axons). Thus the number of axons re-entering host visual centers was small and maximal extent of outgrowth was limited. Furthermore, in many studies PN grafts were inserted after a several week delay into *ipsilateral* visual centers, and regenerative growth via PN grafts was enhanced by removal of the remaining normal eye, a procedure that obviously cannot be applied in any clinical context.

In adult rat studies, by combining pharmacotherapy and AAV gene therapy to deliver a secretable form of ciliary neurotrophic factor (CNTF) to injured RGCs, on average about 10,000 RGCs can be induced to regrow axons at least 1–1.5 cm into blind-ended PN grafts (Leaver et al., 2006; Hellström and Harvey, 2011, 2014). In the present study, an autologous sciatic nerve was sutured onto the transected ON, the distal end inserted acutely into contralateral superior colliculus (SC) and AAV–CNTF vectors injected into the eye at the time of surgery (Hellström et al., 2011b). To track and identify retinotopically distinct RGC axons, and thus reveal any topographic order in the regenerate retinal projection, we used three bi-*cis*tronic AAV–CNTF vectors expressing different fluorescent reporters, each injected into different regions of the grafted eye.

AAV–CNTF-injected animals were allocated to 3 groups. The first group received no further intervention. In the other groups, to encourage sprouting and synaptogenesis within the SC (Sawai et al., 1996; Cohen-Cory et al., 2010), the grafted eye was additionally injected with recombinant brain-derived neurotrophic factor (rBDNF) or AAV–BDNF 6 weeks after the initial surgery. For all animals, visual behaviors mediated by the grafted eye were tested at 15 weeks post-transplantation, and the total regenerate projection was visualized by injecting grafted eyes with the anterograde tracer cholera toxin B (CTB).

2. Materials and methods

Forty-six PVG/c rats (female, 8–10 weeks of age, Animal Resources Centre, WA) were used in this study. One additional rat was used as a non-operated control. The animals were housed in standard sized cages with filtered tops along with sterile food and water access ad libitum in a 12-h light–dark cycle (red lighting: 10 am to 10 pm, white lighting: 10 pm to 10 am). Care and treatment of all animals were in strict accordance with NHMRC guidelines and all experimental procedures were approved by The University of Western Australia Animal Ethics Committee. The overall experimental plan, including details of groups, is shown in Fig. 1.

2.1. Viral vectors

Recombinant AAV2 vectors were produced by Vector Biolabs (Philadelphia, USA) from plasmid pTRUF12 containing BDNF and plasmid pTRUF12.1 (gift, Prof. Joost Verhaagen) containing a secretable form of CNTF (Sendtner et al., 1992) linked via IRES to 3 different fluorescent reporter proteins; green fluorescent protein (GFP), mCherry and Venus (gift, RIKEN Brain Science Institute, Japan). Both plasmids contained the cytomegalovirus-chicken beta actin promoter, but pTRUF12.1 lacked an intron sequence to enable packaging of the larger CNTF construct. Successful expression of the three reporters was seen in HEK-293T cells using lipofectamine mediated delivery of each plasmid. A control AAV2–GFP vector was also produced using the pTRUF12.1 plasmid.

The AAV–BDNF–GFP and AAV–CNTF–GFP vectors were successfully characterized previously (Leaver et al., 2006; Hellström and Harvey, 2011). We confirmed functionality of the new AAV-vectors by transducing HEK-293T cells in vitro under standard culturing conditions with reduced fetal calf serum (FCS; 2%). After 5 days, cells were visualized under fluorescence and harvested for mRNA (qPCR) and protein (ELISA) analysis.

Total RNA was extracted from cells using standard Qiagen protocols. Validated primer pairs were used to quantify mRNA transcript expressions of *BDNF*, *CNTF*, *mCherry* and *GFP*, and internal reference genes *Ppia*, *TBP*, and *YWAHZ*. qPCR runs were performed on the Rotor-Gene 6000 (Qiagen, USA), using Bio-Rad iQ SYBR 2× Mastermix (Bio-Rad, Australia). Normalized data were analyzed for changes between groups by Kruskal–Wallis ANOVA, and correlations confirmed using Biogazelle software. ELISA was used to detect BDNF and CNTF release from the cells after AAV transduction. For BDNF detection ChemiKine BDNF Sandwich ELISA (Millipore Bioscience Research Reagents) was used, and for CNTF measurements the TSZ ELISA kit (Waltham. MA Cat No M1473) was used, in both cases following manufacturer's instructions. Optical density was determined in a plate reader set at 450 nm. Sample values were calculated from the standard curve. Optical density was a linear function of concentration between 15 and 1000 pg ml^{-1} with a coefficient of variation <10%.

2.2. Surgical procedures

Rats were anesthetized with a 1:1 mixture (1.5 ml/kg) of ketamine (100 mg/ml) and xylazine (20 mg/ml). Eye ointment containing atropine sulphate (10 mg/g, Troy Ilium) was applied to protect the cornea during surgery. The sciatic nerve was exposed in the left leg and a segment of about 3.5 cm was isolated. The leg wound was moistened with sterile saline and margins temporarily re-opposed to keep the isolated peripheral nerve (PN) in situ until the grafting procedure. The left ON was exposed by protruding the eye and opening the conjunctivum. The ON was completely severed about 1.5 mm from the optic disc, while taking care not to disrupt the blood flow to the retina. The PN segment was removed from the leg and the proximal end was transplanted onto the ON stump (end-to-end) using a single suture (10-0 Ethilon, Johnson & Johnson, North Ryde, NSW, Australia).

The head of the animal was then fitted in a head holder using a maxillary clamp and part of the right posterior hemisphere was exposed and aspirated to reveal the surface of the superior colliculus (SC). The PN was then laid on the skull and the distal end was prepared so that individual nerve bundles protruded from the epineurial sheath. The bundles were teased apart prior to gentle insertion into the rostral part of the right SC just below the pial surface using a sterile glass pipette (Fig. 2A, B). The surgical cavity was packed with small pieces of gelfoam and the overlying scalp was closed with sterile fine sutures (6-0). Then the leg wound was closed with sterile wound clips. Rats also received a subcutaneous injection of buprenorphine (0.02 mg/kg, Temgesic; Reckitt & Colman, Hull, UK) followed by Carprofen (5 mg/kg, Provet, WA an intramuscular injection of Benacillin (0.3 mg/kg, Troy Ilium; NSW, Australia). Rats were placed on a heat pad and monitored until recovery from the anesthesia at regular intervals thereafter. The following parameters were checked regularly post-surgery: signs of post-operative infection or bleeding, patency of sutures, self-grooming, stable or weekly weight gain, general alertness and responsiveness, normal