Neurotherapeutics (2018) 15:489–499
https://doi.org/10.1007/s13311-018-0608-5

ORIGINAL ARTICLE

Alleviation of Trigeminal Nociception Using p75 Neurotrophin Receptor Targeted Lentiviral Interference Therapy

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Published online: 9 February 2018

Abstract

Acute and chronic trigeminal (TG) neuropathies are the cause of considerable distress, with limited treatments available at present. Nociceptive neurons enriched with the vanilloid type 1 receptor (VR1) partake in pain sensation and sensitization in the TG system. While VR1 blockers with anti-nociceptive potential are of substantial medical interest, their use remains limited due to poor selectivity and lack of cell-targeting capabilities. This study describes a methodology for the alleviation of nociception via targeted depletion of VR1 in TG sensory neurons in rats. In cultured TG ganglion neurons, VR1 expression was virtually abolished by lentiviral short hairpin RNA (LV-VR1). By decorating GFP encoding LV (LV-GFP) and LV-VR1 with IgG192 for targeting TG sensory neurons enriched with the p75 neurotrophin receptor (p75NTR), transduction of a reporter GFP and VR1 depletion was achieved after injection of targeted vectors into the whisker pad. In IgG192/LV-VR1-injected rats, the behavioral response to capsaicin exposure as well as Erk 1/2 phosphorylation and VR1 current activation by capsaicin were significantly reduced. This pioneering investigation, thus, provides a proof of principle for a means of attenuating TG nociception, revealing therapeutic potential.

Keywords p75NTR · Capsaicin · TRPV1 · Neuropathic pain · Neurotherapeutics

Introduction

Lesion and functional impairments of peripheral nerves cause an array of poorly manageable medical conditions, affecting the lives of hundreds of thousands worldwide [1]. The rapidly aging population, increased incidents of diabetes, and improved cancer survival after chemotherapy are also major risk factors for different forms of neuropathies and sensitization of peripheral nerves, predicting a dramatic rise in their frequency in the upcoming years. One of the most prevalent diseases of peripheral nerves is trigeminal (TG) neuralgia (TGN), also known as *tic douloureux* [2]. This condition affects mainly women over 50 years of age and is associated with a malfunctioning trigeminal sensory nerve that connects large areas of the face to the brain [2, 3]. Manifesting initially as short-term mild unilateral facial soreness, TGN progresses to bouts of excruciating pain of increased frequency and duration [4]. Amongst the known causes of this condition, stroke, hypersensitization of the trigeminal nerve due to abnormal vascular compression, facial trauma, and tumor associated with focal demyelination are the most widely documented [5, 6].

Three main branches of the TG nerve, the ophthalmic, maxillary, and mandibular, converge on the TG ganglion (TGG) located within Meckel's cave, which contains the cell bodies of sensory nerve fibers projecting to the Sp5 nucleus of the brain stem (Fig. 1a, b). Insights into the molecular processes of painful sensation have been obtained from the cloning and functional characterization of the vanilloid type 1 receptor (VR1), also a receptor for capsaicin, the pungent ingredient in

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13311-018-0608-5) contains supplementary material, which is available to authorized users.

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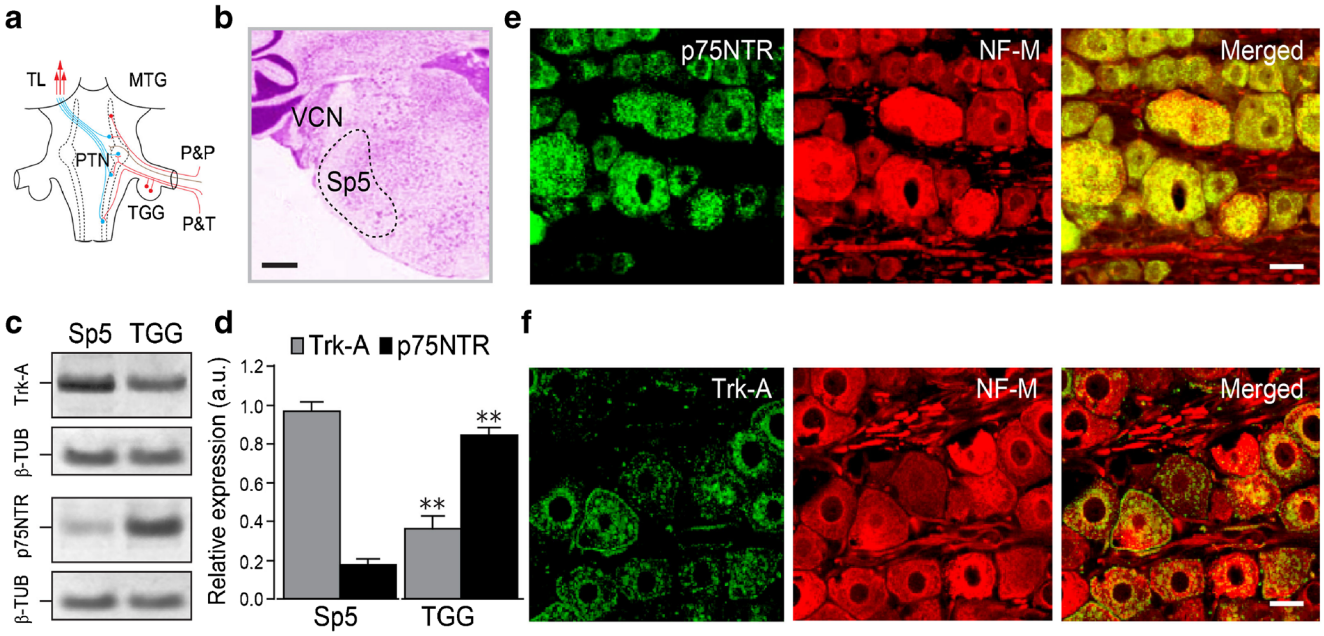

Fig. 1 Enrichment of neurotrophin receptors p75NTR and TrkA in the rat TGG and brain stem. (**a**) Schematic representation of the trigeminal nerve with ganglion and primary sensory nuclei that relay sensory inputs to the rat brain stem. P&T pain and touch, P&P pressure and proprioception, TGG trigeminal ganglion, PTN pontine trigeminal nucleus, MTG motor trigeminal nerve, TL thalamic lemnisci (left). (**b**) Nissl-stained rat BS cross section showing the location of the trigeminal sensory nucleus (Sp5) and the ventral cochlear nucleus (VCN). Scale bar = 0.5 mm. (**c**) Western blots demonstrating the expression of p75NTR and TrkA in the TGG and in the brain stem Sp5 area, with β-tubulin III used as an endogenous loading control. (**d**) Summary histograms of the relative expression of p75NTR and TrkA in the TGG and Sp5 areas following normalization to β-tubulin III loading controls from Western blot quantitative analysis. Significance is represented by asterisks; $^{**}p < 0.01$. (**e**), (**f**) Typical confocal microscopic images of TGG neurons expressing p75NTR (green; upper left) and TrkA (green; lower left). Sensory TGG neurons are labeled with a specific neurofilament medium (NF-M; red; middle) marker. Note the stronger enrichment of TGG sensory neurons with p75NTR. Scale bar = 10 μm

hot chili peppers [7]. Capsaicin and related endogenous vanilloid analogs, as well as high temperature, produce burning pain by depolarizing specific subsets of afferent nociceptors (e.g., C and type 2 A-δ) via activation of the coupled transient receptor potential (TRP) ion channel (or vanilloid) receptor, TRPV1/VR1 [7]. As VR1 is enriched only in this subset of nociceptors and is involved in neuropathic pain, targeted depletion of VR1 is expected to alleviate nociception while preserving tactile and other modalities of sensation (mediated by A-α, A-β, and type 1 A-δ fiber neurons) and motor function [8–10].

Mice lacking the VR1 ion channel not only reveal a loss of capsaicin sensitivity but exhibit a deficit in their ability to detect and respond to pain due to tissue-damaging heat [11, 12]. The unique role of VR1-positive nociceptors in pain sensation was also shown by others reporting the analgesic effects of the VR1 ligand, resiniferatoxin in non-human primates, when administered before capsaicin exposure [13]. The clinically approved pharmaceutical treatments used currently for alleviating distress related to TG sensitization are of low efficacy and specificity, involving anticonvulsant (e.g., carbamazepine, lamotrigine, pimozide) and/or antidepressant (amitriptyline) therapies [14]. Small molecule antagonists of TRPV1 receptors have been of considerable interest recently, with the majority of available formulas however, demonstrating low metabolic stability and pharmacokinetic properties as well as poor selectivity as revealed in rodent studies [15]. Indeed, off-target effects on nicotinic acetylcholine receptors, voltage-gated Ca^{2+} channels, and other members of the TRP receptors render the clinical use of TRPV1 receptor blockers problematic [16–18]. Also, due to the importance of TRPV1 in temperature regulation, the systemic use of antagonists carries the risks of dysregulation of the body temperature as well as burn injuries resulting from an increased thermal threshold [15, 19–21]. Repetitive use of transcranial magnetic stimulation has also been considered for alleviating trigeminal pain but is of low efficiency and requires in-depth mechanistic validation [22]. Thus, all currently available methods for management of the distress associated with TGN sensitization lack desired effectiveness, are short acting, and come with considerable risks and off-target effects [23].

Previously, we took advantage of the physiological enrichment of basal forebrain (BF) cholinergic neurons with the p75 neurotrophin receptor (p75NTR) for their targeting by IgG192-conjugated viral vectors within the intact brain [24]. It was hypothesized that such an approach could be useful for

the transduction of therapeutics to peripheral pain-sensing sensory neurons, also enriched with p75NTR. Herein, p75NTR has been utilized for retro-axonal short hairpin RNA (shRNA) interference and depletion of VR1 in TG nociceptors. This affords delivery of therapeutic vectors to rat TG neurons and provides a means for targeted attenuation of nociception in an experimentally induced neuropathy model in rat, offering the prospect of effective pain management.

Materials and Methods

Experimental Animals

All procedures involving rats conformed to the guidelines approved by the Research Ethics Committee of Dublin City University (DCU) and the animal license issued by the Department of Health and Children, Republic of Ireland. Wistar female rats [postnatal day 5 and 1 month old] were used for all experiments (n = 4–5 per group), which were housed (21 ± 1 °C, humidity 36 ± 2% in a 12-h light/12-h dark cycle) in the Bio-Resource Unit at DCU with food and water provided *ad libitum*.

TGG and Brain Stem Extraction with Processing for Histology or Western Blotting

Under a lethal dose of sodium pentobarbital (200 mg/kg, i.p.), rats were perfused with 1× PBS (100 ml) followed by 4% paraformaldehyde (PFA) in PBS (80 ml). Brain stem (BS) and TGG were dissected and incubated overnight in 4% PFA followed by dehydration for 24 h in 30% sucrose in 0.1× PBS. Afterwards, the tissue was embedded in O.C.T. compound (Tissue-Tek), cryo-sectioned (30 μm) and stained with cresyl violet (Nissl dye; Sigma-Aldrich) or utilized for immunohistochemistry. For Western blotting, unpreserved tissue was extracted and placed in T-PER extraction buffer (Thermo Scientific) with the addition of protease inhibitor cocktail tablets (Roche). The reagent (100 μl) was added to the tissue (100 μg) followed by homogenization and sonication (20 s). After a 5-min centrifugation at 14,000*g*, the supernatant was retained and stored at − 80 °C. Protein concentration was determined using a bicinchoninic acid (BCA) assay (Thermo Scientific).

Electrophoresis and Western Blotting

TGG or BS lysates (30 μg/10 μl) were mixed with 4× NuPage LDS sample buffer (2.5 μl; Life Technologies) and heated for 5 min at 70 °C before loading onto 12% NuPage Bis-Tris gels (Novex Life Technologies) with electrophoresis at 4 °C in 1× MOPS SDS running buffer at 180 V. Separated proteins were transferred onto Nytran membranes under standard conditions followed by blocking in 5% bovine serum albumin in TBS-T [Tris base 0.2 M, NaCl 1.5 M, Tween 20 (0.05%)]. TrkA, p75NTR, VR1, vesicular stomatitis virus glycoprotein G (VSV-G), GFP, and phosphorylated Erk 1/2 were detected with overnight incubation of Nytran membranes at 4 °C with the following antibodies (1:500 in blocking reagent): rabbit anti-TrkA (mAb; Abcam), rabbit anti-p75 nerve growth factor (NGF) receptor (Abcam), rabbit anti-rat TRPV1 (extracellular)-ATTO-488 (Alomone Labs), rabbit anti-VSV-G (Abcam), goat anti-GFP (Abcam), and rabbit anti-Erk 1/2 (Cell Signaling) antibodies, respectively. Beta-tubulin III and β-actin were also detected as controls using rabbit anti-β-tubulin III (Sigma-Aldrich) and rabbit anti-β-actin (Abcam) antibodies, respectively. Following extensive washing with TBS-T, the membranes were exposed to alkaline phosphatase-conjugated goat anti-rabbit (1:1000; Sigma-Aldrich) secondary antibody for 1 h at room temperature (RT). Proteins (TrkA, p75NTR, and β-tubulin III) were visualized using a mix of 5-bromo-4-chloro-3′-indolyl-phospate *p*-toluidine and nitro blue tetrazolium chloride solution (Sigma-Aldrich). Western blots were photographed with a FluorChem HD2 gel visualization system (Alpha Innotec, Germany) with specific protein band intensities quantified using ImageJ (NIH, Bethesda, MD, USA).

Immunofluorescent Staining and Confocal Microscopy

TGG sections (30 μm) were post-fixed for 1 h in PFA (4%), permeabilized with PBS-T (0.5% triton X-100) for 1 h, and blocked for 1 h in 5% BSA in 1× PBS-T supplemented with 2% goat serum. Sections were subsequently exposed overnight at 4 °C to appropriate TrkA, p75NTR primary or VR1 antibodies (indicated above), rabbit anti-neurofilament medium (NF-M; Millipore), or mouse monoclonal SNAP-25 (Covance; 1:500 in blocking reagent). Following extensive washing (three times for 15 min in 1× PBS), sections were exposed to secondary antibodies labeled with Alexa Fluor 488 goat anti-rabbit (1:1000 in blocking reagent; Life Technologies), Alexa Fluor 568 goat anti-rabbit (1:1000 in blocking reagent; Life Technologies), or Alexa Fluor 568 goat anti-mouse (1:1000 in blocking reagent; Abcam) with incubation for 1 h at RT. Control sections were incubated with the appropriate secondary antibody only. Additional washing steps were performed as described above with sections subsequently mounted in Vectashield HardSet with DAPI (Vector Labs) and covered with cover slips. Fluorescence images were acquired using a LSM 710 confocal microscope (Carl Zeiss). Argon and helium/neon lasers provided the 488-nm (GFP) and 568-nm line for excitation with emitted signals sampled in a frame mode at a spatial resolution of 30 nm per pixel and a

dwell time of 1.5 μs through a Plan-Apo × 40/0.95 NA oil immersion objective.

Isolation and Culturing of Rat TGNs

Previously described procedures were used for tissue culture [25–27]. In brief, TGG sections were dissected from PD5 female Wistar rats after being deeply anesthetized with an injection of Dolethal (50 mg/kg, i.p.). The tissue was placed in ice-cold L15 medium (Gibco) and washed twice in ice-cold sterile Hank's buffered salt solution (Ca^{2+} and Mg^{2+} free; CMF-HBSS; Gibco) before centrifugation at 170*g* for 1 min. After chopping into small pieces and passing through 10-ml Falcon pipettes pre-coated with L15 medium, the tissue was incubated while shaking at 37 °C for 30 min in a 1:1 mixture of CMF-HBSS containing Dispase II (2.4 U/ml) and collagenase I (1 mg/ml). The suspension was then gently triturated through 10-ml Falcon pipettes pre-coated with L15 medium until cloudy, before adding DNase I (1 mg/ml) for 15 min. Following centrifugation at 170*g* for 5 min, the pellet was suspended and washed thrice in culture medium [Ham's F12 solution containing 10% (*v*/*v*) heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin]. Cells were seeded onto 24-well plates pre-coated with poly-L-lysine (0.1 mg/ml) and laminin (20 g/ml) in F12 medium supplemented with NGF (50 ng/ml; Sigma-Aldrich), and maintained in a CO_2 incubator at 37 °C. After 24 h and every other day thereafter, the culture supernatant was replaced with a medium containing the anti-mitotic agent cytosine-D-arabinofuranoside (10 mM).

Lentiviral Production

A replication-deficient lentivirus was produced by transfecting HEK-293FT cells with pWPT-GFP, pMD-2G, and pCMV-d-8.74 using polyethylenimine as previously described [28]. Protein content of the resultant GFP encoding lentivirus (LV-GFP) was measured using bicinchoninic acid and titers determined by p24 ELISA (Aalto) [18].

Lentiviral shRNA Transfection of TGNs

TGNs were transfected overnight in antibiotic/antimycotic-free media with replication-deficient LV-GFP, Mission® shRNA lentiviral transduction particles (LV-VR1; cat no. SHCLNV; Sigma-Aldrich), or Mission® pLKO.1-puro non-mammalian shRNA negative control transduction particles (control; cat no. SHC002V; Sigma-Aldrich) at a multiplicity of infection (MOI) ratio of 5. Following refreshment of growth media, proteins were extracted for immunoblotting or cells fixed with PFA for immunohistochemistry.

Lentivirus-IgG192 Conjugation with Biotin-Streptavidin

The targeting methodology utilized was previously reported [24]. In brief, sulfo-NHS-SS-biotin (Thermo Fisher) was used for biotinylation of LV particles, in accordance with the manufacturer's instructions. Biotin was dissolved in ultrapure dH_2O (10 μg/1 μl) and added to LV particles at a 40-fold molar excess based on VSV-G (70 Kd) with incubation at 4 °C for 2 h. Non-reacted sulfo-NHS-SS-biotin was removed by dialysis (12–14 k molecular weight cut-off) in 1× PBS (pH 7.4) at 4 °C overnight. The amount of biotin bound to the lentivirus was estimated with 4′-hydroxyazobenzene-2-carboxylic acid avidin reagent (Sigma-Aldrich, St. Louis, MO, USA), using a Pierce calculator (http://www.piercenet.com/haba/habacalcmp.cfm). The conjugation kit (Innova Biosciences) was used to link streptavidin (10 μg) to anti-p75NTR antibody (IgG192, 10 μg; Millipore) in 100 μl amine-free buffer (10–50 mM, pH 6.5–8.5). Following overnight incubation at RT, quencher reagent [1 μl; supplied in kit (Innova Biosciences)] was added for 30 min at RT. Biotinylated LV (5×10^6 particles) was combined with IgG192-conjugated streptavidin (10 μg) and incubated for 5 h at RT. The effectiveness of conjugation was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting (12% polyacrylamide gels) as previously reported [24].

Injection of Capsaicin into the Rat Whisker Pad

Rats (1 month old) were placed briefly in an induction chamber supplied with isoflurane via a vaporizer set to 5%. When sedated, animals were removed from the chamber and exposed to the gas (2%) via a nose cone. Lentiviral injection (2 μg, 5×10^6 lentiviral particles/10 μl injection) into the whisker pad was undertaken within 2–3 min before the animal would awaken. Respiration and response to toe pinch stimulation were monitored throughout the procedure with vaporizer adjustment if required. Upon completion of the procedure, the vaporizer was turned off and the animal was placed in a recovery area with thermal support. After 4, 8, and 12 days, rats were euthanized as indicated above, followed by TGG isolation for analysis.

Behavioral Response to Capsaicin Following VR1 Knockdown

The experimental procedure used complemented that previously reported [11]. In brief, rats (1 month old) were injected with Mission shRNA lentiviral transduction particles (against VR1 and negative control as indicated above) conjugated to IgG192 (10 μg IgG192, 2×10^9 infectious units/20 μl peripheral injection) into the whisker pad. Eight days later, capsaicin

(25 or 75 μg) was injected into the whisker pad. Facial grooming time was recorded within 5-min intervals for up to 20 min post capsaicin injection (n = 4–5 rats per group). Data was calculated as the percentage of seconds spent for grooming per 5-min interval.

Determination of Erk 1/2 Phosphorylation as a Biomarker Readout of Nociception

Five minutes after the monitoring of the behavioral response to capsaicin (25 and 75 μg) exposure (indicated above), rats were euthanized and TGG proteins extracted for nociception assessment via phosphorylation of Erk 1/2 normalized against β-actin as loading control (antibody details provided above).

Neurophysiological Recordings

Recordings were made from dissociated TGG neurons as described [27, 29]. In brief, rats injected with targeted LV-VR1 or negative control were anesthetized with ketamine (150 mg/kg, i.p.) and then decapitated. The TGG sections were removed bilaterally (kept separate) and dissociated in a 15-ml centrifuge tube in CMF-HBSS with 1 mg/ml trypsin and 1 mg/ml collagenase. After a 30-min incubation at 37 °C in a shaking water bath, the resulting cell suspension was centrifuged at 300g for 5 min. The supernatant was discarded and the tissue pellet resuspended in 2 ml of Neurobasal medium (Gibco) containing B27 and L-glutamine. Centrifugation was repeated and the resuspended cells titrated with a Pasteur pipette until cells were dispersed evenly. Neurons were plated on a glass cover slip coated with poly-D-lysine and kept in an incubator at 37 °C with 98% O_2 blown over the solution until use. Extracellular (cell-attached patch) and whole-cell recordings were made in the recording solution [in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 1.5 $CaCl_2$, 2 $MgCl_2$, and 25 glucose (bubbled with 95% O_2, 5% CO_2)] at 32–34 °C. Voltage clamp measurements were made using an EPC 10 amplifier (HEKA), with pipettes filled with internal solution [in mM: 140 KCH_3O_3S, 10 KCl, 5 NaCl, 2 MgATP, 0.01 EGTA, and 10 HEPES, pH 7.3] with 280 mOsm/l, R_{inp} of 3–7 MΩ. C_m and R_s compensation were canceled using a protocol built-in PatchMaster as described [30]. Capsaicin stock solutions were made in 100% ethanol, with a final concentration of 10 μM. Pressure was applied on neurons over 5 s through a micropipette (~ 10 μm tip diameter) using the Picospritzer III (FMI).

Statistical Analysis and Data Presentation

Data were calculated and graphs generated by GraphPad Prism 4.0 or Igor Pro; each point represents the mean ± SEM. Statistical significance was assessed using paired or unpaired Student's two-tailed t tests, with $p < 0.05$ defining a significant difference.

Results

Physiological Enrichment of p75NTR in Rat Trigeminal Nociceptors

Expression and functioning of p75NTR and Trk neurotrophin receptors (A–C) in various neuronal groups have been well documented in the central and peripheral nervous systems [31–33]. While in young adult rats (aged 1 month), p75NTR and TrkA receptors are prevalent in neurons of the TG sensory brain stem and the TGG, their relative expression level is not equal (Fig. 1). Western blot analysis revealed much higher expression of TrkA in the trigeminal sensory nucleus (Sp5 region) of the brain stem [~ 5-fold, 1.05 ± 0.03 arbitrary units (AU)] as compared to p75NTR (0.17 ± 0.01 AU) after normalization against the endogenous control β-tubulin ($p < 0.01$; Fig. 1b–d). The expression levels of neurotrophin receptors in the TGG showed the opposite trend, with the levels of p75NTR (0.82 ± 0.02 AU) exceeding those of TrkA (0.37 ± 0.02 AU) when normalized (~ 2.2-fold; $p < 0.01$; Fig. 1c, d). These results agree with the outcome from histochemical studies with microscopic analysis of the expression of p75NTR and TrkA in the TGG (Fig. 1e, f). The strong expression of p75NTR in sensory neurons of TGG renders it suitable as a receptor candidate for targeting these nociceptive neurons with IgG192-conjugated viral vectors.

VR1 Knockdown in Rat-Cultured TG Neurons Using shRNA Interference

In transgenic mice, a shRNA construct can interfere with VR1 expression induced by lentiviral transgenesis [34]. A similar approach was applied in this study to deplete VR1 in cultured TGG neurons using transduction with LV-VR1 (Fig. 2). A considerable reduction in VR1 expression (0.8-fold, $p = 0.006$) was noted 2 days post transfection, with an even further decrease detected by 4 days (eightfold decrease, $p < 0.01$) as revealed with Western blotting in comparison to control cultures transfected with a negative control shRNA lentivirus (Fig. 2a, b). Immunohistochemical staining for the presence of VR1 in TGG neuronal cultures revealed a strong depletion of VR1 expression by 4 days post LV-VR1 transfection (89 ± 8% decrease, $p < 0.01$; Fig. 2c, d). These findings demonstrate that VR1 expression could be strongly suppressed upon VR1 shRNA lentiviral transduction and offered a proof of principle for progressing with targeted *in vivo* experimentation with TG nociceptors.

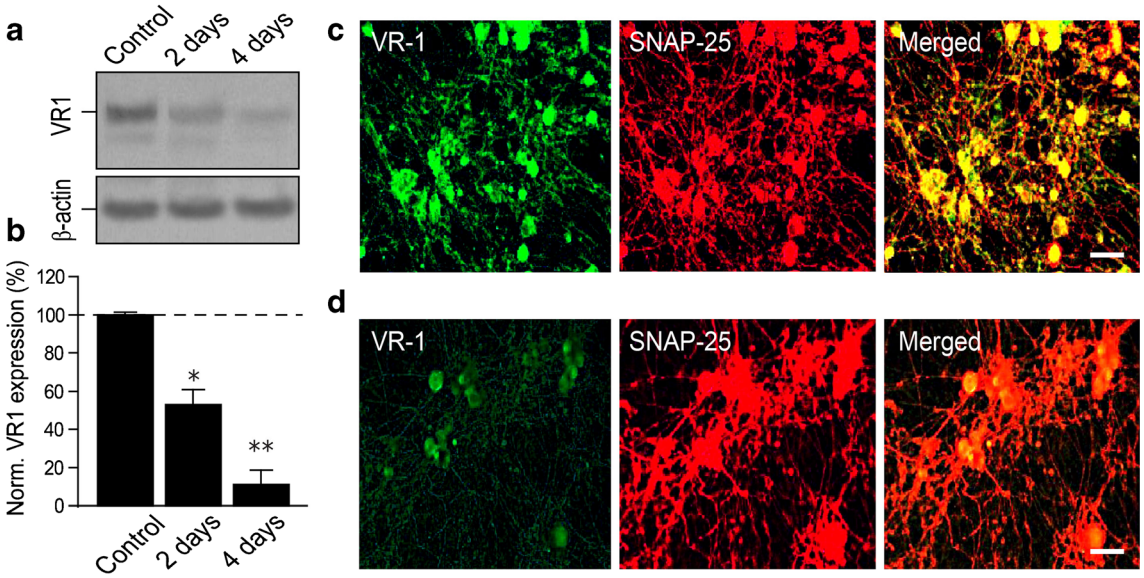

Fig. 2 VR1 knockdown with shRNA lentiviral transduction of TGG neuronal cultures. (**a**) Representative Western blots showing decreased VR1 expression over time in comparison to β-actin as loading control. Protein extracted from TG neurons. Lane 1, 4 days post transfection with negative control; lanes 2 and 3, 2 and 4 days post transfection with Mission shRNA lentiviral particles against LV-VR1 (190 Kd). (**b**) Histograms showing the relative expression of VR1 normalized to β-actin post transfection with LV-VR1. Significance is represented by asterisks; $*p < 0.05$; $**p < 0.01$; $n = 4$. (**c**, **d**) Representative immunofluorescence micrographs of TG neurons stained for VR1 (left) or SNAP-25 (middle) 4 days post transfection with LV-VR1 (left, lower) or negative control (left, upper). Merged images (right). Scale bars = 20 μm

Long-Range Transduction of Reporter GFP in TGG with Targeted p75NTR/LV Vectors

The mechanisms governing the internalization and transport of ligands bound to p75NTR have been analyzed in detail and exploited for targeting viral vectors [24, 35, 36]. Selective and long-range transduction of BF cholinergic neurons was achieved with IgG192-conjugated LV [24], considered otherwise to restrict transduction to the injection site [37]. In young adult rats, injection of Cy3-labeled IgG192 into the whisker pad (Fig. 3a) caused retro-axonal transport with robust labeling of TGG neurons *in vivo* after 2 days (Fig. 3a, b). Subsequent immunostaining with anti-VR1 antibodies revealed a strong cellular association between p75NTR (identified by IgG192/Cy3) and VR1 expression (Fig. 3c, d) up to 4 days post injection.

In the follow-up experiments with non-targeted and targeted GFP encoding LV vectors, while no GFP signal could be detected in the TGG in rats injected in the whisker pad with non-targeted LV-GFP (i.e., non-decorated with IgG192), injection of IgG192/LV-GFP at the same location was followed 4–12 days later by TGG GFP expression (Fig. 3e–g). GFP expression was readily detectable only on the IgG192/LV-GFP-injected side (Fig. 3g). Transgene enrichment increased fourfold by 8 days with expression and plateauing at later time points (12 days; $p < 0.05$ and $p < 0.01$, respectively; Fig. 3g, h). These experiments revealed the readily detectable expression of retrogradely transported transgenes to the TGG from 4 to 8 days post injection as seen from GFP Western blot analysis in this tissue (Fig. 3h). Of note, based on the VSV-G level, the enrichment of the TGG by this replication-deficient LV significantly diminished by 8 days post injection, becoming barely detectable at 12 days (Fig. 3g, h). Considering that VSV-G pseudotyped lentivirus on its own is incapable of retro-axonal spread and transduction of remote neurons, these results demonstrate that such long-range trafficking ability of LV vectors to the TGG has been endowed by p75NTR conjugation.

Depletion of VR1 in the TGG with shRNA Interference

To test whether reduced expression of VR1 in TG nociceptors could be achieved using IgG192-decorated lentivirus encoding shRNA against transcripts of this receptor, IgG192/LV-VR1 was injected in the right whisker pad of rats. Immunostaining with confocal microscopic imaging of TGG slices showed markedly reduced VR1 expression (71 ± 3% reduction by 12 days) post IgG192/LV-VR1 injection (10 μg IgG192; 2×10^9 LV infectious units) as compared to a likewise targeted negative shRNA control ($p < 0.01$, Fig. 4a, b, d). VR1 depletion (62 ± 2% reduction) was also evident in neurites when these sample groups were compared at 12 days post injection ($p < 0.05$, Fig. 4c, d).

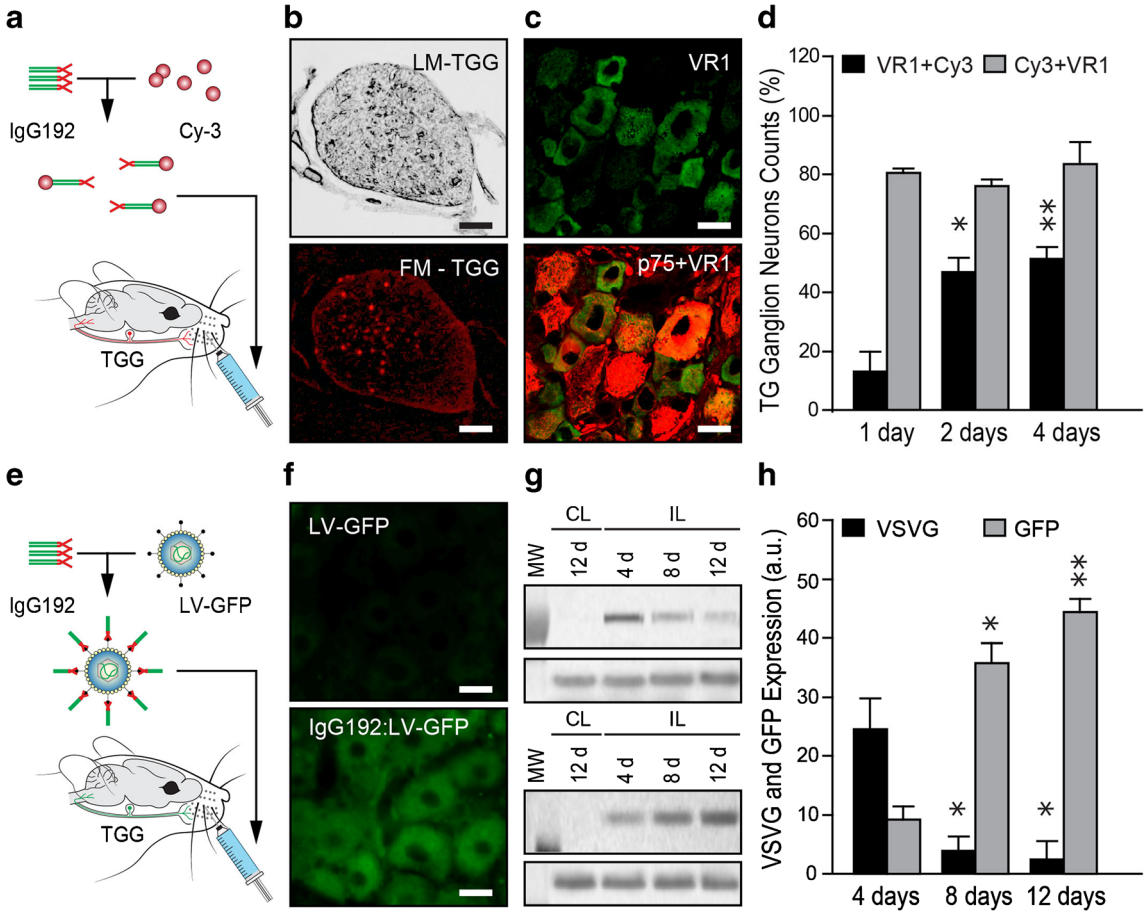

Fig. 3 IgG192-targeted lentivirus affords transduction of TGG neurons post peripheral injection. (**a**) Schematic illustrating a rat whisker pad injection of IgG192 conjugated to Cy3. TGG trigeminal ganglion. (**b**) Representative bright-field light micrograph (LM; upper) and fluorescence micrograph (FM; lower) of a TGG slice. Red fluorescence indicates enrichment of IgG192/Cy3 in the TGG 2 days post injection into the rat whisker pad. Scale bar = 150 μm. (**c**) Representative confocal micrograph of the TGG immunostained for VR1 (green; upper) and the p75 receptor (red; lower merged image). Scale bar = 15 μm. (**d**) Histograms of the co-labeling of TGG neurons with VR1 and IgG192/Cy3 at 1, 2, and 4 days post injection of the latter into the whisker pad ($n = 4$). Scale bar = 20 μm. (**e**) Schematic illustrating a rat whisker pad injection of IgG192 conjugated to lentivirus encoding the green fluorescent protein (LV-GFP). (**f**) Representative epifluorescence micrographs of GFP expression in the TGG 12 days post injection of LV-GFP (upper) or IgG192/LV-GFP (lower) into the rat whisker pad. Scale bar = 25 μm. (**g**) Western blots of lentiviral vesicular stomatitis virus glycoprotein (VSV-G; upper) and GFP (lower) expression at the TGG ipsilateral side (IL; 4, 8, and 12 days) and contralateral side (CL; 12 days) post injection of IgG192/LV-GFP. Western blots of β-actin shown below as a loading control. (**h**) Histograms of the relative expression of VSV-G and GFP up to 12 days post injection of IgG192/LV-GFP into the rat whisker pad normalized against β-actin ($n = 4$). Significance is represented by asterisks; *$p < 0.05$; **$p < 0.01$

Neurophysiological Functional Profiling and Behavioral Monitoring Reveal Nociceptive Alleviation from VR1 Depletion

Having obtained molecular evidence for amelioration of VR1 expression in the TGG within 4–12 days post peripheral injection of IgG192/LV-VR1, both functional and behavioral readouts were sought. The same targeting strategy was adopted as described above (Fig. 5a), followed by TGG neuronal dissociation from the IgG192/LV-VR1- or IgG192-conjugated negative control's injected side. The electrophysiological response of neurons to a direct puff of capsaicin via a micropipette was determined with extracellular and whole-cell electrophysiological recordings. In TG neurons of the IgG192/LV-VR1-injected side (12 days post injection), both the frequency of transient spiking and the amplitude of the inward current in response to capsaicin were significantly reduced as compared to neurons of rats similarly injected with the targeted negative control (IgG192/LV, Fig. 5b–d). Behavioral tests revealed a curbed response of rats to capsaicin in the IgG192/LV-VR1-injected group in comparison to negative IgG192/LV-injected controls (Fig. 5e, f). In the IgG192/LV-VR1-injected rats, the average duration of facial grooming between 5 and 20 min post capsaicin injection was strongly reduced ($p < 0.01$, Fig. 5e, f). The attenuated responsiveness of TG neurons to capsaicin revealed in behavioral experiments agreed with decreased Erk 1/2 phosphorylation in the same group. Western blot analysis showed inhibition of

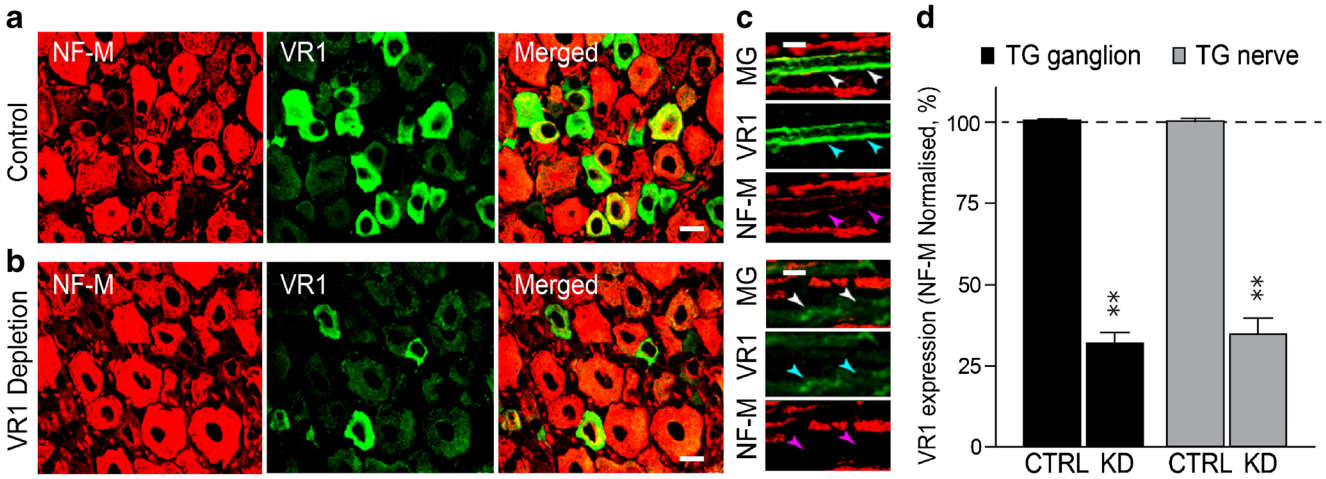

Fig. 4 VR1 depletion by IgG192/LV-VR1 injection into the rat whisker pad. (**a**–**c**) Representative confocal micrographs of the TG ganglion (**a**, **b**) or axons of the TG nerve (**c**) immunostained for neurofilament medium (NF-M; sensory neuron marker; red), VR1 (green), and merged (yellow) 12 days post injection with targeted lentivirus encoding shRNA negative control (upper) or shRNA against VR1 (IgG192/LV-VR1; lower). Scale bar = 10 μm. **d** Histograms showing the significantly decreased relative expression of VR1 in the TG ganglion (black bars) and nerve (gray bars) normalized to NF-M 12 days following IgG192-targeted delivery of lentivirus encoding shRNA negative control (CTRL) or shRNA against VR1 (KD). Significance is represented by asterisks; $^{**}p < 0.01$

capsaicin-induced Erk 1/2 phosphorylation in the TG neurons in rats injected with targeted IgG192/LV-VR1 12 days prior to the test (25 μg, 51 ± 3%, $p = 0.02$; 75 μg, 48 ± 4%, $p = 0.01$; Fig. 5g). Overall, the results of these experiments provide direct evidence for depletion of VR1 and attenuation of nociception in rats injected with therapeutic IgG192/LV-VR1.

Discussion

This study introduces a therapeutic targeting strategy that provides a means to post-transcriptionally downregulate VR1 in TG nociceptors of adult rats *in vivo*. Advantage has been taken of the physiological enrichment of TG sensory neurons with the low-affinity p75NTR, which was exploited to facilitate the long-range retro-axonal delivery of LV-VR1 vectors. VR1 depletion in nociceptive neurons of the TG was confirmed *in vitro* and *ex vivo* through immunoblotting and histochemistry, supported also by the results of electrophysiological, behavioral, and molecular signaling assays.

Alleviation of nociception in rats using the method presented here facilitates future research and therapeutic translation, offering the prospect of improving the management of TGN through relieving neuropathic pain and peripheral sensitization. TG nociception is also amongst the most common causes of facial pain prevalent in dental and neurological practice, causing considerable suffering due to a lack of effective and sustained therapies [38–40]. Currently, antiepileptic drugs are considered as the first line of treatment for TGN and TG pain, with carbamazepine used as the medicine of choice [41, 42]. Surgical interventions, which include microvascular decompression, percutaneous TG rhizotomies, and Gamma Knife radiosurgery, have also been considered but are typically reserved for patients who do not respond to non-invasive primary medication [38, 43]. Nevertheless, numerous refractory cases remain along with patients unable to tolerate the side effects of systemic administration of carbamazepine and other antiepileptic drugs [44, 45]. Non-invasive targeted intervention directed at modulating the excitability of TG nociceptive neurons, thus, might be of major benefit, with selectivity of viral vectors for specific neuronal types offering the ability to provide sustained relief without the need for repeated dosing and related side effects [46, 47]. It is noteworthy, however, that while the described-here method is capable of abolishing the VR1 signal in over 70% of TGG neurons, a significant fraction of cells remains irresponsive to shRNA intervention. This limited efficiency could reflect the heterogeneity of VR1-positive TG neurons, with a significant fraction devoid of p75NTR, similar to that of basal forebrain cholinergic neurons [48]. Another factor that might contribute towards an incomplete knockdown of VR1 in TGG neurons is that a local injection of IgG192/LV in the whisker pad, while targeting axons and neurons innervating this region through the maxillary branch of the TG (V) nerve, is likely to fail to reach and interfere with the axons of the ophthalmic and mandibular branches, leaving their VR1 expression levels intact. These limitations, together with the carcinogenic and immunogenic potentials of retroviral therapy [49, 50], should be considered in future applications of the herein-described approach.

The reliance of this method on (1) p75NTR as a targeting moiety and (2) the intrinsic propensity of lentiviruses for retention at the site of injection [37, 51] underpins its exquisite suitability for vector delivery and targeted transduction of TGG neurons. We have recently demonstrated that lentiviral

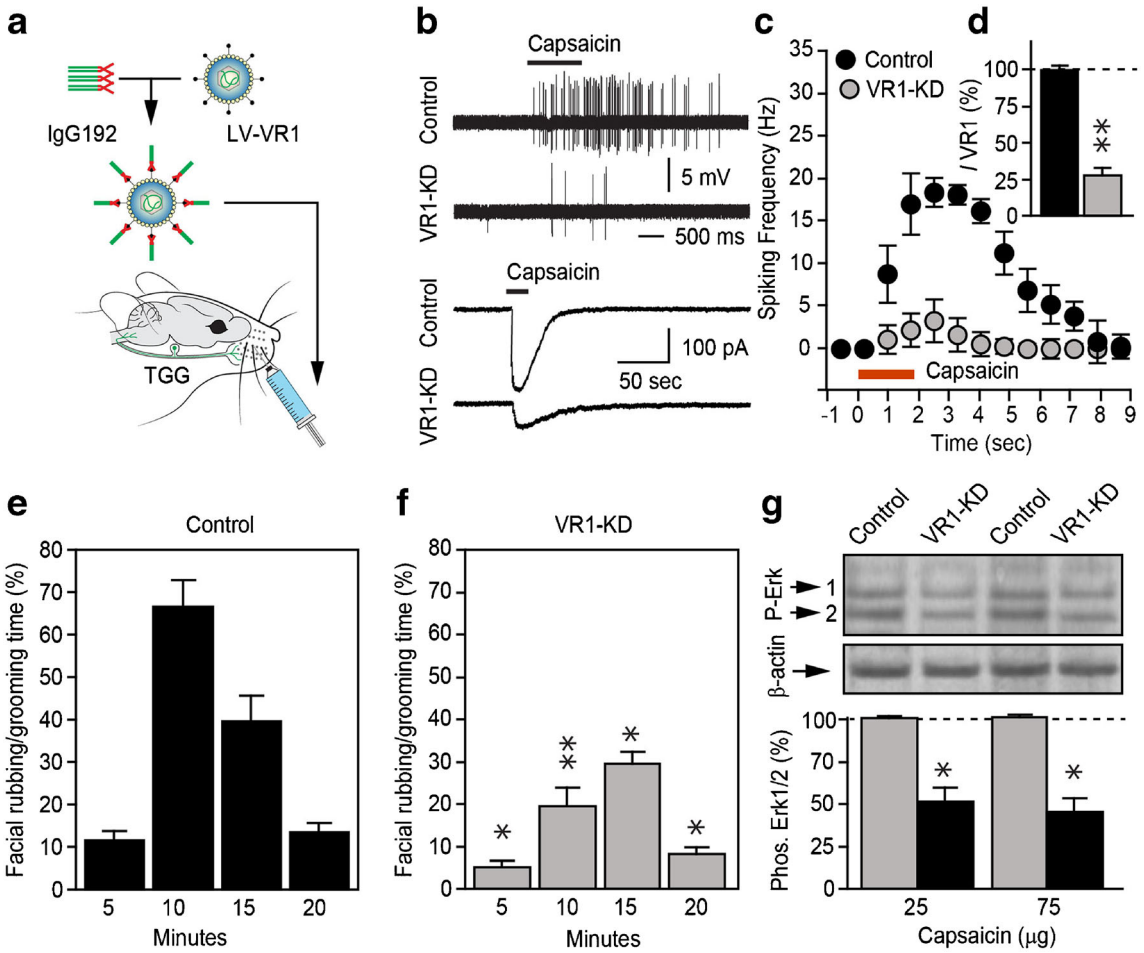

Fig. 5 VR1 depletion diminishes capsaicin-induced transient membrane currents and behavioral/molecular readouts of pain response. (**a**) Schematic illustrating a rat whisker pad injection of IgG192 conjugated to lentivirus (LV) encoding shRNA against VR1 (LV-VR1). (**b**) Electrophysiological responses of TGG-dissociated neurons extracted from rats 12 days post whisker pad injection with IgG192-targeted lentivirus encoding shRNA negative control (control; upper) or shRNA against VR1 (IgG192: LV-VR1; lower). Extracellular recordings of TG neuron spiking (top traces) and whole-cell recordings of capsaicin-induced inward currents (bottom traces). (**c**, **d**) Summary graphs of the spiking frequency dynamics over a 10-s time period in response to a 1-s application of 10 μM capsaicin (**c**) and inward current amplitude activated by capsaicin (**d**. **e**, **f**) Histograms showing changes in the duration of facial grooming (seconds; grooming shown as percentage) measured in 5-min intervals after capsaicin (25 μg) injection in control and IgG192/LV-VR1 rats (12 days prior to the test; $n = 5$). Significance is represented by asterisks; $*p < 0.05$; $**p < 0.01$. (**g**) Representative Western blots of phosphorylated Erk 1 and 2 (upper and lower arrows, respectively) after capsaicin (25 and 75 μg) exposure in control and IgG192/LV-VR1 rats as indicated. The β-actin loading control is shown below for each lane ($n = 5$). Histograms (below) representing Western blot analysis of phosphorylated Erk 1/2. Gray and black show data after capsaicin (25 and 75 μg) exposure in control and IgG192/LV-VR1 rats, respectively ($n = 5$)

vectors can be rendered capable of spreading over long distances via their retargeting with a proteolytically inactive mutant of tetanus toxin (TeTIM), tetanus toxin-binding H_C fragments, or IgG192 [24, 52–54]. With such an approach, effective transduction of spinal cord motor neurons and BF cholinergic neurons has been achieved from a remote central and peripheral injection site [24, 52–54]. Extending the spreading capabilities of a lentiviral vector via conjugation to anti-p75NTR (IgG192) and TeTIM is likely to result from sorting of targeted vectors to intracellular carriers specialized for long-range retrograde transport. This is not surprising considering p75NTR is known to enable internalization and retro-axonal trafficking of neurotrophins, pathogens, toxins, and viral vectors from the periphery to the central nervous system [36, 55–57]. Reassuringly, the extracellular epitope of the p75NTR receptor targeted with IgG192 differs from that binding NGF, with no interference of IgG192 with NGF-p75NTR binding, internalization, or intracellular transport reported [58, 59]. Moreover, targeting of p75NTR receptors in basal forebrain cholinergic neurons has been widely utilized for their *in vivo* pre-labeling, with no toxicity or changes in their electrophysiological profile or viability reported [60–63]. Overall, the pioneering data presented here demonstrates that distal delivery with transduction of TGG neurons can be achieved by conjugating lentiviral vectors to IgG192, affording post-transcriptional VR1 depletion with shRNA interference.

The superbly stable covalent linkage offered by streptavidin-biotin conjugation (Kd = 10^{-15} M) that ensures antibody plus lentiviral bondage has already been utilized for targeted transfer of viral and non-viral cargo [64–66]. Reports of the successful use of streptavidin-bound conjugates of retroviruses with antibodies in other systems [67, 68] unveiled its suitability for our applications, extending the use of this technology for long-range targeted transduction of TG sensory neurons. As pointed out earlier, TGN and sensitization of TG nociceptors can be associated with molecular alterations within these neurons, causing therapy-resistant distress [69]. Targeted modulation of TG functions as demonstrated herein, therefore, offers an innovative and viable strategy for overcoming both functional and molecular impairments, enabling effective modulation of TG nociception with anticipated therapeutic benefits.

Funding Information This work was supported by the Program for Research in Third Level Institutions Cycle 4 grant from the Republic of Ireland Higher Educational Authority for the neuroscience section of "targeted-driven therapeutics and theranostics" (JOD and SVO).

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