Bimodal regulation of axonal transport by the GDNF-RET signalling axis in healthy and diseased motor neurons

Elena R. Rhymes, Andrew P. Tosolini, Alexander D. Fellows, William Mahy, Neil Q. McDonald and Giampietro Schiavo

Deficits in axonal transport are one of the earliest pathological outcomes in several models of amyotrophic lateral sclerosis (ALS), including SOD1<sup>G93A</sup> mice. Evidence suggests that rescuing these deficits prevents disease progression, stops denervation, and extends survival. Kinase inhibitors have been previously identified as transport enhancers, and are being investigated as potential therapies for ALS. For example, inhibitors of p38 mitogen-activated protein kinase and insulin growth factor receptor 1 have been shown to rescue axonal transport deficits in vivo in symptomatic SOD1<sup>G93A</sup> mice. In this work, we investigated the impact of RET, the tyrosine kinase receptor for glial cell line-derived neurotrophic factor (GDNF), as a modifier of axonal transport. We identified the fundamental interplay between RET signalling and axonal transport in both wild-type and SOD1<sup>G93A</sup> motor neurons in vitro. We demonstrated that blockade of RET signalling using pharmacological inhibitors and genetic knockdown enhances signalling endosome transport in wild-type motor neurons and uncovered a divergence in the response of primary motor neurons to GDNF compared with cell lines. Finally, we showed that inhibition of the GDNF-RET signalling axis rescues in vivo transport deficits in early symptomatic SOD1<sup>G93A</sup> mice, promoting RET as a potential therapeutic target in the treatment of ALS.

INRODUCTION

Axonal transport, the process by which mRNAs, protein complexes and organelles move between the soma and synaptic terminals and vice versa, is fundamental for neuronal survival [1]. Anterograde transport towards axonal terminals is mediated by members of the kinesin motor protein superfamily, whereas retrograde transport is driven by cytoplasmic dynein. Human motor neuron (MN) axons can be over a metre long, necessitating efficient axonal transport. Accordingly, deficits in this process occur in many neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) [2, 3], for which impairment in axonal transport is one of the earliest pathological outcomes [4–7].

Signalling endosomes are generated following the activation and internalisation of neurotrophin receptors, such as TrkB and p75<sup>NTR</sup>, and predominantly undergo retrograde axonal transport [8]. Analysis of signalling endosome dynamics in the sciatic nerve of SOD1<sup>G93A</sup> mice, an established animal model of ALS, revealed a significant slowing of retrograde transport compared to wild type (WT), both at pre-symptomatic and symptomatic disease stages [7, 9]. Axonal transport deficits have also been observed in primary MN cultures from SOD1<sup>G93A</sup> [5] and TDP-43<sup>Δ757V</sup> [10] embryos, cultured SOD1<sup>G93A</sup> spinal cord explants [11], induced pluripotent stem cell (iPSC)-derived MNs harbouring ALS causative mutations in SOD1 [12], FUS [12] and TDP-43 [13], as well as in vivo, in transgenic TDP-43<sup>Δ757V</sup> mice [6]. Therefore, rescuing these early deficits is a key priority for therapeutics in ALS.

Protein kinases regulate axonal transport [13] and as such, kinase inhibitors represent a class of pharmacological compounds with the potential to boost axonal transport. While until recently the main focus of translational efforts in the protein kinase area has been the role of these enzymes in cancer [14], kinase overactivation has also been identified in neurological diseases [15], and linked to alterations in axonal transport. For example, GSK3-β has been shown to phosphorylate kinesin and induce a significant slowing of anterograde axonal transport [16]. Interestingly, aberrant kinesin phosphorylation occurs after treatment with mutant SOD1 protein [17]. GSK3-β has also been shown to inhibit retrograde transport through phosphorylation of the dynein intermediate chain, reducing dynein’s force generation [18]. Since many kinase inhibitors have been already approved for cancer treatment [14], the repurposing of some of these drugs for neurodegenerative diseases may offer faster patient-access time. Importantly, rescuing axonal transport deficits can significantly improve ALS phenotypes. For example, inhibitors of histone deacetylase 6 (HDAC6) have been shown to increase axonal transport speeds [12], and HDAC6 deletion in SOD1<sup>G93A</sup> mice increases neuromuscular junction innervation and extends survival [19].

In line with this, we performed a pilot screen by treating mouse embryonic stem cell-derived (ES) MN with a small kinase inhibitor library, and identified several potential enhancers of axonal transport [4]. Two of these hits have since been validated, with acute inhibition of both p38 mitogen-activated protein kinase (MAPK) and insulin-like growth factor 1 receptor (IGF1R) rescuing...
Additional hit from this screen, the compound C3 (Fig. 1A), which inhibits RET, a tyrosine kinase receptor for the glial cell-line derived neurotrophic factor (GDNF) family of ligands, including GDNF itself. Together with its co-receptor, GDNF-family receptor (GFR) α1, GDNF triggers RET dimerisation, and activation of downstream signalling cascades, including PI3K-AKT and MAPK-ERK1/2, which promote MN development and survival [21].

Fig. 1 A kinase inhibitor screen identified C3, a RET inhibitor, as an enhancer of axonal transport. A Summary of results from a kinase inhibitor pilot screen [4]. The graph represents the accumulation of a fluorescent binding fragment of tetanus neurotoxin (H4/T) and a fluorescently conjugated antibody directed against the p75NTR neurotrophin receptor (α-p75NTR) in ES-derived MNs following incubation with 2 μM of the different compounds. The yellow box shows a threefold average standard deviation of the entire data set. Compounds falling outside the yellow box in the upper right quadrant significantly enhanced accumulation. A1, A4 [4], E4 [20] and C3 were identified as potential transport enhancers. B Representative western blot of SH-SY5Y cell lysates following treatment with recombinant GDNF and GFRα1, the RET inhibitors (C3 and LOXO-292), and their vehicle control, DMSO. SH-SY5Y cells were pre-treated with DMSO, 1 or 2 μM C3 or LOXO-292 for 1 h, and stimulated with 100 ng/ml GDNF and GFRα1 for the final 10 min of incubation.

ALTERATIONS IN GDNF-RET SIGNALLING ARE ASSOCIATED WITH ALS PATHOLOGY, WITH INCREASED MICROGLIAL RET IMMUNOREACTIVITY IN SOD1G93A MICE [22], AND INCREASED GDNF LEVELS IN ALS PATIENT MUSCLE BIOPSYs [23]. One of only two FDA-approved ALS therapeutics, edaravone, has been shown to increase RET and GFRα1 protein levels, and increase AKT and ERK1/2 activation in MNs derived from ALS-patient iPSCs [24]. However, although GDNF-based therapies have shown promise, they have so far failed to progress from pre-clinical testing or produce significant clinical improvements [25, 26], with some studies even demonstrating opposite effects [27]. This highlights a complex relationship between the GDNF-RET signalling axis and ALS. Altogether, these findings support further testing of the hypothesis that altering RET signalling may rescue axonal transport deficits in ALS.

In this work, we identified a new functional interaction between the RET-GDNF signalling axis and axonal transport of signalling endosomes, and revealed profound differences in RET activity between WT and ALS neurons. We showed that pharmacological inhibition and knockdown of RET significantly enhance signalling endosome speed in WT MNs, and rescue in vivo axonal transport deficits in the SOD1G93A mouse model of ALS.

European Community Council Directive of 24 November 1986 (86/609/EEC). Animal experiments were performed under license from the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by the UCL Institute of Neurology Ethical Review Committee. Transgenic mice heterozygous for the mutant human SOD1 (G93A) on a C57BL/6-J/SJL mixed background (B6SJLt/G [SOD1*G93A]1Gur/J) were acquired from The Jackson Laboratory. Colonies were maintained at the Queen Square Institute of Neurology Biological Services Unit. Heterozygous males were mated with WT C57BL/6-J/SJL F1 generation females to obtain experimental colonies. Mouse genotypes were confirmed using ear biopsies and PCR amplification of the human SOD1G93A transgene as described by The Jackson Laboratory. Animals were housed in a controlled temperature and humidity environment and maintained on a 12 h light/dark cycle with access to food and water provided ad libitum.

Plasmids and reagents
Chemicals were from Sigma-Aldrich unless otherwise stated. Compound C3 (GW440139A), originally provided by GSK (UK), was re-synthesised at the ARUK Drug Discovery Institute at UCL [28]. LOXO-292 was purchased from MedChemExpress (USA). Recombinant human GDNF and BDNF were purchased from PeproTech (UK), and GFRα1 was purchased from R&D Systems (USA). RET shRNA constructs were purchased from Genecopoeia in psi-LVRUGP plasmids with eGFP fluorescent Protein (GFP) reporter genes and puromycin stable selection markers (MHS025822-LVRUGP, USA). Antibodies used in immunofluorescence (IF) and western blot (WB) experiments were as follows: RET (C31B4, WB 1:1000, IF 1:250, RET pY905 (3221 1:1000), ERK1/2 (9102 1:1000), pERK1/2 (9101 1:1000), AKT (9272 1:1000), pAKT (S473) (4060 1:1000), GAPDH (mab374 1:5000, Merck Millipore, Germany); βIII-tubulin (IF 1:500, 302305, Synaptic Systems, Germany), GFP (GFP-1010, IF 1:1000, Aves Labs, USA). The α-p75NTR (60-5724) used in the kinase inhibitor screen was previously generated and characterised by the authors [8, 29, 30].

Cell line culture
SH-SY5Y and N2A cells were sourced from the Cancer Research UK London Research Institute (now The Francis Crick Institute) Cell Service. Lenti-X HEK293-T cells were sourced from ClonTech, Mountain View, CA. SH-SY5Y, N2A and Lenti-HEK293 cells were all cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS). Cells were split every 2 days. N2A and SH-SY5Y cells were used when at least 70% confluence.
2–3 days, once they reached 80–90% confluency. For experiments, cells were plated onto PDL-coated coverslips or straight onto plastic, cultured for 1 DIV, and either left undifferentiated (HEK-293), or differentiated using 10 μM retinoic acid (Sigma) for 12–48 h (SH-SYSY and N2A).

Motor neuron cultures

HB9-GFP ES-derived MNs were cultured as previously described [31]. GFP expression, driven by the HB9-homeobox gene enhancer allowed confirmation of MN identity. Mixed ventral horn cultures, referred to in this study as primary MNs, were isolated from E11.5–13.5 mouse embryos as previously described [20]. Briefly, ventral horns from E11.5–13.5 SOD1G93A and WT mice were dissociated, centrifuged at 1 500 rpm for 5 min, seeded into microfluidic chambers [32], coverslips or wells, and maintained in MN media (Neurobasal (Gibco) with 2% v/v B27 (Gibco), 2% heat-inactivated horse serum, 1× Glutamax (Invitrogen), 24.8 μM β-mercaptoethanol, 10 ng/ml ciliary neurotrophic factor (CNTF; R&D Systems, USA) 0.1 ng/ml GDNF, 1 ng/ml BDNF and 1× penicillin–streptomycin (Thermo Fisher)) at 37 °C and 5% CO2.

Lentiviral particle production and PMN transduction

RET shRNA and scrambled control viral particles were made by co-transfecting shRNA, packaging and envelope plasmids into Lent-i-X HEK293 T cells with Lipofectamine 3000 (Thermo Fisher). The medium was collected at 48 and 72 h after transfection. Medium containing lentiviral particles was concentrated using a Lenti-X concentrator (Takara, Japan) and resuspended in Opti-MEM (Thermo Fisher), shRNA viral particles were stored at −80 °C until needed. Neurons were transduced on DIV3 by adding viral particles directly to the medium. Viral particles remained for 72 h, and neurons were lysed or assessed for transport on DIV6.

In vitro retrograde transport assay

At 6–7 DIV, 30 nM AlexaFlour555-labelled H-T was added to the axonal compartment [20], with key reagents (BDNF for SOD1G93A primary MN experiments, GDNF, GFRα1, DMSO, LOXO-292, C3) or vehicle control (PBS) added to the culture media in both axonal and somatic compartments for 45 min. Media was then replaced with fresh MN media containing 20 mM HEPEs-NaOH (pH 7.4) and the experimental compound for live imaging on an inverted Zeiss LSM 780 confocal microscope using a ×40 oil-immersed objective. The microscope and stage were pre-warmed to 37 °C. Videos were taken at 0.500 s frame rate for >2.5 min using a resolution of 1024 × 1024 pixels.

In vivo retrograde transport assay

The in vivo axonal transport was performed as previously described [33, 34]. Briefly, P7 female WT (non-transgenic) and SOD1G93A mice were anaesthetised using isoflurane (National Veterinary Services) and AlexaFlour555-conjugated H-T (13 μM) or BDNF or GDNF (25 μg) injected intramuscularly into the exposed tibialis anterior and lateral gastrocnemius muscles of the right hind leg and the wounds then sutured (for GDNF experiments, only tibialis anterior was injected). At this time, for C3 vs MC experiments only, mice were also intraperitoneally injected (for GDNF experiments, only tibialis anterior was injected). At this time, for C3 vs MC experiments only, mice were also intraperitoneally injected with either 10 mg/kg C3 or an equivalent volume of vehicle control (1% methyl cellulose (MC) (Sigma 274429)). Mice were assigned to control and treatment groups at random, nevertheless ensuring an even spread of treatments between different experimental days. A sample size of 6 or 7 mice per condition (27 in total) was selected based on results from previous in vivo transport experiments, where groups of 5-6 animals per condition were sufficient to identify small, yet significant changes in transport speeds [6, 7, 9]. After injection, mice were then left to recover and kept under standard conditions with unlimited food and water supply. After 4–7 h, mice were re-anaeathetised using isoflurane and the sciatic nerve of the right leg was exposed. The anaesthetised mouse was placed on a heated stage in an environmental chamber and axonal transport was imaged in the intact sciatic nerve by time-lapse confocal microscopy as previously described [33]. The in vivo axonal transport was performed as previously described [33, 34]. Brieﬂy, ventral horns from E11.5–13.5 SOD1G93A and WT mice were dissociated, centrifuged at 1 500 rpm for 5 min, seeded into microfluidic chambers [32], coverslips or wells, and maintained in MN media (Neurobasal (Gibco) with 2% v/v B27 (Gibco), 2% heat-inactivated horse serum, 1× Glutamax (Invitrogen), 24.8 μM β-mercaptoethanol, 10 ng/ml ciliary neurotrophic factor (CNTF; R&D Systems, USA) 0.1 ng/ml GDNF, 1 ng/ml BDNF and 1× penicillin–streptomycin (Thermo Fisher)) at 37 °C and 5% CO2.

Immunofluorescence

Cells on coverslips or in MFCs were fixed using 4% paraformaldehyde and 5% sucrose in PBS for 10 min then permeabilised in blocking solution (10% heat-inactivated horse serum in PBS, 0.5% BSA) with 0.2% Triton-X100. Permeabilised cells were incubated in primary antibody (detailed in Plasmids and Reagents) in block for 1 h at room temperature, washed five times in PBS, and then incubated in fluorescently-conjugated secondary antibodies (Invitrogen, ThermoFisher UK: 1:1000) and DAPI (1:2000) for 1 h at room temperature in the dark. Coverslips/MFCs were washed 5 times in PBS, then coverslips were mounted on glass slides using DAKO Mounting Medium, and imaged using an inverted Zeiss LSM 510 or 780 confocal microscope, on a ×63, 1.4 NA DIC Plan-Apochromat oil-immersion objective.

Western blotting

Primary MN lysates were prepared in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM EGTA) with Halt™ protease and phosphatase inhibitor cocktail (1:100, Thermo Fisher), and incubated on ice for 30 min. Lysates were spun at 14,800 rpm, 4 °C for 15 min, the supernatant collected, setting some aside to quantify protein concentrations. To the remaining lysate, 4x Laemmli sample buffer (15% SDS, 312.5 mM Tris-HCl pH 6.8, 50% glycerol, 10% β-mercaptoethanol, 0.1% bromophenol blue) was added, and samples were boiled for 5 min at 100 °C. Pierce™ BCA protein Assay Kit (Thermo Fisher) was used to quantify the protein concentration. Protein separation was carried out using 4–15% Mini-PROTEAN® TGX Stain-Free™ protein gels (Bio-Rad, USA), and transferred onto 20% ethanol-soaked polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA). Membranes were blocked in 5% BSA dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, then incubated with primary antibody diluted in 5% BSA overnight at 4 °C. Membranes were washed 4 × 5 min in TBST, then incubated with secondary antibody (HRP-conjugated, DAKO, Denmark) diluted in 5% BSA and washed again. Immunoreactivity was detected using chemiluminescent substrates (Luminata Crescendo/Classico, Merck Millipore) and the ChemiDoc™ Touch Imaging System with Bio-Rad ImageLab software. For blots where total and phospho-proteins of the same molecular weight were analysed, samples were run twice on two gels and normalised to the corresponding GAPDH loading control (phospho “p” and total “t”). Original western blots are shown in Supplemental Material—Western Blot Originals.

Image analysis

Image analysis was performed using Fiji (NIH, MD). For the analysis of signalling endosome transport dynamics, the Fiji plugin TrackMate [36] was used to manually track cargo. For both in vitro and in vivo transport assays, the experimentalists performing the axonal transport assays and post-imaging quantification were kept blinded until after the completion of tracking. TrackMate outputs “csv” files, which provide information on the average and maximum speed of each endosome, as well as data describing single endosomal movements to determine instantaneous velocities and percentage pausing (frame–frame movements <0.2 μm/s). In vitro, all retrogradely moving endosomes were tracked. Due to the large volume of endosomes in in vivo videos, 25 endosomes were tracked per axon, in 3 axons per mouse. For both in vitro and in vivo experiments, endosomes pausing for >15 s were excluded from analyses. In vitro and in vivo speed distribution curves were generated by categorising instantaneous velocities into 0.2 μm/s bins.

Statistical analysis

Statistical analysis was performed in Graphpad Prism (San Diego, CA). To ensure the selection of the appropriate statistical test, the normality of data was confirmed using the D’Agostino–Pearson test. The equal variance between groups was confirmed using the F-test. Two-tailed Student’s t tests (paired or unpaired) were used when comparing two groups. One- or two-way analysis of variance (ANOVA), followed by Tukey’s or Holm–Šidak’s multiple comparisons tests were used when multiple groups were present. Significance is noted as follows: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Independent experiments are described in this study as biological replicates. A biological replicate is defined throughout this manuscript as a set of results obtained using primary neurons isolated from mouse embryos sourced from independent litters, or in the case of experiments performed using cell lines, results attained using cell cultures from different passages.
RESULTS
A kinase inhibitor screen identified RET inhibitors as potential axonal transport enhancers
To identify potential axonal transport modulators, we screened a small molecule library of kinase inhibitors in ES-MNs [4]. The readout for the screen was somatic accumulation of two fluorescent markers of signalling endosomes: the non-toxic heavy chain fragment of tetanus neurotoxin, conjugated to Alexa-Fluor555 (H$_2$T-T-555); and a fluorescently conjugated antibody against the extracellular domain of p75$_{NTR}$ (a-p75$_{NTR}$) [8, 9]. An increase in the somatic accumulation of these probes in ES-MNs reflects a rise in the retrograde transport flow. Compounds that significantly increased the somatic accumulation of these retrograde probes fall outside of the yellow box in the upper right quadrant, which represents threefold average standard deviations of the entire data set (Fig. 1A). Previous validation of p38MAPK (A1 and A4) and IGF1R (E4) inhibitors has revealed that these molecules are able to rescue retrograde transport deficits in vivo in symptomatic SOD1G93A mice [4, 20].

Another hit from the screen was the RET inhibitor C3 (GW440139A; red box, Fig. 1A). Although initial investigation confirmed that 10 μM C3 significantly enhanced in vitro signalling endosome transport (data not shown), this concentration also induced neuronal blebbing (Supplementary Fig. 1A). Using C3 at 2 μM stopped axonal blebbing, but at the same time abolished any significant effect on axonal transport speeds (data not shown).

To circumvent this problem, we synthesised a new batch of C3 compounds and first assessed RET activation in the lysates of differentiated SH-SYSY cells, which express high levels of RET [37]. We examined RET activation following treatment with C3, as well as LOXO-292, a commercially available RET inhibitor currently in clinical trials as an anti-cancer therapy [38]. As shown in Fig. 1B, both C3 and LOXO-292 inhibited RET activation to a similar extent (Fig. 1B and Supplementary Fig. 1B), thus validating the new batch of C3. Importantly, at 1 μM, newly synthesised C3 did not cause significant neuronal blebbing in mixed ventral horn spinal cord cultures (hereafter referred to as primary MNs) in compartmentalised or mass cultures (Supplementary Fig. 1C), suggesting that this phenomenon arose from impurities in the original C3 batch used in the kinase inhibitor screen (Fig. 1A).

RET inhibition enhances axonal transport in primary WT motor neurons
To assess the effect of RET inhibition on axonal transport, we adapted an alternative receptor in these cells. These findings highlight a divergence in the signalling response to GDNF in MNs compared to cancer cell lines (e.g., SH-SYSY cells), which are commonly used to investigate RET signalling [42, 43].

GENETIC DOWNREGULATION OF RET MIMICS THE EFFECT OF RET INHIBITION ON AXLONAL TRANSPORT
The neglibible effect of LOXO-292 on signalling endosome transport, together with the significant enhancement observed with GDNF stimulation raises the possibility that the effect of C3 on axonal transport may result from off-target effects of this inhibitor. To confirm that the reduction of RET signalling enhances retrograde transport, we assessed transport dynamics following lentiviral-mediated shRNA knockdown of RET in MNs.

Knockdown of RET was first established in mouse N2A neuroblastoma cells using a panel of shRNAs, from which we selected the two most effective to prepare lentiviral vectors in Lenti-HEK293 cells (Supplementary Fig. 2A, B). We then determined the optimal viral titre for a knockdown in primary MN cultures (Supplementary Fig. 2C). Lentiviral expression of both shRNAs induced robust RET knockdown in primary MNs in mass culture (Fig. 4A–C). These RET-targeting shRNAs increased the average (Fig. 4D, E) and maximum (Fig. 4D, F) signalling endosome transport speeds compared to scrambled control, without significantly altering the pausing of the organelles (Fig. 4G), as seen using the C3 inhibitor (Fig. 2D). These findings validate C3 as a specific RET inhibitor and support the role of this class of compounds as retrograde axonal transport enhancers.
Having shown that blockade of RET signalling enhances signalling endosome transport in WT neurons, we next investigated the effect of RET inhibition in SOD1G93A MNs. We observed no significant difference in AKT or ERK1/2 activation between WT and SOD1G93A neurons (Fig. 5A, C, D). However, RET activation levels in SOD1G93A MNs were significantly reduced compared to WT (Fig. 5A, B, complete representative WT blot shown in Fig. 2G). This evidence suggests the presence of altered RET signalling in SOD1G93A neurons, warranting further investigation of RET signalling in ALS.

We next assessed signalling endosome transport dynamics in SOD1G93A primary MNs. The experimental set-up is shown in Fig. 5E. Neurons were treated with 1 µM C3 in the presence of 50 ng/ml BDNF to determine whether RET inhibition rescues the BDNF-dependent retrograde transport deficits previously described in SOD1G93A primary MNs [5, 7]. C3 treatment had no significant effect on average or maximum endosome speeds, although there is a trend for an increase in both, as well as a reduction of pausing (Fig. 5F–I).

**RET signalling in the SOD1G93A mouse model of ALS**

Having shown that blockade of RET signalling enhances signalling endosome transport in WT neurons, we next investigated the effect of RET inhibition in SOD1G93A MNs. We observed no significant difference in AKT or ERK1/2 activation between WT and SOD1G93A neurons (Fig. 5A, C, D). However, RET activation levels in SOD1G93A MNs were significantly reduced compared to WT (Fig. 5A, B, complete representative WT blot shown in Fig. 2G). This evidence suggests the presence of altered RET signalling in SOD1G93A neurons, warranting further investigation of RET signalling in ALS.

To independently assess the effect of RET inhibition on axonal transport, we tested the C3 inhibitor in vivo by measuring the rate of transport of signalling endosomes in the intact sciatic nerve of early symptomatic (~P73) SOD1G93A mice, a time point that corresponds with ~20% loss of lumbar motor neurons [9]. Using a previously optimised experimental design [33, 34], HcT-555 and...
25 ng BDNF were injected into the tibialis anterior and gastrocnemius muscles in the lower limb of anaesthetised female WT and SOD1G93A mice. For in vivo delivery, we suspended C3 in 1% methyl cellulose (MC) and injected it intraperitoneally at a final concentration of 10 mg/kg. Since no pharmacokinetic or pharmacodynamic data for C3 is currently available, we selected 10 mg/kg based on previous findings showing that two p38MAPK inhibitors administered at both 10 mg/kg and 100 mg/kg significantly enhanced signalling endosome transport in SOD1G93A mice in vivo [4]. Approximately 4 hours after injection of HCT-555, mice were re-anaesthetised and the sciatic nerve of the injected limb was exposed for intravital imaging.

Fig. 3 Stimulation with GDNF enhances WT axonal transport. A Schematic representation of GDNF and GFRα1 transport experiments in three-compartment MFCs. Primary ventral horn neurons were cultured in the central (somatic) compartment of 3-channel MFCs, with axons projecting to two lateral fluidically isolated axonal chambers. 100 ng/ml GDNF, GFRα1, or both together were added to one axonal compartment, with PBS added to the other as an internal control. B–D Average endosome speeds following the addition of GDNF (B), GFRα1 (C), and GDNF + GFRα1 (D) in the right compartment and PBS in the left compartment (B–D). Each point represents a biological replicate. Connecting lines represent speeds observed in control and experimental condition-treated axons from the same cultures. Speeds were significantly increased with GDNF alone (*p = 0.02) and GDNF with GFRα1 (*p = 0.04), but not GFRα1 alone (p = 0.6799), as determined by two-tailed paired t tests. E Representative kymographs showing signalling endosome transport in axons of WT primary MN cultures following treatment with PBS, GDNF, GFRα1 or GDNF together with GFRα1. N = 3–5 biological replicates. Bars represent mean ± SEM. PBS results were collected from 443 endosomes in 42 videos for a total of 45 569 single movements (n = 4). GDNF results were collected from 218 endosomes in 22 videos for a total of 17 938 single movements (n = 5). GDNF and GFRα1 results were collected from 400 endosomes in 29 axons for a total of 31 809 single movements (n = 5). F Representative western blot of primary MN lysates following starvation in Neurobasal and Glutamax, and treatment with 50 ng/ml BDNF, or 100 ng/ml GDNF, and GDNF and GFRα1 (both 100 ng/ml). G–I Western blot quantification of RET, AKT and ERK1/2 activation after starvation and stimulation with BDNF, GDNF and GFRα1. Starvation significantly decreased activation of AKT (****p < 0.0001) and ERK (****p < 0.0001), but not RET (one-way ANOVA with Dunnett’s multiple comparison test).
previously reported deficit in signalling endosome transport (Fig. 6B and Supplementary Fig. 3A, B) [4, 9, 20]. Interestingly, we saw a significant increase in average endosome speed upon treatment of SOD1G93A mice with 10 mg/kg of compound C3 (Fig. 6B). However, this compound did not modify the transport speed of signalling endosomes in WT mice. Accordingly, C3 induced a significant reduction in retarding axonal transport speeds in SOD1G93A mice, without any significant effect in WT animals (Fig. 6C). We also observed a slight shift to the right of the speed distribution curve of SOD1G93A mice treated with the C3 inhibitor (Supplementary Fig. 3D), indicating a general increase in axonal transport speeds upon RET inhibition. Surprisingly, however, C3 had no statistically significant effect on maximum endosome speed in WT or SOD1G93A sciatic nerves (Fig. 6D). Although there was no difference in average endosome speed between control and C3-treated WT mice, we did observe a slight shift to the left of the speed distribution curve following C3 treatment (Supplementary Fig. 3C), which suggests that in WT mice, RET inhibition may trigger a subtle slowing down of axonal transport in vivo. Interestingly, we found that GDNF has no effect on WT or SOD1G93A signalling endosome transport speeds in vivo (Supplementary Fig. 4A–C), indicating that in vivo, increasing GDNF signalling has no impact on axonal transport, and RET inhibition only enhances transport speeds where GDNF-RET signalling is pathologically altered. These results further support dampening rather than boosting GDNF signalling for the treatment of ALS.

Altogether, these results suggest that RET inhibition has the potential to rescue retrograde transport deficits in the SOD1G93A mouse model of ALS. Optimisation of in vivo dose and route of delivery of C3 may potentiate the effect observed, promoting RET inhibition as a possible therapeutic approach in the treatment of ALS.
**DISCUSSION**

Deficits in the retrograde axonal transport of signalling endosomes and other organelles are one of the earliest changes observed in several models of ALS [6, 9]. Protein kinases are known to influence axonal transport [13], and we have previously shown that inhibition of both IGF1R and p38MAPK is effective in rescuing these deficits [4, 20]. The literature investigating GDNF-RET signalling predominantly originates from experiments in cancer cell lines, such as SH-SY5Y neuroblastoma lines, which express RET at high levels following retinoic acid differentiation [42]. Whereas this is informative to determine the mechanism by which RET mutations and altered GDNF-RET signalling impact cancer initiation and progression [40, 44, 45], a comprehensive analysis of RET signalling in neurons is needed to further our understanding of the implications of deregulation of RET signalling in neuromuscular diseases. In this study, we explored the role of the GDNF-RET signalling axis on axonal transport in MNs and found that both GDNF stimulation and RET inhibition boost axonal transport of signalling endosomes. We have validated RET inhibitors as axonal transport enhancers with the potential to rescue disease-dependent axonal transport deficits in vivo and revealed a fundamental function of GDNF-RET signalling in the axonal transport dynamics of signalling endosomes.

Reducing RET activation, either through pharmacological or genetic approaches, increases retrograde axonal transport dynamics. Despite inducing significant increases in axonal transport speeds in WT neurons in vitro, C3 had no significant effect on in vivo transport speeds in WT mice at the concentration investigated in this study. Interestingly, we also saw no increase in signalling endosome transport speeds in vivo following GDNF treatment in WT or SOD1G93A mice. We showed that RET inhibition in SOD1G93A primary MNs is not sufficient to completely overcome the transport impairment observed in vitro. The increased variability of endosome speeds in vivo following GDNF treatment in WT or SOD1G93A mice, which we validated in in vitro experiments [40, 41], therefore, activation of TrkB by BDNF might further mask the effect of RET inhibition on axonal transport. In fact, several signalling pathways downstream of RET are activated upon BDNF stimulation, such as AKT and ERK1/2 [40, 41]. Therefore, activation of TrkB by BDNF may hide any positive effect on transport induced by RET inhibition. Furthermore, the significant
reduction in baseline RET signalling in SOD1<sup>G93A</sup> MNs compared to WT observed in vitro could also contribute to the differential response to RET inhibitors of WT and SOD1<sup>G93A</sup> neurons, providing an explanation as to why RET inhibition has a reduced impact on these neurons. Interestingly, however, acute treatment of symptomatic SOD1<sup>G93A</sup> mice does rescue in vivo retrograde transport deficits monitored in the sciatic nerve. These findings highlight a divergence in the signalling response between primary MNs and the adult sciatic nerve, which could be attributed to the increased duration of C3 treatment in in vivo experiments (~4 h in vivo vs. 1 h in vitro), as well as the developmental stage of the models.

In order to determine whether the rescue in signalling endosome transport speeds observed after acute RET inhibition translates to improvements in pathology or progression in ALS, longitudinal experiments are necessary. Thorough safety and pharmacological analysis of C3 is still required before the long-term effects of RET inhibition can be established. A better understanding of the structural determinants driving RET inhibition by both C3 and LOXO-292 would be required to explain their differing effects on axonal transport in our experimental system. This information would in turn guide us in the identification of suitable RET inhibitors and their appropriate delivery protocols for long-term in vivo testing in ALS animal models, both at pre-symptomatic and early symptomatic stages of disease progression. Furthermore, both the disease stage and delivery method of the RET inhibitor should be carefully considered. In this study, C3 was administered via intraperitoneal injection, however intramuscular or intrathecal injection might ensure more robust delivery of the compound into the motor neuron-muscle system. A longer pre-treatment with the compound or treatment at an earlier disease stage is likely to potentiate the effect of RET inhibition on axonal transport speeds and pathology.

Additionally, these findings suggest that in vivo, C3-mediated RET inhibition selectively targets pathologically altered RET signalling. Changes in GDNF and RET signalling have been previously reported in ALS, with increased immunoreactivity of RET in activated microglia, and reduced expression of GFRα1 mRNA in the spinal cord of SOD1<sup>G93A</sup> mice [22, 46]. Although edaravone, one of only two FDA-approved therapeutics for ALS, has been shown to increase RET, GDNF and GFRα1 protein levels [24], enhancing GDNF signalling in ALS has so far either induced adverse effects [27] or failed to translate to significant clinical improvements [26]. These negative outcomes align with independent clinical trials in Parkinson’s disease [47]. Together with our results, these findings support the conclusion that dampening rather than enhancing RET signalling may have therapeutic promise in ALS.

Neither C3 nor the commercial RET inhibitor LOXO-292 significantly inhibited RET phosphorylation in primary MNs; however, C3 does halt AKT and ERK1/2 activation in WT and SOD1<sup>G93A</sup> neurons. We have previously shown that direct inhibition of AKT is sufficient to increase signalling endosome transport speeds [20], therefore it is possible that C3 enhances transport through indirect inhibition of AKT. Along the same lines, the failure of LOXO-292 to induce a significant enhancement of signalling endosome transport could result from its inability to inhibit AKT activation in primary MNs.
RET was first identified as a proto-oncogene [48], and activating-mutations drive neoplastic processes [43]. Notably, RET rearrangements occur in multiple endocrine neoplasia type 2A/B and familial medullary thyroid carcinoma [49], emphasising the therapeutic importance of targeting this kinase receptor. Accordingly, many RET inhibitors have been developed and are currently being tested for safety and efficacy [50] or are in clinical trials, including LOXO-292 [38]. Repurposing these agents for use in neurodegeneration would provide an opportunity for significantly faster access of ALS patients to novel therapeutics.

However, a conclusion emerging from our studies is that GDNF-RET signalling in MNs is more complex than in cancer cell lines. Both GDNF and RET clearly play a fundamental role in MN development and survival [21, 51, 52], as evidenced by the inclusion of GDNF in standard MM media. GDNF has a robust effect on axonal transport speeds in WT neurons in vitro, however, this appears to be independent of its canonical receptor RET. GDNF stimulation of primary MNs fails to activate Akt and ERK1/2, two key signalling proteins switched on downstream of GDNF-RET activation in several cell lines [53]. GDNF stimulation of MNs also fails to increase RET phosphorylation, suggesting that in these neurons, GDNF enhances WT signalling endosome transport through a RET-independent mechanism.

Interestingly, GDNF-GFRA1 can signal independently of RET. For example, GDNF induces axonal outgrowth in hippocampal neurons, which do not express RET [54]. This response occurs alongside an increase in fibroblast growth factor receptor activation, a kinase receptor activated downstream of neural cell adhesion molecule (NCAM) [55]. Furthermore, GDNF-mediated chemotraction of Schwann cells, which express GFRA1 but not RET, was abolished in the presence of NCAM-blocking antibodies [56]. This effect required downstream activation of the tyrosine kinase, Fyn [57]. Interestingly, Fyn has been shown to promote anterograde transport through phosphorylation of the microtubule-associated protein tau [58], releasing its inhibitory effect on kinesin [59, 60]. Furthermore, dorsal root ganglion neurons from Fyn−/− knockout mice show impairments in both anterograde and retrograde transport [58]. Therefore, it is possible that stimulation of MNs with GDNF potentiates retrograde signalling endosome transport through activation of NCAM signalling.

These findings highlight a divergence of RET and GDNF signalling in MNs, promoting both enhancement of GDNF signalling and RET inhibition as possible therapeutic approaches, and perhaps explaining the uncertain outcomes of GDNF-based therapies. Further investigation of both GDNF and RET signalling cascades is required to determine the physiological outcomes of manipulating these pathways in neurons, which would enable new translational efforts in ALS and other neurodegenerative conditions.

DATA AVAILABILITY

The authors confirm that all data supporting the findings of this study are available within this article and its Supplementary Information.

REFERENCES

1. Maday S, Twelvetrees AE, Mouhaghan AJ, Holzbauf ELF. Axonal transport: cargo-specific mechanisms of motility and regulation. Neuron. 2014;84:292–309.
2. Sleigh JN, Rosser AM, Fellows AD, Tosolini AP, Schiavo G. Axonal transport and neurological disease. Nat Rev Neurol. 2019;15:691–703.
3. De Vos KJ, Hafefzarast M. Neurobiology of axonal transport defects in motor neuron diseases: opportunities for translational research? Neurobiol Dis. 2017;102:283–99.
4. Gibbs KL, Kalmar B, Ryhmes ER, Fellows AD, Ahmed M, Whiting P, et al. Inhibiting p38 MAPK alpha rescues axonal retrograde transport defects in a mouse model of ALS. Cell Death Dis. 2018;9:596.
5. Kieran D, Hafefzarast M, Bohnert S, Dick JRT, Martin J, Schiavo G, et al. A mutation in dynein rescues axonal transport defects and extends the life span of ALS mice. J Cell Biol. 2005;169:561–7.
6. Sleigh JN, Tosolini AP, Gordon D, Devoy A, Fratta P, Fisher EMC, et al. Mice carrying ALS mutant TDP-43, but not mutant FUS, display in vivo defects in axonal transport of signaling endosomes. Cell Rep. 2020;30:3655.e2–62.e2.
7. Tosolini AP, Sleigh JN, Surana S, Ryhmes ER, Cahalan SD, Schiavo G. BDNF-dependent modulation of axonal transport is selectively impaired in ALS. bioRxiv 2021;2021.12.06.471484 [Preprint]; 2021 [cited 2021 Dec 7]; [11 p]. Available from: https://www.biorxiv.org/content/10.1101/2021.12.06.471484v2.
8. Deinhardt K, Salinas S, Verastegui C, Watson R, Worth D, Hanahan S, et al. Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. Neuron. 2006;52:293–305.
9. Bililng GD, Sahai EI, Kelly G, Golding M, Greensmith L, Schiavo G. Deficits in axonal transport precede ALS symptoms in vivo. Proc Natl Acad Sci USA. 2010;107:20523–8.
10. Alami NH, Smith RB, Carrasco MA, Williams LA, Winborn CS, Han SSW, et al. Axonal transport of TDP-43 mRNA granules is impaired by ALS-causing mutations. Neuro. 2014;81:536–43.
11. Ionescu A, Gradus T, Altiman M, Maimon R, Saraf Avraham N, Geva M, et al. Targeting the sigma-1 receptor via pridopidine ameliorates central features of ALS pathology in a SOD1 G93A model. Cell Death Dis. 2019. https://doi.org/10.1038/s41419-019-1451-2.
12. Guo W, Naujock M, Fumagalli L, Vandoorne T, Baatsen P, Boon R, et al. HDAC6 inhibition reverses axonal transport defects in motor neurons derived from FUS-ALS patients. Nat Commun. 2017;8:1–15.
13. Gibbs KL, Greensmith L, Schiavo G. Regulation of axonal transport by protein kinases. Trends Biochem Sci. 2015;40:597–610.
14. Bhullar KS, Lagarón NO, McGowan EM, Parmar J, Jha A, Hubbard BP, et al. Kinase-targeted cancer therapies: progress, challenges and future directions. Mol Cancer. 2018;17:48.
15. Mueller BK, Mack H, Teusch N. Rho kinase, a promising drug target for neurode- logical disorders. Nat Rev Drug Discov. 2005;4:387–98.
16. Morfini G, Szebenyi G, Elluru R, Ratnner N, Brady ST. Glycogen synthase kinase 3 β phosphorylates kinesin light chains and negatively regulates kinesin-based motility. EMBO J. 2002;21:281–93.
17. Morfini GA, Bosco DA, Brown H, Gatto R, Kaminska A, Song Y, et al. Inhibition of fast axonal transport by pathogenic SOD1 involves activation of p38 MAP kinase. PLoS ONE. 2013;8:e65235.
18. Gao FJ, Hebbel S, Gao XA, Alexander M, Pandey JW, Walla MD, et al. gsk-3β phosphorylation of cytotoxic motor neuron dysfunction Ndn1 binding to intermediate chains and alters dynein motility. Traffic. 2015;16:941–61.
19. Tae S, Timmers M, Hersmus N, Bento-Abreu A, Van Den Bosch L, Van Damme P, et al. Hdac6 deletion delays disease progression in the SOD1G93A mouse model of ALS. Hum Mol Genet. 2013;22:1783–90.
20. Fellows AD, Ryhmes ER, Gibbs KL, Greensmith L, Schiavo G. IGF 1R regulates retrograde axonal transport of signalling endosomes in motor neurons. EMBO Rep. 2020. https://doi.org/10.15252/embr.201949129.
21. García A, Haase G, Airaksinen MS, Livet J, Filippi P, DeLapeyrière O. GFRα1 is required for development of distinct subpopulations of motoneuron. J Neurosci. 2000;20:4992–5000.
22. Ryu H, Jeon GS, Cashman NR, Kowall NW, Lee J. Differential expression of c-Ret in motor neurons versus non-neuronal cells is linked to the pathogenesis of ALS. Lab Invest. 2011;91:342–52.
23. Yamamoto M, Mitsuma N, Inukai A, Ito Y, Li M, Mitsuma T, et al. Expression of GDNF and GDNF-R a mRNAs in muscles of patients with motor neuron diseases. Neurochem Res. 1999;24:785–90.
24. Li Q, Feng Y, Xue Y, Zhan X, Fu Y, Gui G, et al. Edaravone activates the GDNF/RET neurotrophic signaling pathway and protects mRNA-induced motor neurons from IPS cells. Mol Neurodegener. 2021;17. https://doi.org/10.1186/s13238-021-00510-y.
25. Manabe Y, Nagano I, A Gazi MS, Murakami T, Shiote M, Shoji M, et al. Adenosinemediated gene transfer of glial cell line-derived neurotrophic factor prevents motor neuron loss of transgenic model mice for amyotrophic lateral sclerosis. Apoptosis. 2002;7:329–34.
26. Tosolini AP, Sleigh JN. Motor neuron gene therapy: lessons from spinal muscular atrophy for amyotrophic lateral sclerosis. Front Mol Neurosci. 2017;10:405.
27. Thomsen GM, Alkaslasi M, Vit JP, Lawless G, Godoy M, Gowing G, et al. Systemic injection of AAAV-GDNF provides modest functional improvements in the SOD1 G93A ALS rat but has adverse side effects. Gene Ther. 2017;24:245–52.
28. Graham Robinett R, Freemaner AJ, Skinner MA, Shevchuk L, Lackey K. The discovery of substituted 4-(3-hydroxymethyl)-quinolines as potent RET kinase inhibitors. Bioorg Med Chem Lett. 2007;17:5886–93.
29. Deinhardt K, Reversi A, Berninghausen O, Hopkins CR, Schiavo G. Neurotrophins redirect p75NTR from a clathrin-independent to a clathrin-dependent endocytic pathway coupled to axonal transport. Traffic. 2007;8:1736–49.
30. Vilà M, Charalampopoulos I, Kenchappa RS, Reversi A, Klos-Applequist JM, Karaca E, et al. Ligand-independent signaling by disulfide-crosslinked dimers of the p75 neurotrophin receptor. J Cell Sci. 2009;122:3351–7.
31. Arce V, Goldberg M, Schiavo G. siRNA screen of E5 cell-derived motor neurons identifies novel regulators of tetanus toxin and neurotrophin receptor trafficking. Front Cell Neurosci. 2014;8:140.

30. Restani L, Giribaldi F, Manich M, Bercsenyi K, Mendezene G, Rossetto O, et al. Botulinum neurotoxins A and E undergo retrograde axonal transport in primary motor neurons. PLoS Pathog. 2012;8:e1003087.

29. Oppenheim RW, Houenou LJ, Parsadanian AS, Prevette D, Snider WD, Shen L. Expression of glial cell-line derived neurotrophic factor (GDNF) in tumour anogenesis. Methods Mol Biol. 2020;2143;271–92.

28. Tosolini AP, Villaroel-Campos D, Schiavo G, Sleigh JN. Expanding the toolkit for in vivo imaging of axonal transport. J Vis Exp. 2011;78:e3471. https://doi.org/10.3791/3471.

27. Gibbs RL, Kalmar B, Sleigh JN, Greensmith L, Schiavo G. In vivo imaging of axonal transport in murine motor and sensory neurons. J Neurosci Methods. 2016;257;26–33.

26. Tinevez JY, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, et al. TrackMate: an open and extensible platform for single-particle tracking. Methods. 2017;115:80–90.

25. Kovalevich J, Langford D. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. Methods Mol Biol. 2013;1078;9–21.

24. Drilon A, Ondr G, Tan DSW, Loong HHF, Johnson M, Gainer J, et al. Efficacy of seliparstinib in RET fusion–positive non–small–cell lung cancer. N Engl J Med. 2020;383:813–24.

23. Subbiah V, Velcheti V, Tuch BB, Ebita K, Busaidy NL, Cabanillas ME, et al. Selective RET kinase inhibition for patients with RET–altered cancers. Ann Oncol. 2018;29:1869.

22. Mulligan LM, RET revisited: expanding the oncogenic portfolio. Nat Rev Cancer. 2014;14:173–86.

21. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci. 2003;4:299–309.

20. Oppenheimer O, Cheung N-K, Gerald WL. The RET oncogene is a critical component of transcriptional programs associated with retinoic acid–induced differentiation in neuroblastoma. Mol Cancer Ther. 2007. https://doi.org/10.1158/1535-7163.MCT-06-0357.

19. Mulligan LM. GDNF and the RET receptor in cancer: new insights and therapeutic potential. Front Physiol. 2019. https://doi.org/10.3389/FPHYS.2018.01873.

18. Plaza-Menacho I, Bona J, Barry R, Borg A, Orme M, Chauhan R, et al. RET functions as a dual-specificity kinase that requires allosteric inputs from juxtaprotein elements. Cell Rep. 2016;17:3191–32.

17. Romei C, Ciampi R, Elisei R. A comprehensive overview of the role of the RET proto–oncogene in thyroid carcinoma. Nat Rev Endocrinol. 2016;12:192–202.

16. Zhang J, Huang EJ. Dynamic expression of neurotrophic factor receptors in postnatal spinal motorneurons and in mouse model of ALS. J Neurobiol. 2006;66:882–95.

15. Kambe PY, Kanwore K, Ayanlaja AA, Nadeem I, Du YZ, Bubewa W, et al. Failure of glial cell–line derived neurotrophic factor (GDNF) in clinical trials orchestrated by reduced NR4A2 (NURR1) transcription factor in Parkinson’s disease. A systematic review. Front Aging Neurosci. 2021;13:59.

14. Takahashi M, Ritz J, Cooper GM. Activation of a novel human transforming gene, ret, by DNA rearrangement. Cell. 1985;42:581–8.

13. Plaza-Menacho I. Structure and function of RET in multiple endocrine neoplasia type 2. Endocr Relat Cancer. 2018;25:779–90.

12. Takamori S, Matsubara T, Harakata N, Toyokawa G, Fujishita T, Toyozawa R, et al. Neurite outgrowth of glial cell and regeneration axonal transport in rodent peripheral nerves. Methods Mol Biol. 2020;2143;271–92.

11. Tosolini AP, Villaroel-Campos D, Schiavo G, Sleigh JN. Expanding the toolkit for in vivo imaging of axonal transport. J Vis Exp. 2011;78:e3471. https://doi.org/10.3791/3471.

10. Gibbs RL, Kalmar B, Sleigh JN, Greensmith L, Schiavo G. In vivo imaging of axonal transport in murine motor and sensory neurons. J Neurosci Methods. 2016;257;26–33.

9. Tinevez JY, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, et al. TrackMate: an open and extensible platform for single-particle tracking. Methods. 2017;115:80–90.

8. Kovalevich J, Langford D. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. Methods Mol Biol. 2013;1078;9–21.

7. Drilon A, Ondr G, Tan DSW, Loong HHF, Johnson M, Gainer J, et al. Efficacy of seliparstinib in RET fusion–positive non–small–cell lung cancer. N Engl J Med. 2020;383:813–24.

6. Subbiah V, Velcheti V, Tuch BB, Ebita K, Busaidy NL, Cabanillas ME, et al. Selective RET kinase inhibition for patients with RET–altered cancers. Ann Oncol. 2018;29:1869.

5. Mulligan LM, RET revisited: expanding the oncogenic portfolio. Nat Rev Cancer. 2014;14:173–86.

4. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci. 2003;4:299–309.

3. Oppenheimer O, Cheung N-K, Gerald WL. The RET oncogene is a critical component of transcriptional programs associated with retinoic acid–induced differentiation in neuroblastoma. Mol Cancer Ther. 2007. https://doi.org/10.1158/1535-7163.MCT-06-0357.

2. Mulligan LM. GDNF and the RET receptor in cancer: new insights and therapeutic potential. Front Physiol. 2019. https://doi.org/10.3389/FPHYS.2018.01873.

1. Plaza-Menacho I, Bona J, Barry R, Borg A, Orme M, Chauhan R, et al. RET functions as a dual-specificity kinase that requires allosteric inputs from juxtaprotein elements. Cell Rep. 2016;17:3191–32.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41419-022-05031-0.

Correspondence and requests for materials should be addressed to Giampietro Schiavo.

Reprints and permission information are available at https://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022

E.R. Rhymes et al.

58. Li C, Sasaki Y, Takei K, Yamamoto H, Shouji M, Sugiyama Y, et al. Correlation between Semaphorin3A–produced facilitation of axonal transport and local activation of a translation initiation factor eukaryotic translation initiation factor 4E. J Neurosci. 2004;24:6161.

57. LaPointe NE, Morfini G, Pigino G, Gaisina IN, Roktsovski AP, Binder LI, et al. The amino terminus of tau inhibits kinesin–dependent axonal transport: Implications for filament toxicity. J Neurosci Res. 2009;87:440–51.

56. Kanaan NM, Morfini GA, LaPointe NE, Pigino GF, Patterson KR, Song Y, et al. Pathogenic forms of Tau inhibit kinesin–dependent axonal transport through a mechanism involving activation of axonal phototransferases. J Neurosci. 2011;31:9858–68.

Acknowledgements
We thank James Dick and the personnel of the Denny Brown Laboratories for assistance in maintaining the mouse colonies (Queen Square Institute of Neurology, University College London) and James N. Sleigh (Queen Square Institute of Neurology, University College London) for critical reading of the manuscript.

Author contributions
Conceptualisation: ERR and GS. Investigation: ERR, APT, and ADF. Provision of essential resources: WM. Writing: ERR and GS, with input from all authors. Funding: GS. All authors approved this submission. The funders had no role in study design, data collection and analysis, decision to publish or manuscript preparation.

Funding
This work was supported by a Medical Research Council PhD Studentship (ERR); a Junior Non–Clinical Fellowship from the Motor Neuron Disease Association (Tosolini/Oct/20/973–799) (APT); a Leonard Wolfson PhD studentship (ADF); the ARUK UCL Drug Discovery Institute is core funded by Alzheimer’s Research UK (520909) (WM); the Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001115), the UK Medical Research Council (FC001115) and the Wellcome Trust (FC001115) (NQM), Wellcome Senior Investigator Awards (107116/Z/15/Z and 223022/Z/21/Z) (GS), and a UK Dementia Research Institute Foundation award (UKDRI–1005) (GS).

Cell Death and Disease (2022) 13:584