PTEN deletion in spinal pathways via retrograde transduction with AAV-retro enhances forelimb motor recovery after cervical spinal cord injury; sex differences and late-onset pathophysiologies

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Conflict of interest: OS is a co-founder and has economic interests in the company *Axonis Inc*, a biotechnology which is seeking to develop therapies for spinal cord injury and other neurological disorders.

ABSTRACT

Spinal cord injuries (SCI) cause severe and long-lasting functional impairments due to interruption of motor and sensory pathways. Regeneration of axons does not occur due to intrinsic growth limitations of adult neurons and extrinsic inhibitory factors, especially at the injury site, but some regeneration can be achieved via deletion of the phosphatase and tensin homolog (PTEN). Here, deployed an AAV variant that is retrogradely transported to deliver (AAV-retro) to deliver gene modifying cargos to the cells of origin of pathways interrupted by SCI, testing whether this promoted recovery of motor function. We injected different titers of AAV-retro/Cre into the cervical spinal cord at C5 in PTEN$^{f/f}$;Rosa^{tdTomato} mice and control Rosa^{tdTomato} mice at the time of a C5 dorsal hemisection injury. Forelimb grip strength was tested over time using a grip strength meter. PTEN$^{f/f}$;Rosa^{tdTomato} mice injected with AAV-retro/Cre exhibited substantial improvements in forelimb gripping ability in comparison to controls. Of note, there were major sex differences in the extent of recovery, with male mice exhibiting greater recovery than females. The values for male mice largely account for the overall differences between PTEN-deleted and controls. However, some PTEN-deleted mice began to exhibit pathophysiologies involving excessive scratching and rigid forward extension of the hindlimbs which we term “dystonia”. These pathophysiologies increased over time. Our results reveal that although intra-spinal injections of AAV-retro/Cre in PTEN$^{f/f}$;Rosa^{tdTomato} mice can enhance forelimb motor recovery after SCI, there are late-developing functional abnormalities with the experimental conditions used here. The mechanisms underlying these late-developing pathophysiologies remain to be defined.

INTRODUCTION

Spinal cord injuries (SCI) disrupt ascending and descending pathways in the spinal cord, compromising motor function below the site of injury and sensory signals that go to the brain. Damaged connections within the spinal cord do not regenerate and the prevailing concept is that regeneration failure is due to lack of intrinsic growth capacity of adult neurons and extrinsic inhibitory factors present in the mature nervous system, especially at the injury site.

Neuron-intrinsic signaling pathways that enable a regenerative response have been identified. In this context, the phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway, plays an important role for regulating cell size, protein synthesis, survival and cytoskeleton formation necessary for axon extension after injury (Leibinger, Hilla, Andreadaki, & Fischer, 2019; Proud, 2002; Testa & Tsichlis, 2005; Zhou & Snider, 2006). A well-established step regulating axon growth, is the activation of PI3K via receptor tyrosine kinase (RTK) (Song, Ouyang, & Bao, 2005). The activation of this lipid kinase results in the activation of serine/threonine protein kinase AKT, leading to the subsequent activation of the mTOR pathway. Activation of mTOR leads to the phosphorylation of different substrates, including ribosomal protein S6 (pS6), one consequence of which is increased translation of 5' TOP mRNAs important for cell growth (Guertin & Sabatini, 2007). Of note, however, there are likely to be other important consequences of mTOR activation in general and S6 phosphorylation in particular including alterations in gene transcription in the nucleus (Proud, 2002).

An important negative regulator of the AKT/mTOR pathway is phosphatase and tensin homolog (PTEN), a phosphatase that converts inositol triphosphate (IP3) to inositol biphosphate (IP2) reversing the action of PI3K. Overall activation of mTOR depends on the balance between PI3K and PTEN, so PTEN inactivation drives the mTOR pathway. In terms of axon regeneration, studies from multiple labs have revealed that turning on mTOR activity by genetic deletion or knockdown of PTEN enables regeneration of CNS axons and this regeneration-enabling effect is blocked by rapamycin, confirming mTOR as the critical molecular signaling pathway (Park, Liu, Hu, Kanter, & He, 2010).

Cre-driven deletion of PTEN in central nervous system neurons (CNS) reliably enables corticospinal tract (CST) regeneration after SCI in $PTEN^{f/f}$ mice (Danilov & Steward, 2015; Geoffroy, Hilton, Tetzlaff, & Zheng, 2016; K. Liu et al., 2010; Willenberg, Zukor, Liu, He, & Steward, 2016). Furthermore, knockdown of PTEN via short-hairpin RNA (shRNA) in CNS neurons, improves long-distance regeneration and functional recovery after injury (Lewandowski & Steward, 2014; Zukor et al., 2013). Regardless, the extent of axonal regeneration is still limited, as many CST axons that are growing robustly after PTEN deletion fail to penetrate into or get stuck at the lesion site and do not grow past it.

Adeno-associated virus (AAVs) are an effective platform for *in vivo* gene therapy, as they mediate high levels of transgene expression in dividing and non-dividing cells, are non-toxic and present low innate immunity (Kaplitt et al., 2007). AAV-based delivery of cargos can be effective in enabling regeneration after SCI, but the strategy has been to target one pathway at a time. For example, regeneration of CST axons is enabled by targeting the sensorimotor cortex (K. Liu et al., 2010) and regeneration of rubrospinal

tract axons is enabled by targeting the red nucleus (Challagundla et al., 2015). This approach does not address the critical unmet need for regeneration of multiple pathways following SCI.

A potential tool to anatomically target cells of origin of multiple spinal pathways, in widely-distributed brain regions come from the development of the retrogradely transported AAV variant: AAV-retro (Tervo et al., 2016). When injected into the spinal cord, these vectors are retrogradely transported to the cells of origin of multiple spinal pathways (Tervo et al., 2016), enabling delivery of gene modifying cargos to the cells of origin of multiple pathways that are interrupted by SCI (Metcalfe et al., 2022; Wang, Maunze, Wang, Tsoulfas, & Blackmore, 2018).

Here we tested AAV-retro as a platform to delete PTEN in multiple pathways after a cervical SCI, to test whether cell-specific deletion promoted regeneration of descending tracts and recovery of motor function. For this, we injected AAV-retro/Cre into the cervical spinal cord at C5 in PTEN$^{f/f}$;Rosa^{tdTomato} mice and control Rosa^{tdTomato} mice at the time of a C5 dorsal hemisection injury and we then assessed forelimb gripping ability with a grip strength meter. Our results revealed that PTEN$^{f/f}$;Rosa^{tdTomato} mice exhibited transient improvements in forelimb gripping ability in comparison to controls. We discovered, however, that at around 2-3 months post-injury, some mice began to exhibit pathophysiologies involving excessive scratching and hindlimb dystonia with persistent extension, which increased over time. To begin to assess mechanisms of pathophysiologies, and whether motor recovery could be achieved without adverse effects, additional studies were to define conditions leading to pathophysiologies. Our studies revealed that pathophysiogies were not observed in un-injured PTEN$^{f/f}$;Rosa^{tdTomato} mice or control SCI mice that received intra-spinal cord injections of AAV-retro/Cre, indicating that the pathophysiologies are not due to PTEN deletion alone, or AAV itself.

Our findings document that although intra-spinal injections of AAV-retro/Cre in PTEN$^{f/f}$;Rosa^{tdTomato} mice can lead to initial improvements in forelimb motor recovery after SCI, there are late-developing functional abnormalities with the experimental conditions used here. The mechanisms underlying late-developing pathophysiologies remain to be defined.

MATERIALS AND METHODS

Experimental Animals

Animal use and care. All procedures involving animals were approved by the Institutional Animal Care and use Committee (IACUC) of the University of California Irvine in compliance with the National Institute of Health guidelines. Mice were maintained on a 12h light/dark cycle at 25°C. All procedures were done during the light portion of the cycle.

Mice. Our studies were done using two lines of transgenic mice that we developed in our lab (Gallent & Steward, 2018). One line was created by crossing Rosa26^{tdTomato} mice obtained from the Jackson Laboratory (JAX: 007905) with PTEN$^{f/f}$ mice from our breeding colony to create a new transgenic strain that was homozygous at both loci

($PTEN^{f/f}$;$Rosa^{tdTomato}$). $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice have a lox-P flanked STOP cassette in the ROSA locus and lox-P flanked exon 5 of PTEN so AAV-mediated expression of Cre recombinase deletes PTEN and induces robust expression of tdTomato (tdT) in the same neurons. In creating this new transgenic strain, we also selected for mice that were homozygous at the tdT locus as controls. Because the resulting $Rosa^{tdTomato}$ mice were derived from the crossing, they have a different genetic background from the original, $Rosa^{tdTomato}$ mice obtained from JAX Labs (genetic background B6;129S6).

AAV vectors: To retrogradely transduce neurons in the brain that project to the spinal cord, we used AAV-retro/Cre (Addgene viral prep #24593-AAVrg) or AAV-retro/shPTEN/GFP. To transduce cells locally within the spinal cord with minimal retrograde transport, we used AAV2/Cre (Vector Biolabs viral prep #7011).

Spinal cord injuries

Surgical procedures for intra-spinal cord AAV-retro and AAV2 injections at C5 were as previously described (Steward et al., 2021). Mice were anesthetized with Isoflurane (2-3%), their eyes were swabbed with Vaseline, and the skin at the incision site was shaved and cleaned with Betadine. A laminectomy was performed at C5 to expose the underlying spinal cord. Injections (0.3 µl per side) were made using a Hamilton microsyringe fitted with a pulled glass micropipette at 0.5 mm lateral to the midline and 0.5 mm deep.

C5 dorsal hemisections were performed under the same procedure as intraspinal AAV-retro or AAV2 injections. To create a dorsal hemisection, a 15-degree angled Microscalpel (Electron Microscopy Sciences #72046-15) was inserted into the spinal cord at the midline to a depth of 0.5 mm and drawn laterally, then the blade was turned around and drawn in the opposite direction. This was repeated several times to assure completeness of the dorsal transection. After the spinal cord lesion was created, the mouse was passed to a separate station for AAV injections. After completion of the hemisection and intra-spinal cord AAV injections, muscle layers were sutured and the skin was secured with wound clips.

Post-operative care

Following the surgeries, animals were immediately placed on water-circulating jacketed heating pads at 37°C until they fully recovered from the anesthetic. When able to move around in their cage and feed themselves, animals were housed 2-4 per cage on Alpha-Dri bedding. For 3-5 days post-injury, mice received lactated Ringer solution (1ml/20g, sub-cutaneously) for prophylactic treatment against urinary tract infections. Animals were monitored twice daily for general health, coat quality (indicative of normal grooming activity) and mobility within the cage.

Order of studies to test recovery of function

The overall study of consequences of PTEN deletion on recovery following spinal cord injury (SCI) includes 3 separate iterations carried out at different times. Each experiment involved delivery of different doses of AAV-retro/Cre [dose = number of genome copies (GC) injected per mouse]. For each study, surgeries and AAV-retro/Cre injections were done by the same surgical team and were blind with respect to strain

and vector. Different experimenters were responsible for the functional analyses in each study.

Study 1 was performed to determine if PTEN deletion via intra-spinal injection of AAV-retro/Cre would promote functional recovery after SCI. Study 1 involved 15 PTEN$^{f/f}$;Rosa^{tdTomato} mice (n=11 male; n=4 female) and 14 Rosa^{tdTomato} mice (n=4 male; n=10 female). Mice were 11 weeks old at the start of the study and were handled for 2 weeks. GSM testing began when mice were 13 weeks of age and continued for 4 weeks to collect baseline GSM values. Dorsal hemisection injuries and intra-spinal cord injections of AAV-retro/Cre were done when mice were 17 weeks of age. Mice received 4 bilateral injections, 2 above and 2 below the injury (total of 12E9 genome copies (GC) per mouse, 0.3 µl/injection in the same surgery as a dorsal hemisection injury at C5 (hemisections were done first). Of those, 3 PTEN$^{f/f}$;Rosa^{tdTomato} mice and 1 Rosa^{tdTomato} mouse died 1-2 days post-surgery. Thus after attrition, there were 12 PTEN$^{f/f}$;Rosa^{tdTomato} mice (n=8 male; n=4 female) and 13 Rosa^{tdTomato} mice (n=4 male; n=9 female).

Mice were monitored daily by research staff during the first 2 weeks post-injury, but after health had stabilized, monitoring was by vivarium staff. Starting 4 weeks post-injury, vivarium staff began noting wounds behind the ears of many mice. Details on these are described in the Results, as well as our determination of the cause (incessant scratching). Despite treatment, wounds increased in severity to the point that some mice were euthanized. Some mice began to exhibit abnormal forward extension of the hindlimbs, which we term dystonia. Because of the increasing severity of the pathophysiologies, the study was terminated and mice were euthanized 9 weeks post AAV-retro/Cre injections plus SCI.

Importantly, mice were group housed (up to 4 mice/cage), treatment groups were not separated, and both vivarium staff and individual doing GSM testing were blind to genotype. As a result, we did not detect the fact that these unusual events were occurring only in PTEN$^{f/f}$;Rosa^{tdTomato} mice with SCI and AAV-retro/Cre until the study was terminated and blinding was removed. Overall, 9/13 PTEN$^{f/f}$;Rosa^{tdTomato} mice with SCI and AAV-retro/Cre exhibited wounds and scratching and some hindlimb dystonia

To begin to define causes and mechanisms of the pathophysiologies, we carried out follow-up studies. In Study 2, we reduced AAV-retro doses (6E9 GC/animal) to determine if this was related to the occurrence of pathophysiologies. Injections were rostral to the injury. Study 2 involved a total of 26 PTEN$^{f/f}$;Rosa^{tdTomato} mice that were 11 weeks old at the start of the study. Mice were handled for 2 weeks, GSM testing began when mice were 13 weeks old and continued for 2 weeks to collect baseline GSM values. Mice received dorsal hemisection injuries and two bilateral intra-spinal cord injections of AAV-retro/Cre, above the injury, when mice were 15 weeks of age. 16 mice (n=9 male; n=7 female) received 6E9 GC per mouse of AAV-retro/Cre. 10 mice (n=6 male; n=4 female) received 6E9 GC of AAV-retro/GFP (0.3 µl/injection). Of those, 9 mice injected with AAV-retro/Cre and 3 mice injected with AAV-retro/GFP died on the first 1-2 days post-surgery. Thus after attrition, there were a total of 18 PTEN$^{f/f}$;Rosa^{tdTomato} mice; 11 mice (n=7 male; n=4 female) injected with AAV-retro/Cre and 7 mice (n=4 male; n=3 female) injected with AAV-retro/GFP.

Two PTEN$^{f/f}$;Rosa^{tdTomato} mice injected with AAV-retro/Cre began to exhibit pathophysiologies 5 weeks post injections/SCI, although pathophysiologies were not as

severe as seen with higher doses of AAV-retro in Study 1, and we were able to conduct the experiment to its completion. Animals were perfused 16 weeks post AAV-retro plus SCI.

In Study 3 we further reduced AAV-retro doses (3E9 GC/animal). Here, injections were done rostral to the injury, but only on one side. Onset and nature of pathophysiologies was monitored carefully. Study 3 involved a total of 30 PTEN$^{f/f}$;Rosa^{tdTomato} mice (n=14 male; n=16 female) and 16 Rosa^{tdTomato} mice (n=8 male; n=8 female). Mice were 25 weeks old at the start of the study and were handled for 2 weeks. When mice were 27 weeks old, GSM testing began and baseline values were collected for 9 weeks. When mice were 36 weeks of age, they received dorsal hemisection injuries and unilateral intra-spinal cord injections of AAV-retro/Cre above the lesion (0.3 µl/injection). 5 PTEN$^{f/f}$;Rosa^{tdTomato} mice and 1 Rosa^{tdTomato} mouse died 1-2 days post-surgery, thus after attrition, total animals were 25 PTEN$^{f/f}$;Rosa^{tdTomato} mice (n=10 male; n=15 female) and 15 Rosa^{tdTomato} mice (n=8 male; n=7 female).

Despite lower dose of AAV-retro/Cre, mice in Study 3 still exhibited pathophysiologies (detailed in Results). Only one mouse was euthanized due to the severity of the pathophysiology, so remaining mice were tested for the planned duration of the study. Mice were perfused 12 weeks post AAV-retro/Cre injections/SCI.

Study 4 assessed consequences of siRNA-based knockdown of PTEN. This study involved 15 Rosa^{tdTomato} mice (n=7 male; n=8 female). Mice were 25 weeks old at the start of the study, were handled for 2 weeks, and then baseline GSM values were collected for 9 weeks. When mice were 34 weeks of age, they received dorsal hemisection injuries and unilateral intra-spinal cord injections of 3E9 GC per mouse of AAV-retro/siPTEN/GFP above the lesion (0.3 µl/injection). Of these, 1 mouse died during the first 2 days post-surgery; thus after attrition, starting animal numbers were 14 (n=6 male; n=8 female).

Of the mice that received AAV-retro/siPTEN/GFP, one male mouse exhibited hindlimb dystonia 1 week post lesion/injection, and then excessive scratching. This mouse was euthanized at this time point (1 week post lesion/injection) due to severity of scratching wounds. One male mouse developed shoulder wounds due to scratching 2.5 weeks post lesion/injection and 6 more (n=3 males; n=3 females) began to exhibit pathophysiologies 5 weeks post lesion/injection. The scratching wounds were not severe enough to require euthanasia and all mice except for 1 continued in the study and were perfused 12 weeks post lesion/injection.

Study 5 tested whether pathophysiologies were due to deletion of PTEN in local spinal cord circuitry in the context of a spinal cord injury. For this, mice received AAV2/Cre rather than AAV-*retro*-Cre at the time of SCI to delete PTEN in the spinal cord around the injection site without retrograde transduction of cells of origin of spinal pathways in the brain. 15 PTEN$^{f/f}$;Rosa^{tdTomato} (n=6 male; n=9 female) and 13 Rosa^{tdTomato} mice (n=8 male; n=5 female) were 12 weeks old before GSM pre-training, were handled for 2 weeks and baseline GSM values were collected for 2 weeks. When mice were 16 weeks of age, they received dorsal hemisection injuries and 2 bilateral intra-spinal cord injections of AAV/Cre above the injury (0.3 µl/injection). Of these, 1 PTEN$^{f/f}$;Rosa^{tdTomato} mouse and 1 Rosa^{tdTomato} mouse died on the first 1-2 days post-surgery; thus after attrition, total animals were 14 PTEN$^{f/f}$;Rosa^{tdTomato} mice (n=5 male;

n=9 female) and 12 Rosa^{tdTomato} mice (n=8 male; n=4 female). No pathophysiologies were seen and these mice were perfused 8 weeks post AAV2/Cre injections plus SCI.

Our previous anatomical studies involved injections of AAV-retro/Cre in PTEN$^{f/f}$;Rosa^{tdTomato} mice and Rosa^{tdTomato} mice without injuries. Thus, we respectively reviewed husbandry reports and survival statistics to determine whether pathophysiologies occurred with AAV-retro/Cre injections in PTEN$^{f/f}$;Rosa^{tdTomato} mice in the absence of spinal cord injury (Metcalfe et al., 2022).

The retrospective review included n=12 PTEN$^{f/f}$;Rosa^{tdTomato} mice (n=8 males were between 14-24 weeks old; n=3 females were between 16-24 weeks old). These mice received two bilateral intra-spinal cord injections of AAV-retro-Cre (6E9 GC/mouse) at C5. No animals died during injection procedures. Mice were allowed to survive for different time periods and were killed for anatomical studies. Two males and 1 female mouse were killed 1 month post AAV-retro/Cre injection; 4 male mice were killed 4 months post AAV-retro/Cre injections; 1 male mouse was killed 7 months post AAV-retro/Cre injection and 2 male and 3 females were killed 12 months post AAV-retro/Cre injection. As detailed in the Results, none of these mice exhibited pathophysiologies.

Details on animal use (genotypes, ages at the time of injection, injection location, survival time are summarized in Table 1.

Grip strength meter (GSM)

The GSM measures grip strength (the maximal peak force in grams) as a mouse is allowed to grip a bar and then is gently pulled away. To test grip strength of one paw, the opposite forepaw is taped with non-stick surgery tape (Danilov & Steward, 2015). For testing, mice were held by the tail and suspended 2-3 inches above the plate of the GSM. Mice were gradually brought toward the bar until they reached out and gripped the bar. Mice were allowed to briefly adjust their paw on the bar to establish grip, then the mice were gently pulled away until the grip was released. The GSM records the maximal force applied at the time grip was released. This procedure was repeated 4 times in each testing session, recording grip strength separately for each paw and then averaging across the session.

For each study, mice were pre-handled for 2 weeks and then pre-trained on the GSM as stated for each study. Mice typically exhibit a stronger grip early in training and then gripping force decreases to a plateau that is maintained over time. After recording the last 5 days of pre-injury values, mice received dorsal hemisections and were then tested post injury on the GSM 3x per week as stated for each study.

Tissue preparation

Mice were transcardially perfused at survival times indicated in Table 1 with 4% paraformaldehyde in 0.1M phosphate buffer (4% PFA). Brains, spinal cords and dorsal roots were dissected, post-fixed in 4% PFA overnight, then immersed in 27% sucrose for cryoprotection overnight, frozen in TissueTek OCT (VWR International) and stored at -20°C until they were sectioned with a cryostat.

Immunostaining to assess AAV transduction

Brain blocks were sectioned by cryostat and free-floating sections were collected. One set of the sections was mounted in serial order for visualization of native tdT

Study	Genotype	Sex	No. animals	Attrition	Virus	Titer (GC/ mouse)	Injection	No. injections	Survival time (weeks)
Study 1	PTEN$^{f/f}$; Rosa^{tdTomato}	Male	11	3	AAV-retro/Cre	12E9	bilateral	4	9
		Female	4	0					
	Rosa^{tdTomato}	Male	4	0					
		Female	10	1					
Study 2	PTEN$^{f/f;}$ Rosa^{tdTomato}	Male	9	2	AAV-retro/Cre	6E9	bilateral	2	16
		Female	7	3					
		Male	6	2	AAV-retro/GFP				
		Female	4	1					
Study 3	PTEN$^{f/f}$; Rosa^{tdTomato}	Male	14	4	AAV-retro/Cre	3E9	unilateral	1	12
		Female	16	1					
	Rosa^{tdTomato}	Male	8	0					
		Female	8	1					
Study 4	Rosa^{tdTomato}	Male	7	1	AAV-retro/ siPTEN/ GFP	3E9	unilateral	1	12
		Female	8	8					
Study 5	PTEN$^{f/f}$; Rosa^{tdTomato}	Male	6	1	AAV2/Cre	6E9	bilateral	2	9
		Female	9	0					
	Rosa^{tdTomato}	Male	8	0					
		Female	5	1					

Table 1: Details on animal use, genotypes, attrition, AAV-retro doses, injection location and survival time per Study.

fluorescence to assess AAV transduction. Other sets of sections were immunostained for tdT (rabbit anti-red fluorescent protein, Rockland Immunochemicals Inc., #600-401-379, RRID:AB_2209751). To assess deletion/knockdown, one set of sections was immunostained for PTEN (rabbit anti-PTEN Cell Signaling Technology #9188L, RRID:AB_2253290). Biotin-conjugated secondary antibodies were used at 1:250 dilution and stained with tyramide-Cy3 (for PTEN).

Quantification of PTEN-deleted CMNs

Brains were sectioned at 30 µm in the coronal plane; sets of sections are collected at 240 µm intervals to generate 10 sets of regularly-spaced sections. One set of sections was prepared for PTEN immunofluorescence to detect PTEN-deleted neurons. PTEN deletion manifests itself as the “ghost-cells” in the staining compared with a relatively uniform neuropil.

Ten sections were used for quantitative analyses of numbers of PTEN-deleted neurons. For animals belonging to the high dose group (12E9 GC/mouse), n=4 mice were chosen taking the first three in the study list that were not used for analyses for other studies (Metcalfe et al., 2022). For mid doses (6E9 GC/mouse) n=2 mice were chosen from the top and the bottom of the study list and that made it to the end of the study. The area of the sensorimotor cortex containing PTEN-deleted neurons was

imaged at 10X. Images of cortex containing ghost cells were obtained by setting edge points encompassing the totality of layer V in that section at 10x magnification using the Keyence microscope “Image Stitch” program. Keyence analysis software stitched the individual frames together and measurements were then exported to ImageJ (Schneider, Rasband, & Eliceiri, 2012) to determine the average number of PTEN-negative neurons (ghost cells) per section and then average of ghost-cell per mouse. Measurements were then exported to Excel (Microsoft) and an average of ghost-cells per mouse for each dose was determined.

Statistical Analyses

For statistical analysis of ghost cells numbers, Prism software was used; the analysis was a one-way ANOVA with a post hoc Tukey’s multiple comparisons test to determine significant differences among groups. For GSM values analyses, Prism software was used; the primary statistical analysis was by a two-way repeated measures ANOVA for each separate study with variables of "groups" and "time post-injury". A mixed effects model analysis was used when values were missing for some timepoints, particularly when animals were euthanized due to health concerns. We also calculated *percent recovery in each group* by comparing average pre-injury GSM values vs. peak post injury values. Statistical analyses then compared groups (PTEN deleted vs. control) by considering the percent recovery for each animal.

RESULTS

Intra-spinal cord injections of AAV-retro/Cre in PTEN$^{f/f}$;Rosa^{tdTomato} mice transduces corticomotor neurons to induce tdT expression and delete PTEN

The present study takes advantage of a model using double-transgenic mice (PTEN$^{f/f}$;Rosa^{tdTomato}) and AAV-retro/Cre, which allows remote transduction of neurons via retrograde transport. We have previously shown that intra-spinal cord injections of AAV-retro/Cre in PTEN$^{f/f}$;Rosa^{tdTomato} mice led to selective transduction of thousands of neurons in layer V of the sensorimotor cortex as revealed by induction of tdTomato (tdT) expression. These are the cells of origin of the corticospinal tract (CST), termed “corticomotor neurons” (CMNs). Cells of origin of other descending spinal pathways were also transduced, including neurons in the brainstem reticular formation, red nucleus, and hypothalamus and some populations of neurons in dorsal root ganglia near the injection are also transduced (Metcalfe et al., 2022). Studies from other groups have revealed that AAV-retro also transduces the cells of origin of some but not all intra-spinal circuits; for example, neurons of the propriospinal pathways are not efficiently transduced (Wang et al., 2018). Finally, some populations of neurons in dorsal root ganglia near the injection are also transduced (Metcalfe et al., 2022).

Results of the present studies were consistent with previous data. In Study 1, PTEN$^{f/f}$;Rosa^{tdTomato} mice received doses of AAV-retro/Cre that were 2-fold higher than in our previous study (total of 12E9 GC/mouse) at the time of a dorsal hemisection. This was delivered in 4 injections, 2 above and 2 below the injury. TdT was visible in the spinal cord at the injection site in intact spinal cords (Fig. 1A), and epi-fluorescence illumination revealed oval areas of tdT fluorescence marking clouds of CMNs

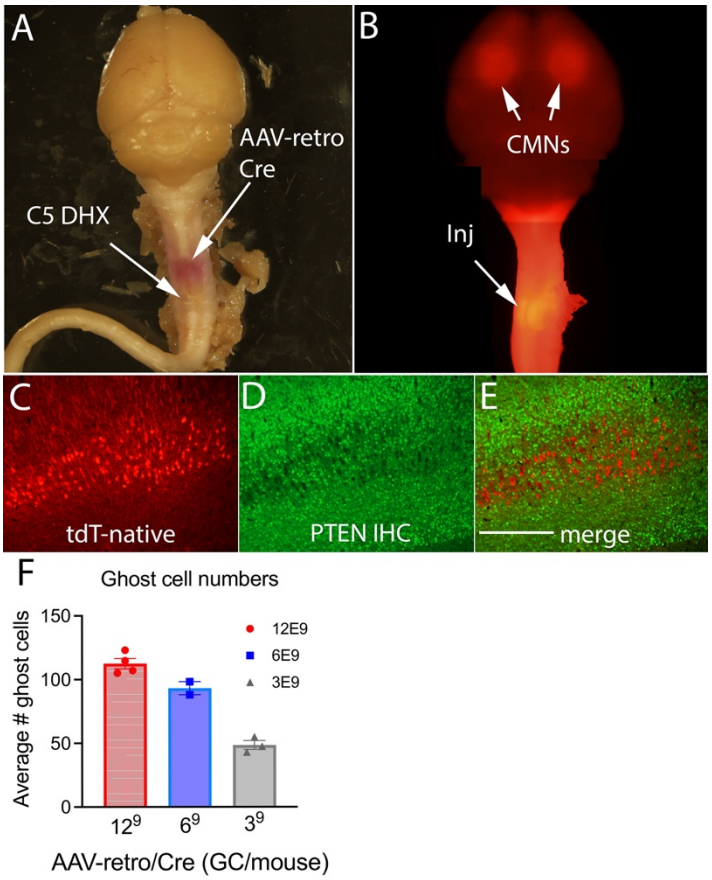

Figure 1. Retrograde transfection of cortical motoneurons (CMNs) with injections of retro-AAV/Cre into the spinal cord of PTEN$^{f/f}$: Rosa^{tdTomato} mice. A) Intact brain and attached spinal cord. tdTomato is visible at the site of injection in the cervical spinal cord as well as the site of injury (arrows) B) Epifluorescence images of the same brain and spinal cord showing oval areas of tdT fluorescence marking clouds of CMNs expressing tdT and site of injection in thecervical spinal cord (arrows). C-E) Coronal sections through the sensorimotor cortex showing large numbers of native fluorescent tdT-positive pyramidal neurons in layer V (C) that were completely negative for PTEN protein (D). Co-imaging for tdT revealed that tdT-positive neurons are negative for PTEN protein (E). Scale bar = 1 mm. F) Different doses of AAV-retro/Cre revealed an average of 112.5 ghost cells/section at the 12E9 GC dose, 93.16 ghost cells per section at the 6E9 GC/mouse dose, and 42.7 ghost cells at the 3E9 dose. One-way ANOVA revealed significant differences between GC doses [F(64.96) DF 2, 6, $p<0.0001$]. Post-hoc Tukey's multiple comparisons test revealed significant differences between high (12E9 GC/mouse) and low GC doses (3E9 GC/mouse) ($p<0.0001$, 95% C.I. = [46.6, 81.27] and between intermediate (6E9 GC/mouse) and low GC doses (3E9 GC/mouse) ($p = 0.0014$, 95% C.I. = [23.88, 65.32]). There were no significant differences between high (12E9 GC/mouse) and intermediate GC doses (6E9 GC/ml) ($p = 0.0532$, 95% C.I. = [-0.32, 39]).

expressing tdT (Fig. 1B). In coronal sections through the sensorimotor cortex, large numbers of tdT-positive pyramidal neurons were evident in layer V without immunostaining (native fluorescence, Fig. 1C).

Confirming previous findings, immunostaining for PTEN in PTEN$^{f/f}$;Rosa^{tdTomato} mice injected with AAV-retro/Cre revealed large numbers of CMNs that were completely negative for PTEN protein, appearing as "ghost cells" (Fig. 1D, same section as Fig. 1C). Co-imaging for tdT revealed that tdT-positive neurons are negative for PTEN protein (No double labeling which would be represented in yellow, Fig. 1E).

Different doses of AAV-retro/Cre were delivered in studies 1, 2, and 3 (12E9 GC/mouse delivered in two injections above the lesion in study 1, 6E9 GC/mouse delivered in two injections above the lesion in study 2, and 3E9 GC/mouse in one unilateral injection in Study 3). Counts revealed an average of 112.5 ghost cells/section at the 12E9 GC dose, 93.16 ghost cells per section at the 6E9 GC/mouse dose, and 42.7 ghost cells at the 3E9 dose (Fig. 1E). One-way ANOVA revealed significant differences between GC doses [F(64.96) DF 2, 6, $p<0.0001$]. Post-hoc Tukey's multiple comparisons test revealed significant differences between high (12E9 GC/mouse) and low GC doses (3E9 GC/mouse) ($p<0.0001$, 95% C.I. = [46.6, 81.27] and between intermediate (6E9 GC/mouse) and low GC doses (3E9 GC/mouse) ($p = 0.0014$, 95% C.I. = [23.88, 65.32]). There were no significant differences between high (12E9 GC/mouse) and intermediate GC doses (6E9 GC/ml) ($p = 0.0532$, 95% C.I. = [-0.32, 39]).

The likely explanation for the small difference between the high and intermediate GC doses in average numbers of PTEN-negative cells/section is that 2 of the injections at the high dose were below the injury. With complete dorsal hemisections, injections below the injury would not be expected to transduce many CMNs because the dorsal and dorso-lateral CST is completely transected. Presumably, only the ne urons that give rise to the sparse ventral CST would be transduced.

<u>Enhanced recovery of forelimb motor function after SCI with intra-spinal cord injections of AAV-retro/Cre</u>

1. <u>Study 1:</u>

Study 1 was undertaken to test AAV-retro as a platform to delete PTEN to promote recovery of function after dorsal hemisection injuries at C5 with bilateral injections of AAV-retro/Cre (total of 12E9 GC/mouse) at the time of the injury. Mice received 2 injections above the level of the dorsal hemisection at approximately C4 and 2 injections below the injury at approximately C6. Controls were $Rosa^{tdTomato}$ mice that received similar injections. Forelimb motor function was assessed over time using a GSM by an individual blinded to treatment group.

Following C5 lesions and AAV-retro/Cre intra-spinal cord injections, GSM values dropped after injury to 5.5% of pre-injury values for $Rosa^{tdTomato}$ mice and 26.9% of pre-injury values for $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice (Fig. 2A). For the control $Rosa^{tdTomato}$ mice GSM values recovered to a plateau at around 14 days (36% of pre-injury control). In contrast, values for $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice increased progressively reaching a plateau at 25 days of 84% of pre-injury control values. At all post-injury time points, average values for the $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice (PTEN-deleted) were higher than $Rosa^{tdTomato}$ controls. Two-way repeated measures ANOVA revealed significant differences over time [F(15.13) DF 18, 414, $p<0.0001$] and between genotypes [F(6.41) = DF 1,23, $p=0.018$].

<u>1.1 Comparisons by sex:</u>

Stratification of GSM data by sex (Fig. 2B &C) revealed no sex differences in the values of the $Rosa^{tdTomato}$ control mice, whereas there were substantial sex differences in $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice (PTEN-deleted). Specifically, enhanced recovery of GSM

values was seen primarily in male mice. GSM values for female PTEN$^{f/f}$;Rosa^{tdTomato} mice (PTEN-deleted) were 77.3% of pre-injury control at the plateau vs. 35.6% for the Rosa^{tdTomato} female control mice. In contrast, GSM values for male PTEN$^{f/f}$;Rosa^{tdTomato} mice were 95.6% of pre-injury control at the plateau vs. 27.1% for the Rosa^{tdTomato} male control mice. Two-way repeated measures ANOVA revealed no significant sex differences for the Rosa^{tdTomato} mice [F(0.63) DF 1,11, p=0.442] but significant sex differences for the PTEN$^{f/f}$;Rosa^{tdTomato} mice [F(4.97) DF 1,10, p=0.05]. Thus, the values for the male PTEN$^{f/f}$;Rosa^{tdTomato} mice largely accounted for the overall difference in the combined values reported above.

1.2 Late-developing pathophysiologies:

Mice were tested with GSM once/week and after the initial post-injury period, mice were not individually examined every day by research staff but were monitored routinely by vivarium staff (routine animal husbandry). At 4 weeks post-injury, vivarium staff reported a mouse with severe skin wounds behind the ears and on the neck. These wounds were initially attributed to “over-grooming”. After careful observation, we determined that the mouse was repeatedly scratching the area of the wound with its hindlimbs. Wounds on this mouse were severe so the mouse was euthanized. At 5 weeks, another mouse was reported as having wounds diagnosed as “ulcerative dermatitis”. Wounds were treated with Silvadeen and toenails were trimmed. Similar wounds began to appear in other mice that were attributed to various causes; in each case, wounds were treated with Silvadeen and toenails were trimmed. At 6 weeks, 3 more mice exhibited wounds that were diagnosed as “skin lesions” and “ulcerative dermatitis”. At 7 weeks, 2 more mice exhibited wounds that were diagnosed as “fight wounds and ulcerative dermatitis”. Finally, at 9 weeks, 2 more mice exhibited wounds diagnosed as “fight wounds” in one case and “ulcerative dermatitis” in the other. Some mice also began to exhibit rigid forward extension of the hindlimbs, which we term dystonia. Overall, 9/13 PTEN$^{f/f}$;Rosa^{tdTomato} mice with SCI and AAV-retro/Cre exhibited wounds and scratching. We had originally planned to inject BDA to trace CST axons, but because of the continued incessant scratching and increasing severity of the wounds, we terminated the experiment and all remaining mice were euthanized and perfused at 64 days post- AAV-retro/Cre injections. When mice were terminated, we did not note the sex of mice that had exhibited pathophysiologies, and whether there were sex differences in the incidence or severity of wounds.

2. Study 2:

To begin to define causes and mechanisms of the pathophysiologies that appeared in study 1, in study 2, we made 2 adjustments to the dosing. First, the overall dose was reduced to 6E9 GC/mouse to determine whether this dose could promote forelimb recovery without occurrence of pathophysiologies. Second, because some mice exhibited rigid forward extension of the hindlimb, we wondered whether this would be reduced or eliminated if AAV-retro/Cre injections were only rostral to the injury. Also, for controls, genotype was held constant; all mice were PTEN$^{f/f}$;Rosa^{tdTomato} but the control group was injected with AAV-retro/GFP rather than AAV-retro/Cre.

One week following C5 lesions and AAV-retro intra-spinal cord injections, GSM values on the first testing day were 1% of pre-injury values for mice injected with AAV-

retro/Cre and to 1.7% of pre-injury values for mice injected with AAV2-retro/GFP. For the control mice injected with AAV-retro/GFP GSM values recovered progressively to a plateau at around 25 days post-injury (9.1% of pre-injury control). In contrast, values for PTEN-deleted mice increased progressively reaching a plateau at 25 days 49.3% of pre-injury control values. A mixed effects model analysis revealed significant differences over time [F(121.1) DF 5.07, 65.4, $p<0.0001$] and between genotypes [F(5.47) = DF 1, 14, $p=0.035$].

Of note, post-injury values for controls were lower in this study (9% of pre-injury) in comparison to study 1 (36% of pre-injury). One possible explanation is that the control group is differs between studies ($Rosa^{tdTomato}$ in study 1 vs. $PTEN^{f/f}$;$Rosa^{tdTomato}$ that received AAV-retro/GFP in study 2). Differences in lesion extent are possible but less likely because the same surgeon did the dorsal hemisections for all studies. The differences observed between study 1 and 2 in the control values allowed us to refine the definition of a scorable grip and determine strict criteria for interrater reliability for studies 3 through 5.

2.1 Comparisons by sex:

Stratification of GSM data by sex (Fig. 2E &F) revealed no sex differences in the values of the AAV-retro/GFP control mice (PTEN intact). However, as in Study 1, there were substantial sex differences in $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice injected with AAV-retro/Cre (PTEN-deleted). Specifically, enhanced recovery of GSM values was greater in male mice. GSM values for female PTEN-deleted were 30.25% of pre-injury control at the plateau vs. 7.86% for the PTEN-intact female control mice. In contrast, GSM values for male PTEN-deleted mice were 61.1% of pre-injury control at the plateau vs. 11.8% for the PTEN-intact male control mice. A mixed effects model analysis revealed no significant sex differences for control mice injected with AAV-retro/GFP [F(1.63) DF 1,5, $p=0.26$] but significant sex differences for the PTEN-deleted mice injected with AAV-retro/Cre [F(8.39) DF 1,7, $p=0.023$]. Thus, as in study 1, the values for the male $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice largely accounted for the overall difference in the combined values reported above.

2.2 Late-developing pathophysiologies:

Although mice that received AAV-retro/Cre exhibited higher GSM scores throughout the testing period, pathophysiologies developed at 5 weeks post injection/SCI in n=2 $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice (n=1 male; n=1 female). The male mouse was reported as having hindlimb dystonia plus autophagia and the female exhibited scratching wounds on shoulders. These mice were euthanized because of the severity of the wounds. Behavioral testing (GSM) continued for 64 days for the remaining mice [7 $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice injected with AAV-retro/Cre (n=5 male; n=2 female) and 7 injected with AAV-retro/GFP (n=4 male; n=3 female)]. We did not record which animal numbers exhibited pathophysiologies, so in this study we didn't document if there were sex differences in the incidence of wounds. None of the $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice injected with AAV-retro/GFP (PTEN-intact) exhibited excessive scratching, wounds, or hindlimb dystonia at any time. Animals were perfused 16 weeks post AAV-retro plus SCI.

3. Study 3:

In study 3 we further lowered AAV-retro/Cre doses (3E9 GC/animal). PTEN$^{f/f}$;Rosa^{tdTomato} mice received 1 unilateral injection above the level of the dorsal hemisection at approximately C4. Controls were Rosa^{tdTomato} mice that received similar injections. Forelimb motor function was assessed over time using a GSM by an individual blinded to treatment group.

Following C5 lesions and AAV-retro/Cre intra-spinal cord injections, (one week post-injury), GSM values dropped to 4.1% of pre-injury values for Rosa^{tdTomato} mice and 6.4% of pre-injury values for PTEN$^{f/f}$;Rosa^{tdTomato} mice. For the control Rosa^{tdTomato} mice GSM values recovered to a plateau at around 25 days post-injury (6.8% of pre-injury control). Values for PTEN$^{f/f}$;Rosa^{tdTomato} mice were slightly higher than control mice, reaching a plateau at 25 days 12.4% of pre-injury control values. A mixed effects model analysis revealed significant differences over time [F(823.0) DF 4.56, 174.6, p<0.0.001] but no significant differences between genotypes [F(0.018) = DF 1, 39, p=0.8944] (Fig. 2G).

3.1 Comparisons by sex:

Stratification of GSM data by sex (Fig. 2H &I) revealed no sex differences in the values of the Rosa^{tdTomato} control mice, and in PTEN$^{f/f}$;Rosa^{tdTomato} mice (PTEN-deleted). Specifically, GSM values for female PTEN$^{f/f}$;Rosa^{tdTomato} mice (PTEN-deleted) were

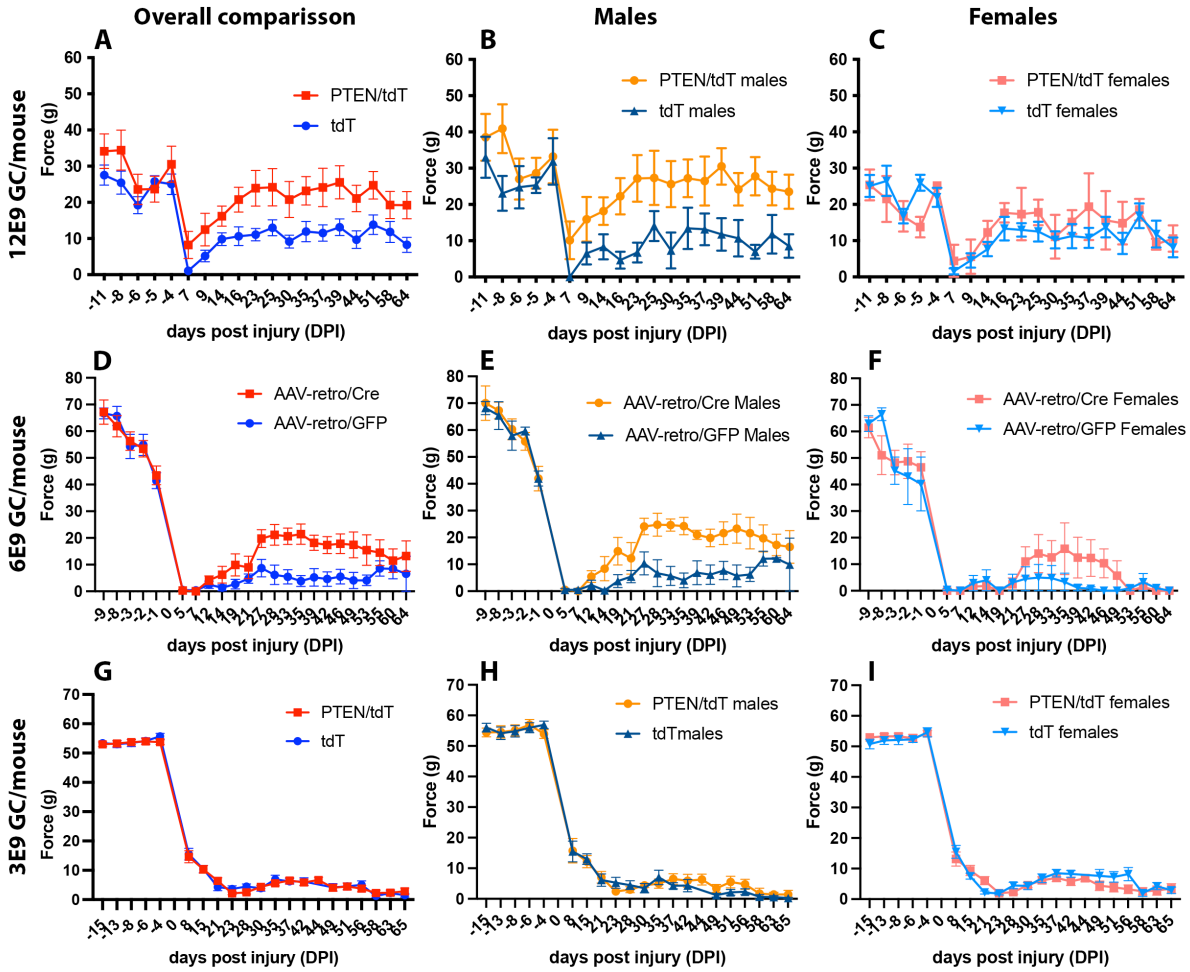

Figure 2. Genetic deletion of PTEN promotes transient functional recovery followed by functional deterioration. Forepaw gripping function as measured by grip strength meter (GSM). A) Rosa^{tdTomato} and PTEN$^{f/f}$;Rosa^{tdTomato} mice were injected with 12E9 GC/mouse of AAV-retro/Cre at the same time as a cervical dorsal hemisection. Two-way repeated measures ANOVA revealed significant differences over time [F(15.13) DF 18, 414, p<0.0001] and between genotypes [F(6.41) = DF 1,23, p=0.018]. B-C) Stratification of GSM data by sex revealed no sex differences in the values of the Rosa^{tdTomato} control mice [F(0.63) DF 1,11, p=0.442], whereas there were substantial sex differences in PTEN$^{f/f}$;Rosa^{tdTomato} mice (PTEN-deleted) [F(4.97) DF 1,10, p=0.05]. D) PTEN$^{f/f}$;Rosa^{tdTomato} mice were injected with 12E9 GC/mouse of AAV-retro/Cre or AAV-retro/GFP at the same time as a cervical dorsal hemisection. A mixed effects model analysis revealed significant differences over time [F(121.1) DF 5.07, 65.4, p<0.0001] and between genotypes [F(5.47) = DF 1, 14, p=0.035]. E-F) Stratification of GSM data by sex revealed no sex differences in the values of the AAV-retro/GFP control mice (PTEN intact) [F(1.63) DF 1,5, p=0.26] but significant sex differences for the PTEN-deleted mice injected with AAV-retro/Cre [F(8.39) DF 1,7, p=0.023]. G) Rosa^{tdTomato} and PTEN$^{f/f}$;Rosa^{tdTomato} mice were injected with 3E9 GC/mouse of AAV-retro/Cre at the same time as a cervical dorsal hemisection. A mixed effects model analysis revealed significant differences over time [F(823.0) DF 4.56, 174.6, p<0.0.001] but no significant differences between genotypes [F(0.018) = DF 1, 39, p=0.8944]. H-I) A mixed model ANOVA revealed no significant sex differences for the Rosa^{tdTomato} mice [F(0.92) DF 1,13, p=0.35] nor for the PTEN$^{f/f}$;Rosa^{tdTomato} mice [F(2.29) DF 1,23, p=0.63].

12.6% of pre-injury control at the plateau vs. 15.6% for the Rosa^{tdTomato} female control mice. GSM values for male PTEN$^{f/f}$;Rosa^{tdTomato} mice were 13% of pre-injury control at the plateau vs. 7.7% for the Rosa^{tdTomato} male control mice. A mixed model ANOVA revealed no significant sex differences for the Rosa^{tdTomato} mice [F(0.92) DF 1,13, p=0.35] nor for the PTEN$^{f/f}$;Rosa^{tdTomato} mice [F(2.29) DF 1,23, p=0.63]. Overall neither group showed improvement in recovery of function after SCI plus AAV-retro/Cre injection.

Again, post-injury values for controls were lower in this study (6.8% of pre-injury) in comparison to study 1 (36% of pre-injury). In this case, the control group is the same (Rosa^{tdTomato}). Differences in lesion extent are possible but less likely because the same surgeon did the dorsal hemisections for all studies. A possible explanation is that the rater in study 1 was different than the rater in study 2 and 3.

3.2 Late-developing pathophysiologies:

In study 3, despite lowering the dose of AAV-retro/Cre 4-fold than in Study 1, so much so that functional recovery due to PTEN deletion was lost, PTEN$^{f/f}$;Rosa^{tdTomato} mice (PTEN-deleted) mice still developed late occurring pathophysiologies. The first incidence of pathophysiology was reported 3 weeks post AAV2-retro/Cre injection and SCI in n=4 mice (all males). N=3 of those showed injuries behind their ears due to chronic scratching and n=1 exhibited hindlimb dystonia. Out of those, 1 of the animals that were scratching subsequently developed hindlimb dystonia at 7 weeks post AAV-retro/Cre injections plus SCI. This mouse euthanized due to severity of injuries at this time point. New cases of pathophysiologies appeared at 7 weeks post AAV-retro/Cre injections plus SCI in n=9 mice (n=5 males; n=4 females). Of these, n=4 showed only scratching behind the ears, n=1 only hindlimb dystonia and n=4 exhibited both scratching and hindlimb dystonia. Injuries were not as severe as in study 1, so no other animals were euthanized due to severity of pathophysiologies. Thus out of n=26 PTEN$^{f/f}$;Rosa^{tdTomato} mice (n=10 males; n=16 females), n=13 developed pathophysiologies starting 3 weeks post AAV-retro/Cre injections plus SCI. Animals were perfused 12 weeks post AAV-retro/Cre injections plus SCI.

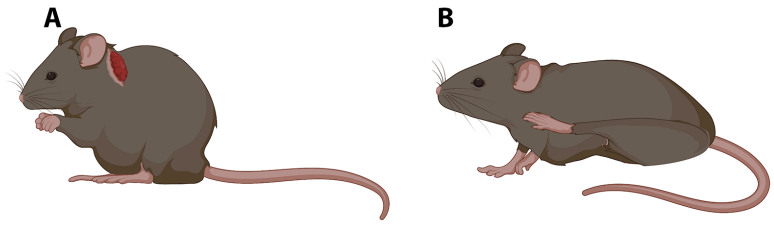

Figure 3. Pathophysiology with AAV-retro mediated PTEN deletion and SCI. At 2-3 months post-injury animals develop excessive scratching and hindlimb dystonia. PTEN$^{f/f}$;Rosa^{tdTomato} injected with AAV-retro/Cre show severe scratching (A) and/or hindlimb dystonia (B).

4. Study 4: AAV-retro/siRNA-based knockdown of PTEN:

Study 4 assessed whether PTEN knockdown via AAV-retro/siPTEN would enhance motor recovery or lead to pathophysiologies. Rosa^{tdTomato} mice received unilateral intraspinal cord injections of AAV-retro/siPTEN/GFP above the injury (3E9 GC/mouse). Controls were Rosa^{tdTomato} mice that received similar injections of AAV-retro/Cre. Forelimb motor function was assessed over time using a GSM by an individual blinded to treatment group.

Following C5 lesions and AAV-retro intra-spinal cord injections, GSM values were 8% of pre-injury values at 21 days post-injury for mice injected with AAV-retro/Cre (PTEN-intact controls). Earlier time points are not shown because of errors in scoring. Values for mice that received AAV-retro/siPTEN were 5% of pre-injury control. For the control PTEN-intact mice GSM values recovered to a plateau at around 37 days post-injury of 11.8% of pre-injury control, whereas values for Rosa^{tdTomato} mice that received AAV-retro/siPTEN were 6.7% of pre-injury control. A mixed effects model analysis revealed significant differences over time [F(535.8) DF 25, 375, p<0.0.001] and but no significant differences between genotypes [F(0.8753) = DF 1, 15, p=0.4301] (Fig. 4A).

4.1 Comparisons by sex:

Stratification of GSM data by sex (Fig. 4B &C) revealed no sex differences in the values of the Rosa^{tdTomato} control mice injected with AAV-retro/Cre and injected with AAV-retro/siPTEN/GFP. GSM values for female mice that received AAV-retro/siPTEN were 4.68% of pre-injury control at the plateau vs. 18.9% for the Rosa^{tdTomato} female control mice. GSM values for male mice that received AAV-retro/siPTEN were 9.57% of pre-injury control at the plateau vs. 4.8% for the Rosa^{tdTomato} male control mice. A mixed effects model analysis revealed no significant sex differences for the control Rosa^{tdTomato} mice [F(0.079) DF 1,13, p=0.78] nor for the Rosa^{tdTomato} mice injected with AAV-retro/siPTEN/GFP (PTEN-knockdown) [F(0.11) DF 1,12, p=0.74]. Overall neither group showed improvement in recovery of function after SCI plus AAV-retro/siPTEN/GFP injection.

4.2 Late-developing pathophysiologies:

Although mice with AAV-retro/siPTEN/GFP (PTEN knock-down) did not show improvements in motor function in comparison to controls, they did develop pathophysiologies. One male mouse that received AAV-retro/siPTEN/GFP exhibited

hindlimb dystonia 1-week post-surgery. This animal was euthanized due to lack of mobility and health concerns. One male mouse that received AAV-retro/siPTEN/GFP exhibited shoulder wounds due to scratching 2.5 weeks post AAV-retro/shPTEN intraspinal injection plus SCI. N=6 mice showed pathophysiologies 5 weeks post AAV-retro/siPTEN/GFP intraspinal injection plus SCI. Out of those 6, n=5 (n=3 males; n=2 females) exhibited only dystonia and n=1 (female) exhibited both scratching and dystonia. Out of those 6 animals one mouse was euthanized due to the severity of the scratching wounds. Scratching wounds were treated with Silvadeen and toenails were trimmed. From n=14 $Rosa^{tdTomato}$ mice (n=6 males; n=8 females), n=8 developed pathophysiologies starting 1 week post AAV-retro/siPTEN/GFP intraspinal injection plus SCI, out of those 8, n=5 were males and n=3 were females, therefore there was no apparent sex difference in incidence of pathophysiologies with AAV-retro/siPTEN/GFP injections plus SCI. Animals were perfused 12 weeks post AAV-retro/siPTEN/GFP injections plus SCI.

5. Study 5: PTEN deletion in local spinal circuitry:

Study 5 tested whether pathophysiologies were due to deletion of PTEN in local spinal cord circuitry in the context of a spinal cord injury. Mice received dorsal hemisection injuries and intra-spinal cord injections of AAV2/Cre rather than AAV2-*retro*-Cre (6E9 GC/mouse). Intra-spinal injections of AAV2/Cre delete PTEN in a local region of the spinal cord around the injection site (Fig. 4D) but there is no retrograde transduction of cells of origin of spinal pathways in the brain. $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice received 2 injections above the level of the dorsal hemisection at approximately C4. Controls were $Rosa^{tdTomato}$ mice that received similar injections. Forelimb motor function was assessed over time using a GSM by an individual blinded to treatment group.

Following C5 lesions and AAV2/Cre intra-spinal cord injections, 7 days post-injury GSM values dropped to 8.3% of pre-injury values for $Rosa^{tdTomato}$ mice and 1.8% of pre-injury values for $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice. Values remained low for the duration of the testing period and they plateaued at around 21 days post injury to 5.4% of pre-injury control and to 2.6% of pre-injury values for $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice. Two-way repeated measures ANOVA revealed significant differences over time [F(988.0) DF 5.13, 123.1, $p<0.0001$] and no significant differences in grip strength between genotypes [F(4.24) = DF 1,24, $p=0.051$].

5.1 Comparisons by sex:

Stratification of GSM data by sex (Fig. 4E &F) revealed no sex differences in the values of the $Rosa^{tdTomato}$ control mice nor in the values of the $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice injected with AAV2/Cre. Specifically, at 21 days post injury, GSM values for female $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice (PTEN-deleted) were 2.7% of pre-injury control at the plateau vs. 7.7% for the $Rosa^{tdTomato}$ female control mice. GSM values for male $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice were 1.7% of pre-injury control at the plateau vs. 1.5% for the $Rosa^{tdTomato}$ male control mice. Two-way repeated measures ANOVA revealed no significant sex differences for the $Rosa^{tdTomato}$ mice [F(3.17) DF 1,10, $p=0.11$] nor for the $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice [F(0.42) DF 1,12, $p=0.53$]. Overall neither group showed improvement in recovery of function after SCI plus AAV2/Cre intraspinal injections.

5.2 Late-developing pathophysiologies:

None of the mice that received dorsal hemisections and intra-spinal injections of AAV2/Cre exhibited pathophysiologies described above. All animals were euthanized 42 days post injury.

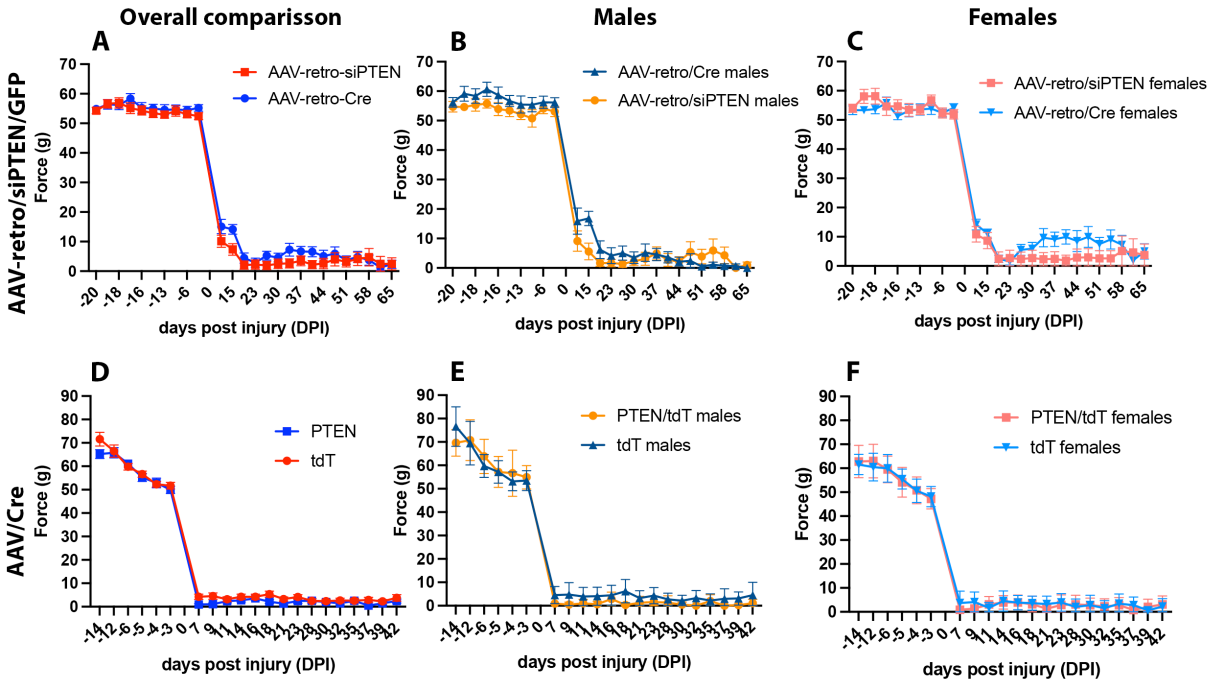

Figure 4. PTEN knockdown with AAV-retro/siPTEN/GFP and PTEN deletion in local spinal circuitry does not promotes functional recovery. Forepaw gripping function as measured by grip strength meter (GSM). A) Rosa^{tdTomato} mice were injected with 3E9 GC/mouse of AAV-retro/siPTEN/GFP or AAV-retro/Cre at the same time as a cervical dorsal hemisection. A mixed effects model analysis revealed significant differences over time [F(535.8) DF 25, 375, p<0.0.001] and but no significant differences between genotypes [F(0.8753) = DF 1, 15, p=0.4301]. B-C) A mixed effects model analysis revealed no significant sex differences for the control Rosa^{tdTomato} mice [F(0.079) DF 1,13, p=0.78] nor for the Rosa^{tdTomato} mice injected with AAV-retro/siPTEN/GFP (PTEN-knockdown) [F(0.11) DF 1,12, p=0.74]. D) Rosa^{tdTomato} and PTEN$^{f/f}$;Rosa^{tdTomato} mice were injected with 6E9 GC/mouse of AAV/Cre. Two-way repeated measures ANOVA revealed significant differences over time [F(988.0) DF 5.13, 123.1, p<0.0001] and no significant differences in grip strength between genotypes [F(4.24) = DF 1,24, p=0.051]. E-F) Two-way repeated measures ANOVA revealed no significant sex differences for the Rosa^{tdTomato} mice [F(3.17) DF 1,10, p=0.11] nor for the PTEN$^{f/f}$;Rosa^{tdTomato} mice [F(0.42) DF 1,12, p=0.53].

6. Retrospective analysis of whether there are pathophysiologies with intra-spinal injections of AAV-retro/Cre without concurrent SCI:

To assess whether pathophysiologies developed with injections of AAV-retro-Cre without concurrent SCI, we reviewed husbandry and survival records from mice in our previous anatomical studies (Metcalfe et al., 2022; Steward et al., 2021) to assess retrograde transduction of CMNs. These studies involved 12 PTEN$^{f/f}$;Rosa^{tdTomato} mice (n=8 males; n=3 females) that were allowed to survive for different intervals after bilateral intra-spinal cord injections of AAV-retro-Cre at C5 (6E9 GC/mouse). Three

mice (2 males and 1 female) survived for 1 month post AAV-retro/Cre injection; 4 males survived 4 months post AAV-retro/Cre injection; 1 male survived 7 months post AAV-retro/Cre injection; 5 mice (n=2 males and n=3 females) survived 12 months post AAV-retro/Cre injection. None of these mice exhibited scratching or hindlimb dystonia or any other adverse condition that led to euthanasia.

DISCUSSION

The results of our study indicate that AAV-retro may serve as a promising platform to promote motor recovery after cervical spinal cord injury (SCI) by deleting PTEN in multiple pathways. We aimed to assess whether cell-specific deletion of PTEN could facilitate regeneration of descending tracts and recovery of motor function. Our results revealed: 1) Intra-spinal cord injections of AAV-retro/Cre at the time of a dorsal hemisection at C5 in $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice enhanced forelimb motor recovery in a dose (GC) dependent fashion. 2) There were major differences between sex in the extent of recovery. Male mice exhibited much greater recovery than females, and the data from males largely accounted for the differences in the combined data. 3) Despite initial recovery, late-onset pathophysiologies involving incessant scratching and hindlimb dystonia developed in many mice, abrogating the motor recovery. 4) Development of pathophysiologies was dose-dependent, but pathophysiologies occurred even at low GC doses that did not enhance recovery. 5) Pathophysiologies were also seen with AAV-retro/siPTEN injections at C5, even though there was no enhanced motor recovery. 6) Pathophysiologies were not seen with injections of AAV/Cre at C5 (not retro) or with injections of AAV-retro/Cre without concurrent SCI. In what follows, we discuss implications and caveats of these results.

Dose-dependence for enhanced recovery:

Previous studies have suggested that PTEN deletion can enhance axonal regeneration and functional recovery after spinal cord injury (Danilov & Steward, 2015; Du et al., 2015; Geoffroy et al., 2015; Lewandowski & Steward, 2014; K. Liu et al., 2010; Zukor et al., 2013). PTEN is a phosphatase that negatively regulates the PI3K/AKT/mTOR signaling pathway, which is crucial for neuronal survival, axonal growth, and regeneration (Leibinger et al., 2019; Proud, 2002; Testa & Tsichlis, 2005; Zhou & Snider, 2006). In the present study, we targeted PTEN deletion specifically to cells of origin of spinal pathways using AAV-retro/Cre injections.

Our study revealed enhanced recovery of forelimb motor function with intra-spinal injections of AAV-retro/Cre at the time of injury. Recovery was seen at higher doses 12E9 and 12E6 GC (genome copies) and was not seen with the lowest dose tested (3E9 GC). Counts of PTEN-deleted cortical motoneurons (ghost cells in layer V of the sensorimotor cortex) revealed dose-dependent differences in the numbers of PTEN-deleted neurons, although there were no significant differences in ghost cell numbers in Layer V between high and intermediate GC doses.

The likely explanation for the small difference in the average numbers of PTEN-negative cells per section between the high and intermediate GC doses is that the high dose involved 4 injections, 2 rostral and 2 caudal to the injury. Since dorsal and dorso-

lateral CST is completely transected in complete dorsal hemisections, injections below the injury would not be expected to transduce many CMNs. We speculate that the better outcome in functional recovery at high doses could be explained because AAV-retro/Cre injections below the injury may be promoting rewiring and synaptic plasticity of intraspinal circuitry, whereas injections above the injury may be deleting PTEN in supra spinal centers that promote CST growth after injury. Recent studies have shown that coordinated growth stimulation in supra spinal centers and in spinal relay stations led to enhancement in neuronal rewiring and improved locomotor recovery (Van Steenbergen et al., 2023). The combination of these effects may elicit a stronger recovery of function than what is observed with intermediate doses of AAV-retro/Cre.

However, we also observed the occurrence of pathophysiologies with all doses used in this study, even low doses that did not elicit functional recovery. One possible explanation is that permanently deleting PTEN is stimulating growth too much, resulting in chaotic connections that lead to pathophysiologies. Other explanations are possible, however, including persistent activation of the mTOR signaling pathway, which can have profound consequences on the physiological properties of neurons, including causing seizures.

Sex differences in functional recovery of PTEN-deleted mice after SCI :

The findings of this study demonstrate major sex differences in the response to AAV-retro-mediated PTEN deletion following dorsal hemisection injury in mice. While no sex differences were observed in control mice, there was a substantial difference in the PTEN-deleted mice that received high and intermediate doses of AAV-retro. Specifically, the enhanced recovery of GSM values was primarily observed in male mice.

Several studies have investigated sex-related differences in functional recovery after SCI in animal models. For instance, studies in female C57Bl/6 mice with thoracic-level compression injuries have shown better locomotor scores and less necrosis and inflammatory cell infiltration than males (Farooque et al., 2006). Additionally, female mice showed significantly better recovery than their male counterparts after a moderate compression SCI (Hauben, Mizrahi, Agranov, & Schwartz, 2002). However, it is worth noting that these studies used compression injuries that can lead to a secondary inflammatory response, which is not observed in transaction models as used in our study. It is possible that the better outcomes observed in female mice in these studies may be attributed to the potential neuroprotective roles of estrogen-like hormones in the CNS (Yune et al., 2004).

Sex differences in neurologic recovery have been observed in clinical studies of humans, with females showing greater natural recovery than males. However, at the time of discharge from rehabilitation, males tend to perform better functionally for a given level and degree of neurologic injury (Sipski, Jackson, Gómez-Marín, Estores, & Stein, 2004). The underlying reasons for these sex differences are not yet fully understood and could involve a range of factors such as hormone levels, genetics, or other factors.

There are inherent differences in mouse strains that affects neuroinflammation, tissue preservation and spontaneous functional recovery following incomplete SCI (Basso et al., 2006; Kigerl, McGaughy, & Popovich, 2006; Lapointe, Ung, Bergeron,

Cote, & Guertin, 2006; Noristani, They, & Perrin, 2018). In Basso et al (Basso et al., 2006), they observed significantly higher recovery in C57BL/10, B10.PL and F1 than the C57BL/6 and BALB/c strains after moderate contusion SCI. in Noristani et al (Noristani et al., 2018), Swiss Webster mice showed reduced lesion volume than C57BL/6 mice after complete transection of the spinal cord. The differing behavioral response to SCI suggests inherent genetic factors significantly impact locomotor recovery and must be considered in studies with inbred or genetically engineered mouse strains. Further studies are needed to elucidate the mechanisms underlying these differences, which could ultimately lead to the development of more effective treatments for spinal cord injury in both males and females.

Conditions for development of pathophysiologies:

Late-onset pathophysiologies occurred only with AAV-retro based delivery of PTEN modifiers: AAV-retro/Cre or AAV-retro/siPTEN/GFP in conjunction with SCI (dorsal hemisection at C5). This finding eliminates several possible explanations. 1) Pathophysiologies are not due to PTEN-deletion in spinal circuitry only. This conclusion is supported by the fact that animals that received intraspinal AAV/Cre injections to delete PTEN in local spinal circuitry ($PTEN^{f/f}$;$Rosa^{tdT}$ + AAV/Cre + SCI) did not show scratching or hindlimb dystonia. 2) Pathophysiologies are not due to PTEN-deletion alone, since PTEN deleted animals without SCI ($PTEN^{f/f}$;$Rosa^{tdT}$ + AAV-retro/Cre - SCI) do not present pathophysiologies.

A possible explanation for late developing pathophysiologies is that these, are due to AAV-retro mediated PTEN deletion in cells projecting to the spinal cord, either from supraspinal structures or from the dorsal root ganglia (DRG) neurons projecting close to the site of AAV2-retro injection.

Transduction of DRG neurons:

In our previous studies involving intra-spinal AAV-retro/Cre injections, we observed retrograde transduction of large DRG neurons in dorsal root ganglia within 1-2 segments of the injection site at C5 (Metcalfe et al., 2022). Sensory representation of the neck and ears is at represented at C3, and it is possible that injections of AAV-retro/Cre at C5 transduce some DRG neurons at C3, therefore eliciting PTEN deletion in sensory neurons that innervate the neck and head.

The incessant scratching that develops suggests the possibility of chronic itch in the region of the neck and head. Rodents and other quadrupeds use their hindlimbs to scratch regions of their upper torso, neck, and head to relieve itch or sometimes in response to other sensations. In some pathological conditions, chronic itch is caused by peripheral and central sensitization of acute itch pathways, with sensitization of itch-related somatosensory pathways in DRG neurons (Akiyama & Carstens, 2013).

For example, in pathological chronic itch stimulation with a pruritogen elicits a stronger itch response than normal (chemical itch sensitization), and light touch can evoke an itch sensation on non-itchy skin areas (mechanical itch), resulting in persistent spontaneous itch (Ikoma, Steinhoff, Ständer, Yosipovitch, & Schmelz, 2006; LaMotte, Dong, & Ringkamp, 2014). Although both mechanical and chemical itch trigger site-directed scratching, these two forms are transmitted through different neural circuits in the spinal cord. Recent studies have found that interneurons in the dorsal spinal cord

expressing urocortin 3 (Ucn3+) (Pan et al., 2019) and neuropeptide Y receptor (Y1R+) (Acton et al., 2019) are essential for transmitting mechanical itch, and they receive inputs from Toll-like receptor 5-positive (TLR5+) Aß low-threshold mechanoreceptors (LTMR). Downstream projection circuits remain unknown. Itch-inducing molecules activate several membrane-bound receptors, such as histamine receptors (Rossbach et al., 2009). Recently, the Mas-related G-protein coupled receptor (Mrgpr) (Q. Liu et al., 2009) and the transient receptor potential (TRP) channel subfamily V member 1 (TRPV1) (Davidson & Giesler, 2010) have been reported to be required for processing the histamine-induced itch response (Davidson & Giesler, 2010). At the spinal level, secondary neurons for pruriceptors express gastrin-releasing peptide (GRP) in the spinal dorsal horn (Bautista, Wilson, & Hoon, 2014; S. Sun et al., 2017; Y. G. Sun & Chen, 2007). Information is then transmitted to spinal tertiary pruriceptors expressing the GRP receptor (GRPR). Finally, this information is transmitted to supraspinal regions via a relay of spinal ascending neurons (Mu et al., 2017). It remains to be seen whether any of these neuron types and pathways are transduced by intra-spinal injections of AAV-retro/Cre, and if they are, how consequences could differ in the context of SCI.

Our previous studies with cervical intraspinal injections of AAV-retro/Cre revealed that retrogradely transduced neurons in the DRG (identified by tdT labeling) are distinct from the population neurons that express calcitonin gene-related peptide (CGRP) that mediate nociception (meaning no-co-expression was detected) (Metcalfe et al., 2022). It remains to be seen whether other neuron types that express itch-related molecules are transduced with AAV-retro/Cre intraspinal injections.

Using a DRG transcriptomic atlas (Sharma et al., 2020) we identified cell populations in somatosensory neurons that express PTEN and determined that about one fifth of these also express the transient receptor potential (TRP) channel subfamily V member 1 (TRPV1). Although this suggests an intriguing hypothesis, further development of this idea requires: 1) confirmation of co-expression of protein by immunohistochemistry; 2) confirmation that neurons expressing TRPV1 are retrogradely transduced by AAV-retro injections into the spinal cord.

A recent study reported that PTEN deletion in non-peptidergic-isolectin B4 (IB4)-positive DRG neurons in adult mice, promotes ectopic TRPV1 and Mrgpr3a expression in DRGs, and upregulation of GRP in the spinal dorsal horn, leading to pathological itch (Hu et al., 2022). This study also reports that PTEN is not expressed in calcitonin gene-related peptide (CGRP)- positive neurons, that mediate nociception. In our published studies, we reported retrograde transduction of DRG neurons with cervical injections of AAV-retro/Cre in $PTEN^{f/f}$;$Rosa^{tdT}$ mice, showing that tdT is not expressed CGRP-positive neurons (Metcalfe et al., 2022). Therefore, it is likely that AAV-retro/Cre injected in the spinal cord could be taken up by sensory neuron axons that terminate in the spinal cord and transported back to the DRG. There, PTEN deletion presumably could induce the ectopic expression of TRPV1 and/or Mrgpr3a, as it has been observed in a DRG-specific PTEN knock-out model (Hu et al., 2022) consequently triggering itch and scratching. Previous studies have shown that PTEN deletion in DRG neurons has no effect in mechanical nociceptive responses (Hu et al., 2022). On the other hand, TRPV1 has been involved in the transmission of chemical itch (Shim et al., 2007), and if PTEN deletion in DRG neurons increase ectopic TRPV1 expression, chemical itch responses could be altered in PTEN-deleted mice.

Only animals that received AAV-retro/Cre to delete PTEN plus injury scratched. Injured animals with control AAVs and animals with PTEN deletion without injury, didn't scratch, unlike to what was reported in Hu et al, where researchers observed scratching with just PTEN deletion (Hu et al., 2022). Consequently, PTEN deletion plus SCI could be causing aberrant axonal extension and maladaptive synaptic connectivity, contributing to the development of itch syndromes. However future studies are needed to confirm these hypotheses.

Besides itch, most PTEN-deleted animals that received cervical SCI also exhibited persistent forward extension of the hindlimb (dystonia). Some dystonias are caused by loss of descending inputs (upper motor neurons lesions), which cause loss of inhibition of lower motor neurons, resulting in hyperactivity (Bakheit, Fheodoroff, & Molteni, 2011; Sheean & McGuire, 2009). Intraspinal spinal AAV-retro/Cre injections transduce CMNs (Metcalfe et al., 2022), and it is possible that this disrupts their physiological activity, in ways that produce functional phenotypes similar to "upper motoneuron lesions".

Additionally, hindlimb dystonias could be driven by new connections established in the spinal cord after PTEN deletion. It is plausible that what we are observing is an "itch phenotype", for example hindlimb dystonia is initiated independently of stimulation, possibly driven by the lumbar spinal cord. This could be caused by intraspinal connections being made after AAV-retro injections with spared fibers that project to lumbar segments. Future studies will be focused on determining these mechanisms.

Conclusion

Our results support the strong potential of AAV-retro for enhancing motor recovery after SCI. However, our findings also highlight the potential for late-developing pathophysiologies with this approach. Although initial improvements in forelimb gripping ability were observed in $PTEN^{f/f}$;$Rosa^{tdTomato}$ mice, some mice exhibited un-remitting scratching and hindlimb dystonia with persistent extension. Additional studies are necessary to elucidate the mechanisms underlying these late-developing functional abnormalities and to determine whether motor recovery can be achieved without these late-onset adverse effects.

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