Restoration of skilled locomotion by sprouting corticospinal axons induced by co-deletion of PTEN and SOCS3

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The limited rewiring of the corticospinal tract (CST) only partially compensates the lost functions after stroke, brain trauma and spinal cord injury. Therefore it is important to develop new therapies to enhance the compensatory circuitry mediated by spared CST axons. Here by using a unilateral pyramidotomy model, we find that deletion of cortical suppressor of cytokine signaling 3 (SOCS3), a negative regulator of cytokine-activated pathway, promotes sprouting of uninjured CST axons to the denervated spinal cord. A likely trigger of such sprouting is ciliary neurotrophic factor (CNTF) expressed in local spinal neurons. Such sprouting can be further enhanced by deletion of phosphatase and tensin homolog (PTEN), a mechanistic target of rapamycin (mTOR) negative regulator, resulting in significant recovery of skilled locomotion. Ablation of the corticospinal neurons with sprouting axons abolishes the improved behavioural performance. Furthermore, by optogenetics-based specific CST stimulation, we show a direct limb motor control by sprouting CST axons, providing direct evidence for the reformation of a functional circuit.
Spontaneous sprouting of spared axons may innervate denervated targets and contribute to naturally occurring functional recovery after injury. However this appears to be very limited in the adult central nervous system (CNS)\(^1\). A number of studies have attempted to explore the underlying mechanisms and several manipulations have also been developed to enhance such collateral sprouting\(^1\). For example, both neutralizing extrinsic inhibitory activities and task-specific rehabilitation training are able to promote functional recovery by increasing axonal sprouting in different injury models\(^5\)–\(^7\). However, how the sprouting response is initiated after injury remains poorly understood. On the other hand, observed motor function recovery after these treatments are still at most partial. Very often, only the trained motor tasks, but not other motor behaviours, are improved with such manipulations\(^6\)–\(^12\). At some cases, training one task can even have detrimental effects on another task\(^6\)–\(^12\). A possible explanation is that rehabilitation training might enhance the functional performance of task-specific circuits at the expense of reducing spared axons available for other behaviours. Therefore, available sprouting axons might be a likely limiting factor in mediating functional recovery.

To develop new strategies of boosting robust sprouting of corticospinal tract (CST) axons, we here attempted to explore how spared (intact) axons sense injury and respond with extensive sprouting. In this study, we show that ciliary neurotrophic factor (CNTF) and perhaps other cytokines, are induced in denervated neurons, might serve as molecular triggers of CST sprouting. By manipulating CNTF and other pathways, we achieved robust CST sprouting, forming new circuits almost as strong as intact ones and significant behavioural functional recovery. Furthermore, by both optogenetic and behavioural approaches, we demonstrated that stimulating CST axons could relay the cortical signals to the spinal cord, controlling the limb movement.

## Results

### SOCS3 deletion in cortical neurons promotes CST sprouting.

Our previous studies showed that deleting SOCS3 in retinal ganglion neurons promotes regeneration of injured optic nerve axons after injury\(^1\)\(^4\)\(^,\)\(^5\). We thus asked whether SOCS3 deletion could affect the sprouting of CST axons after unilateral pyramidalotomy (Fig. 1a). In this injury paradigm, CST axons from a unilateral cortical hemisphere are transected at the medullary pyramid above the pyramidal decussation. To monitor collateral sprouting from uninjured CST axons, biotinylated dextran amines (BDAs) are injected into the intact side of sensorimotor cortex at indicated post-injury time points, and transverse sections from different levels of the spinal cord are analysed after an additional 2 weeks (Fig. 1a). In control mice, most of the labelled axons are on the contralateral side of the spinal cord, with minimal labelling on the ipsilateral side\(^7\)\(^,\)\(^10\)\^-\(^18\). Thus, the number of labelled axons on the denervated side of the spinal cord originating from the intact CST in the contralateral side can be used to quantify the extent of CST compensatory sprouting.

To delete SOCS3 in cortical neurons, we first injected Cre-expressing adeno-associated virus (AAV2-Cre) into the intact side of the sensorimotor cortex (contralateral to the injury) of homozygous conditional SOCS3 mutants (SOCS3\(^\text{f/f}\)), ref. 19) on postnatal day 1 (P1). This approach has been previously shown to induce efficient Cre-dependent recombination in neurons throughout the sensorimotor cortex\(^18\). Deletion of SOCS3 at this stage did not appear to change the pattern of CST projections in the adult (Fig. 1b). A unilateral pyramidalotomy was performed at 8 weeks of age, and sprouting responses were analysed 6 weeks post injury. SOCS3 deletion significantly increased sprouting from the spared (intact, left) half of the CST into the denervated (right) side of the spinal cord (Fig. 1d,e), comparing to the controls (Fig. 1c,e). In the denervated (right) side of the spinal cord, the labelled axons could be seen in different regions of the grey matter, with the most abundant projections in the intermediate and dorsal spinal cord (Fig. 1d–g). The density of compensatory/collateral sprouting axons amounted to 25% of the uncrossing CST (Fig. 1e). These results suggest that the signalling pathway(s) regulated by SOCS3 regulate the capacity for compensatory sprouting of spared CST axons.

### Spinal CNTF upregulation after unilateral pyramidalotomy.

SOCS3 is a negative regulator of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which is often activated by cytokines such as CNTF\(^20\). Enhanced CST sprouting from intact cortical neurons after SOCS3 deletion suggests that axonal sprouting responses might be regulated by access to extrinsic cytokines which activate the SOCS3-regulated JAK/STAT pathway. Because the pyramidalotomy is performed on one side of the medullary pyramid, we examined the expression of CNTF in the cortex (where CST axons originate) and in the spinal cord (where CST axons terminate) in both intact and injured wild-type mice.

As shown in Supplementary Fig. 1, no immunoreactivity with anti-CNTF antibodies could be detected in the cortex after a pyramidalotomy on 3 days post injury. However, CNTF immunoreactivity is significantly increased in the spinal cord of the mice with pyramidalotomy, in contrast to the low level seen in the spinal cord of control mice (Fig. 2a). Interestingly, similar levels of the CNTF immunoreactivity were seen in both sides of the spinal cord after such unilateral lesion (Fig. 2a). Additionally, by quantitative PCR with reverse transcription to measure the CNTF mRNA expression in the cervical spinal cord (C4–C7) after pyramidalotomy, we found that compared with the sham control, the CNTF mRNA levels were significantly increased at 1-day post injury (Fig. 2b), suggesting that the CNTF upregulation is likely due to the increased transcription within the denervated spinal cord. Together, these results suggest that a unilateral CST lesion triggers the upregulation of CNTF in the spinal cord.

It is known that glial and inflammatory cells express cytokines such as CNTF after CNS damage such as spinal cord injury\(^21\)\^-\(^22\). Thus the observed CNTF induction might be due to a local inflammation as a result of Wallerian degeneration of lesioned CST axons after unilateral pyramidalotomy. To test this, we assessed the cell types that express CNTF. By immunohistochemistry, CNTF immunoreactivity was not detected in CD68 + microglia or GFAP + astrocytes (Fig. 2c). Instead, most of the CNTF signal was co-localized with NeuN-positive neurons (Fig. 2c), arguing against the possibility that CNTF induction is the direct consequence of glial responses after unilateral pyramidalotomy. Furthermore, the CNTF signal was seen in both sides of the spinal cord (Fig. 2a), in contrast to the mainly contralateral projections of CST axons. Therefore, we propose that CNTF induction is likely to occur mainly in denervated spinal neurons, both direct and indirect synaptic targets of CST axons.

### CNTF triggers CST sprouting in intact spinal cord.

To directly test whether CNTF per se is able to stimulate CST sprouting, we unilaterally injected AAV2-CNTF into the cervical spinal cord (C5–C7) of SOCS3\(^\text{f/f}\) mice. These mice were cortically injected with AAV1 that expresses tamoxifen-inducible CreERT2 (AAV1-CreERT2) or control placental alkaline phosphatase (PLAP) at neonatal age and administrated with tamoxifen (or oil as control) in adults. This strategy allows a timely controllable...
expression of Cre to delete the targeted SOCS3 gene. To validate this, we first injected AAV1-CreERT2 into the cortex of a reporter mouse at neonatal age. Tamoxifen administration resulted in Tomato reporter gene expression in cortical neurons, including pyramidal neurons in layer V (Supplementary Fig. 2). Intraspinal CNTF overexpression (Supplementary Fig. 3) significantly promoted the growth of midline-crossing corticospinal axons labelled by BDA, when compared with the control mice that were intraspinally injected with AAV-PLAP (Fig. 3a,b,d,e). SOCS3 deletion further enhanced the sprouting of CST axons, increasing the density of collateral sprouting axons to ~20% of the uncrossing CST (Fig. 3b–e). These results suggested that CNTF overexpression in the spinal cord is sufficient to trigger CST sprouting.

Figure 1 | SOCS3 deletion in neonatal cortical neurons increases CST sprouting after unilateral pyramiotomy. (a) Scheme of the experiments. AAVs were injected into the sensorimotor cortex of P1–2 SOCS3f/f mice, which then received a unilateral pyramiotomy (Py) or sham lesion at 8 weeks. BDA was injected into the contralateral sensorimotor cortex at 4 weeks post injury and the mice were terminated 2 weeks later. (b–d) Representative images (n = 3) of cervical 7 (C7) spinal cord transverse sections from SOCS3f/f mice with cortical AAV-Cre injection and a sham injury (b) or with cortical AAV-GFP injection and a unilateral pyramiotomy (c) or cortical AAV-Cre injection and a unilateral lesion (d). (e) Quantification of sprouting axon density index (contralateral/ipsilateral). *P < 0.01, ANOVA followed by Bonferroni’s post hoc test. (f) Scheme of quantifying crossing axons at different regions of the spinal cord (Mid: midline, Z1 or Z2: different lateral positions). (g) Quantification of crossing axons counted in different regions of spinal cord normalized against the numbers of labelled CST axons. *P < 0.01, ANOVA followed by Bonferroni’s post hoc test. For e,g five mice used in each group. Three sections at the C7 level were quantified per mouse. Scale bar, 500 μm.
CST sprouting induced by SOCS3 deletion. Despite our observation of CNTF induction after injury, basal expression of other cytokines such as LIF and interleukin-6 might also contribute to the sprouting phenotypes. Thus, we examined whether CST sprouting induced by SOCS3 deletion in cortical neurons is dependent on gp130, a required receptor component shared by CNTF and other cytokines. In our initial attempts, we injected AAV-Cre into the cortex of the SOCS3\(^{f/f}\)/gp130\(^{f/f}\) mice at neonatal age and performed a unilateral pyramidalotomy in the adult mice. In both gp130\(^{f/f}\) and gp130\(^{f/f}\)/SOCS3\(^{f/f}\) mice with neonatal cortical AAV-Cre injection, we found that few CST axons could be labelled by BDA tracing (Supplementary Fig. 4), which might be due to the fact that the JAK/STAT pathway is required for neuronal survival during development\(^{23,24}\).

We then injected AAV1-CreERT2 and AAV-channelrhodopsin (ChR)-YFP to the cortex of the SOCS3\(^{f/f}\), gp130\(^{f/f}\) or SOCS3\(^{f/f}\)/gp130\(^{f/f}\) mice at a neonatal age, and performed tamoxifen induction at 6 weeks, followed by unilateral pyramidalotomy at 8 weeks. In these experiments, ChR-YFP was utilized as a tracer. In another 3 months, we analysed CST axon sprouting from the intact cortex. As expected, robust CST sprouting was seen in the SOCS3-deleted mice (Fig. 4a,d,e). The observed sprouting was stronger than what seen at 6 weeks after injury (Fig. 1d), suggesting a continuous sprouting response as a result of SOCS3 deletion. However, co-deletion of gp130 and SOCS3 dramatically reduced sprouting (Fig. 4b,d,e). Comparable labelling efficiency of CST by ChR-YFP suggested that neuronal death is unlikely to contribute to different sprouting in these mouse lines (Fig. 4a–c). The residual sprouting seen in mice with gp130 deletion might reflect the contribution of other cytokine-independent mechanisms.

Enhanced CST sprouting induced by PTEN/SOCS3 deletion. The results above suggest that denervation-triggered neuronal CNTF expression might be an important extrinsic regulator of CST collateral sprouting. We next examined whether increasing intrinsic growth capacity in uninjured CST neurons by PTEN deletion could further increase CST sprouting elicited by SOCS3 deletion. Thus, by following the procedure shown in Fig. 1a, we injected AAV-Cre or AAV-GFP to the sensorimotor cortex of PTEN\(^{f/f}\)/SOCS3\(^{f/f}\) mice at P1, performed a unilateral pyramidalotomy at 8 weeks of age, and analysed the extent of CST sprouting at 6 weeks post injury.

In intact PTEN and SOCS3 double-deleted mice, the CST projection pattern remains unaltered (Fig. 5a). However, after unilateral pyramidalotomy, uninjured CST axons underwent dramatically increased sprouting responses (Fig. 5c–e), to an extent significantly stronger than that seen in the single SOCS3-deleted (Fig. 1c) or single PTEN-deleted mice\(^{18}\). In these double mutants, sprouting axons reached almost the entire CST territory in the denervated side of the spinal cord (Fig. 5c,d), although their overall distribution is similar to that seen in the single mutants (Fig. 5e versus Fig. 1g). Most, if not all, of these labelled CST axons are likely to be the midline-crossing axons from the contralateral side, because little staining was observed in the white matter of the denervated side of the spinal cord (Fig. 5c). These results suggest that co-deletion of PTEN and SOCS3 greatly enhances corticospinal neuron sprouting after a unilateral pyramidalotomy.

Improved skilled locomotion by PTEN/SOCS3 deletion. To assess skilled motor outcomes in these animals, we used a horizontal walking assay in which the animals were trained and tested to walk on a horizontal ladder with irregularly spaced rungs\(^{25,26}\). Previous studies suggested that CST-carried cortical
followed by Bonferroni’s post hoc test. For d,e three mice used in each group. Three sections at the C6–7 levels were quantified per mouse.

Figure 3 | CNTF induces CST sprouting in intact spinal cord. (a–c) Representative images (n = 3) from the SOCS3f/f mice with neonatal cortical injection of AAV1-CreERT2 and tamoxifen (a,c) or oil (b) i.p. injection at the age of 6 weeks, intraspinal injection of AAV2-PLAP (a) or AAV2-CNTF (b,c) in the adult (~20 weeks), and unilateral BDA injection at the somatosensory cortex 4 weeks post intraspinal injection. Scale bar, 500 μm. (d) Quantification of sprouting axon density index (contralateral/ipsilateral) in different groups. **P < 0.01, ANOVA followed by Bonferroni’s post hoc test. (e) Quantification of crossing axons counted in different regions of spinal cord normalized against the numbers of labelled CST axons. *P < 0.05, *P < 0.01 (P = 0.038), ANOVA followed by Bonferroni’s post hoc test. For d,e three mice used in each group. Three sections at the C6–7 levels were quantified per mouse.

commands are required for animals to prevent missteps during their walking on such irregularly spaced ladders25–28. In this double-blinded experiment, AAV-Cre or control AAV-GFP vectors were injected to the cortex of neonatal PTENf/f/SOCS3f/f mice (Fig. 6a). At the age of 8 weeks, the animals were allowed to be familiar with the ladder till they crossed the ladder with a forelimb error ~20%, before unilateral pyramidotomy (Fig. 6b,c).

After injury, the animals in both groups showed similar degrees of deficit, but the differences started to become statistically significant ~8 weeks post injury on the injured forelimbs (Fig. 6b), but not hindlimbs (Fig. 6d). Temporally delayed functional recovery might reflect the need of spontaneous synaptic formation and refinement of sprouting axons for their functional integration. Such improvement further increased over 8–16 weeks, but appeared to reach a plateau around the time of 16–20 weeks post injury (Fig. 6b). As a control, the error rates of the uninjured side of both forelimbs and hindlimbs were comparable in these two groups over the entire course of the experiments (Fig. 6c,e).

On the other hand, we scored the unskilled locomotion of the mice in different groups at 20 weeks post injury by Basso Mouse Scale (BMS)30 (Fig. 6f). No significant difference was seen between intact mice and those with unilateral pyramidotomy, consistent with the notion that CST axons have little influence on unskilled locomotor function31. We also failed to see significant difference between the PTENf/f/SOCS3f/f mice with cortical injection of AAV-GFP or AAV-Cre (Fig. 6f). Together, these results suggested that the PTEN and SOCS3 deletion in cortical neurons allow the CST-deprived forelimb to restore skilled locomotor function.

Limb movement induced by optogenetic CST stimulation. Because cortical neurons can control the spinal cord function by direct CST or indirect relay pathways, we next utilized an optogenetic approach to specifically stimulate CST axons to address whether the observed behavioural improvement results from the sprouting CST axons-mediated regained control of the denervated spinal cord.

In control mice, a unilateral cortical injection of AAV1 expressing the fusion protein of channelrhodopsin and YFP (AAV-ChR-YFP) led to the YFP expression in the neuronal soma as well as the CST axons in the spinal cord (Fig. 7a, Supplementary Fig. 5a–c). We first characterized the stimulation protocol in which the blue laser was used to stimulate the CST on the dorsal surface of the C5–7 spinal cord in these mice (Fig. 7b, Supplementary Fig. 6a). The amplitude of the movement increased with the longer stimulation duration, peaked at 200 ms and decreased at 2 s or longer (Supplementary Fig. 6a,b). In contrast, we failed to see movement of the contralateral forelimb or hindlimbs in these trials (Supplementary Fig. 6a). Furthermore, after cutting the dorsal column of the spinal cord at C2–3 level, similar light stimulation at the spinal cord rostral to the lesion level failed to trigger the movement of the ipsilateral forelimb (Supplementary Fig. 6a, the bottom panel), arguing against the possibility that the forelimb movement is due to the antidromic transmission of CST and activation of the other
indirect descending tracts. Together, our results suggest that the forelimb movement after photostimulation is likely due to orthodromic CST transmission, and we therefore selected 200 ms as the stimulation length for further experiments.

Next, we co-injected AAV-Chr-YFP and AAV-CreERT2 into the one side cortex of the neonatal PTENf/f/SOCS3f/f mice. The animals were treated with or without tamoxifen at the age of 6 weeks and a unilateral pyramidotomy was performed at the age of 8 weeks. The mice were sacrificed 12 weeks later. (d) Quantification of sprouting axon density index (contralateral/ipsilateral). *P<0.01, ANOVA followed by Bonferroni’s post hoc test. (e) Quantification of crossing axons counted in different regions of spinal cord normalized against the numbers of labelled CST axons. *P<0.01, ANOVA followed by Bonferroni’s post hoc test.

For d,e five mice used in each group. Three sections at the C7 level were quantified per mouse.

Abolishment of the improved skilled locomotion by selective ablation of corticospinal neurons with sprouted axons. Next, we performed a separate set of experiments with PTEN and SOCS3 co-deletion from the age of 6 weeks and unilateral
For different regions of spinal cord normalized against the numbers of labelled CST axons. *density index (contralateral/ipsilateral). *injection and a unilateral pyramidotomy (Py) (7 (C7) spinal cord transverse sections from SOCS3f/f/PTENf/f mice with cortical AAV-Cre injection and a sham injury (a) or cortical AAV-GFP injection and a Py (b) or cortical AAV-Cre injection and a Py (c). Scale bar, 500 μm. (d) Quantification of sprouting axon density index (contralateral/ipsilateral). *P < 0.01, ANOVA followed by Bonferroni’s post hoc correction. (e) Quantifications of crossing axons counted in different regions of spinal cord normalized against the numbers of labelled CST axons. *P < 0.01, ANOVA followed by Bonferroni’s post hoc correction. For d,e five mice were used in each group. Three sections at the C7 level were quantified per mouse.

Figure 5 | Significant CST sprouting in SOCS3 and PTEN co-deleted mice after unilateral pyramidotomy. (a–c) Representative images (n = 3) of cervical 7 (C7) spinal cord transverse sections from SOCS3f/f/PTENf/f mice with cortical AAV-Cre injection and a unilateral pyramidotomy (Py) (b) or cortical AAV-Cre injection and a Py (c). Scale bar, 500 μm. (d) Quantification of sprouting axon density index (contralateral/ipsilateral). *P < 0.01, ANOVA followed by Bonferroni’s post hoc correction. (e) Quantifications of crossing axons counted in different regions of spinal cord normalized against the numbers of labelled CST axons. *P < 0.01, ANOVA followed by Bonferroni’s post hoc correction. For d,e five mice were used in each group. Three sections at the C7 level were quantified per mouse.

pyramidotomy (Fig. 8a, the same as Fig. 7a) and the mice were assays for their performances in the irregularly spaced horizontal walking assay (Fig. 8). Similar to what seen in the mice with neonatal PTEN and SOCS3 co-deletion (Fig. 6), the injured forelimb showed significant reduction of error rate (Fig. 8b,c). This behavioural improvement occurred gradually and became statistically significant from 12 weeks post injury (Fig. 8b).

To assess the contribution of sprouting CST axons in the observed functional recovery, we adapted an intersectional targeting strategy to ablate the CST neurons with their axons sprouted into the denervated side of the spinal cord. To do this, we first optimized a procedure of stereotaxic injection into the unilateral spinal cord (Supplementary Fig. 9). With this, we injected the highly efficient retrograde gene transfer lentiviral vector (HiRet) carrying the flip-excision (FLEX)-human diphtheria toxin receptor (DTR) into the denervated side of the cervical spinal cord (C5–C7) at 20 weeks post injury. Because these animals were previously injected with AAV-CreERT2 in the ipsilateral cortex (Fig. 8d), only tamoxifen-induced CreERT2-expressing cortical neurons that sprouted midline-crossing axons would express DTR and become susceptible to diphtheria toxin-mediated ablation. We first reassessed animals’ performance on irregularly spaced horizontal ladder and found that the intraspinal virus injection had no overt effect on their skilled limb movement (Fig. 8e). Two weeks after diphtheria toxin administration, the improved performance of denervated forelimbs significantly declined (Fig. 8e), along with a significant reduction of the collaterally sprouted axons in the denervated side (Supplementary Fig. 10). The performance of the uninjured forelimbs was also affected to some extent (Fig. 8f), likely because the ablation of these cortical neurons impacted not only the sprouting axons in the denervated side but also the physiological projections in the intact side of the spinal cord (Supplementary Fig. 10). In contrast, no significant effects were observed in control animals (without tamoxifen-induced Cre expression; Fig. 8d–f). These results suggested that the sprouted axons from intact corticospinal neurons are required for the recovered performance of the precise walking task. Together, our results support the notion that sprouting CST axons induced by PTEN and SOCS3 co-deletion are able to mediate the functional recovery of injured forelimbs.

Discussion

Collateral sprouting of spared axons has been proposed as a key mechanism for spontaneous functional recovery after CNS injuries. However, its underlying mechanisms remain elusive and its precise contribution to functional restoration has not been definitively addressed. While removing axon growth-inhibitory molecules has been shown to promote CST sprouting,14–16, manipulation of intrinsic regenerative ability has emerged as a powerful strategy of enhancing axon regrowth in the adult CNS14,15,18. However, in light of recent finding about neuron type-specific enhancement of axon regeneration by PTEN deletion35, it is unknown whether SOCS3-dependent STAT3 pathway is able to regulate CST regrowth. In addition, since manipulating these pathways in adult neurons might alter their cellular and molecular properties, whether these affected neurons could still form functional neuronal connections remain

NATURE COMMUNICATIONS | DOI: 10.1038/ncomms9074 | www.nature.com/naturecommunications

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unknown. In this study, by using a unilateral pyramidalotomy model, we identified CNTF derived from denervated neurons as an important molecular trigger for CST sprouting. Enhancing this CNTF-dependent STAT3 pathway and co-activating mTOR activity, by co-deletion of SOCS3 and PTEN genes in either neonatal or adult cortical neurons, resulted in robust CST sprouting with an accompanying functional recovery of forelimb skilled movements. Such functional recovery was reversed by selectively ablating corticospinal neurons that collaterally sprouted midline-crossing axons in the spinal cord. By using optogenetics-assisted methods, we were able to achieve specific stimulation of CST axons and demonstrate direct effects on limb movements, suggesting direct control of forelimb movement by sprouting axons.

Previous studies have suggested neurotrophic factors, such as brain-derived neurotrophic factor (BDNF)\(^36\), and axon guidance molecules such as Slit and Ephrins\(^37\), as sprouting inducers. Our results here implicate CNTF and perhaps other cytokines as important endogenous triggers for CST sprouting after unilateral corticospinal lesion. gp130 Deletion abolishes most of observed sprouting growth, suggesting that cytokines play a key role in this process. CNTF itself is sufficient to trigger CST sprouting even in the intact mice. In this regard, cytokine-dependent JAK/STAT pathway has been shown to be critical for regenerative growth, but not developmental growth, in cultured sensory neurons\(^38\). Different from reported expression of cytokines in glial and inflammatory cells\(^21,22\), our results suggest that local spinal neurons might respond to denervation by upregulating CNTF expression, which subsequently stimulate axonal sprouting and possibly functional compensation. Such cytokine upregulation by direct and indirect denervated neurons might suggest a potential molecular mechanism for widely reported post-injury structural and functional reorganizations in the CNS, such as motor cortex reorganization seen after forelimb amputation\(^39,40\).

Furthermore, our results provide direct evidence for the notion that sprouting axons, as a result of manipulating these pathways, are able to form new functional circuits, mediating skilled locomotion functional recovery. In these experiments, the

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**Figure 6** Significant recovery of skilled locomotion in mice with neonatal SOCS3 and PTEN co-deletion after unilateral pyramidalotomy.

(a) Experimental paradigm: mice were injected with AAV-GFP or AAV-Cre at neonatal stage and subjected to unilateral pyramidalotomy in adults. (b–e) Performance on irregularly horizontal ladder of ipsilateral (b,d) and contralateral (c,e) forelimbs (b,c) and hindlimbs (d,e) to the lesion, respectively. P values: repeated-measures ANOVA, *P* < 0.05 (P = 0.039, 0.019, 0.005, 0.015 for 8, 12, 16 and 20 week respectively), Bonferroni’s post hoc correction (n = 11 and 12 for AAV-GFP and AAV-Cre injected group, respectively). (f) BMS scores of intact mice and the PTEN\(^{-}\)/SOC3\(^{-}\) mice with AAV-GFP or AAV-Cre injection at 20 weeks after Py. P = 0.25, one-way ANOVA (n = 12, 11 and 12 for intact, PTEN\(^{-}\)/SOC3\(^{-}\) mice with AAV-GFP or AAV-Cre injection, respectively).

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(c) Contralateral forelimb

(d) Ipsilateral hindlimb

(e) Contralateral hindlimb

(f) BMS scores

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observed partial and delayed functional recovery might reflect inaccurate synaptic connections. In light of recent studies that rehabilitation could promote the functional refinement of sprouting\(^6,^8\)–\(^12\), future studies should be aimed at deciphering other accessory treatments that maximize the extent of functional restoration induced by manipulating SOCS3 and mTOR-dependent pathways.

**Methods**

**Mice and surgeries.** All experimental procedures were performed in compliance with animal protocols approved by the Institutional Animal Care and Use Committee at Children’s Hospital, Boston. For cortical AAV injection, neonatal PTEN\(^{fl/fl}\) , SOCS3\(^{fl/fl}\) or PTEN\(^{fl/fl}\)/SOCS3\(^{fl/fl}\) mice (both sexes, P1) were anaesthetized and injected with 2 \(\mu\)l of either 10\(^12\) GC/ml AAV-Cre or AAV-GFP into four sites of the sensorimotor cortex contralateral to the pyramidotomy side using a nanolitre injector attached to a fine glass pipette. Mice were then placed on a warming...
respectively. diphtheria toxin was administrated (i.p.). For pyramidotomy, mice (both sexes, 8 weeks) were anaesthetized with ketamine/xylazine. The procedure is similar to that described previously18. Briefly, an incision was made at the one side of the trachea. Blunt dissection was performed to expose the skull base and a craniotomy allowed for access to the medullary pyramids. The pyramid was cut with a fine scalpel medially up to the basilar artery. Pyramidotomy (2) Tamoxifen/oil i.p. administration

For pyramidotomy, mice (both sexes, 8 weeks) were anaesthetized with ketamine/xylazine. A minimally invasive laminectomy was made at the cervical level C5–C7. Virus was loaded into a pulled glass capillary attached to the nanolitre. The nanolitre was then mounted to the stereotaxic frame and the glass capillary was inserted into the cervical spinal cord. Virus was injected at 3 points (100 nl per point) through ventral to dorsal spinal cord per injection tract. Three injection tracts (from middle to lateral) were made on individual side of the spinal cord. For unilateral spinal cord injection (both sexes, 12 weeks), CTB was co-injected with virus to monitor the injection locations.

pad and returned to their mothers after regaining normal colour and activity. For pyramidotomy, mice (both sexes, 8 weeks) were anaesthetized with ketamine/xylazine. The procedure is similar to that described previously18. Briefly, an incision was made at the one side of the trachea. Blunt dissection was performed to expose the skull base and a craniotomy allowed for access to the medullary pyramids. The pyramid was cut with a fine scalpel medially up to the basilar artery. BDA tracing was performed 4 weeks later (see below). For cervical spinal injection of AAVs expressing PLAP/CNTF (both sexes, 12–14 weeks) or HiRet-flex-DTR (both sexes, 30–32 weeks), we followed the procedure described elsewhere8. Briefly, mice were anaesthetized with ketamine/xylazine. A minimally invasive laminectomy was made at the cervical level C5–C7. Virus was loaded into a pulled glass capillary attached to the nanolitre. The nanolitre was then mounted to the stereotaxic frame and the glass capillary was inserted into the cervical spinal cord. Virus was injected at 3 points (100 nl per point) through ventral to dorsal spinal cord per injection tract. Three injection tracts (from middle to lateral) were made on individual side of the spinal cord. For unilateral spinal cord injection (both sexes, 12 weeks), CTB was co-injected with virus to monitor the injection locations.

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BDA tracing. To label CST axons by anterograde tracing, we injected a total of 2.0 μl of BDA (10%, Invitrogen, D-1956) into the right sensorimotor cortex at five sites (anterior–posterior coordinates from Bregma in mm: 1.0, 0.3, 0, −0.5, −1.0, all at 1.0 mm lateral and at a depth of 0.5 mm). Mice were kept for an additional 2 weeks before being sacrificed.

Histology and immunohistochemistry. Mice were given a lethal dose of anaesthesia and transcardially perfused with 4% paraformaldehyde. Brains and spinal cords were isolated and post-fixed in the same fixative overnight at 4 °C. Tissues were cryoprotected via increasing concentrations of sucrose. After embedding into OCT compound, the samples were snap frozen in dry ice. Serial sections (25 μm) were collected and stored at −20 °C until processed. Coronal sections of the lower medulla were cut for counting BDA-labelled CST fibres. For assessing the extent of CST sprouting, serial sections at C7 and other levels of the spinal cords were cut in the transverse plane. Immunostaining was performed following standard protocols. All antibodies were diluted in a solution consisting of

**Figure 8 | Ablation of cortical neurons with sprouting axons abolishes recovered skilled locomotion performance. (a) Experimental paradigm: mice received cortical injection of AAV-CreERT2 at neonatal stage, tamoxifen at the age of 6 weeks, and unilateral pyramidotomy at 8–10 weeks, the same as Fig. 6a. (b,c) Performance on irregularly horizontal ladder of ipsilateral (b) and contralateral (c) forelimbs, respectively. P values: repeated-measures ANOVA, *P<0.05 (P = 0.03), Bonferroni’s post hoc correction (n = 9 and 10 for without and with tamoxifen injected group, respectively). (d) Experimental paradigm: the same group of animals used in (a) were further analysed here. At 20 weeks after pyramidotomy, tamoxifen was administrated again and lentivirus (HiRet-FLEX-DTR) was intraspinally injected into the denervated side of the cervical spinal cord (C5–C7). After 2 weeks, diphtheria toxin was administrated (i.p.). (e,f) Performance of irregularly horizontal ladder of ipsilateral (e) and contralateral (f) forelimbs, respectively. **P<0.01, NS, not significant, Student’s t test (n = 6 and 4 for without and with tamoxifen injected group, respectively).
5% normal goat or donkey serum and 1% Triton X-100 in phosphate-buffered saline (PBS). We used goat antibody to CNTP1 (5 μg ml−1), RBD-Systems, AF-555 rabbit anti-CNTF (1:400, ref. 11) and rabbit anti-β-galactosidase antibody to GFP (1:200, Wako, Zf334) and mouse antibody to NeuN (1:100, Millipore, MAB377). Sections were incubated with primary antibodies overnight at 4°C and washed three times for 10 min with PBS. Secondary antibodies (biotin-conjugated donkey antibody to goat and Alexa 488-conjugated goat antibody to rabbit, rabbit and mouse) were then applied and incubated for 1 h at 20–25°C. For CNTPF staining, Elite Avidin Biotin Conjugate (ABC, Vector Lab) was applied, followed by TSA Cy3ane 3 (Perkin Elmer). To detect BDA-labelled fibres, BDA staining was performed by incubating the sections in PBS containing streptavidin–horserdsh peroxidase. The remaining staining procedure was performed according to the protocol provided by TSA Cy3anc 3 system (Perkin Elmer).

mRNA quantification. Adult mice with pyramidal injury or sham surgery were euthanized at 1 day or 3 days (n = 3 for each condition) post surgery. Tissue from the cerebral spinal cord (C4–C7) were dissected out immediately in cold PBS. After homogenization, total RNAs were extracted (Trizol, Invitrogen), reversely transcribed (Superscript III, Invitrogen) and subject to quantitative PCR (CFX Connect, BioRad) to measured CNTPF mRNA expression levels. mRNA quantification was used for loading control. The primers used for CNTF & Gapdh were: forward 5′-GGTACCTCCTACATCGCAATG-3′; reverse 5′-GCATCCATCAGGCTCTTTT; forward 5′-AACAGCAGAATCCACCTCTCCT; reverse 5′-CCTGGTGCCTGATTGCGGATTT, respectively.

Axon number and fluorescence intensity quantification. Digital images of C5–7 levels of the spinal cord transverse sections were collected using a Nikon fluorescence microscope. Densitometry measurement on each side of the grey matter was taken using Metamorph software, after being subthresholded to the background and normalized by area. The outcome measure of the sprouting density index was the ratio of contralateral and ipsilateral counts. To quantify the number of sprouting axons, we followed the methods used in our previous studies. Briefly, we drew a horizontal line through the central canal and across the lateral rim of the grey matter. Three vertical lines were then drawn to divide the horizontal line into three equal parts, starting from the central canal to the lateral rim. Only axons crossing the three lines were counted in each section. The results were presented after normalization with the number of counted CST axons at the medulla level. At least three sections were counted for each mouse. To compare the expression of chR2-YFP in the cortex layer V and in the CST axons in the spinal cord, we measured the averaged fluorescent intensities in transverse sections from the animals (three for each group) that were used for the optogenetic stimulation experiment. For layer V cortex, five sections that cover the chR2-YFP expressed areas were selected per animal. For CST axons in the spinal cord, three sections from the cerebral spinal cord C5–C7 were selected per animal. Image was used to calculate the mean fluorescent intensities.

Photostimulation of CST axons with kinematic analysis. A mixture of AAV1-CNTF-YFP and AAV1-CreER72 was injected into the motor and somatosensory cortex of mice at the age of P1. The mice were divided into two groups. The experimental group received tamoxifen injection 6 weeks after the surgical procedure, while the control group was injected with corn oil. The pyramydotomy was performed at 8 weeks. Twelve weeks later both groups of mice were anesthetized with ketamine and xylazine and the cervical segments (C3–C7) of the spinal cord were exposed. To ensure that the animals were not in the midline, we counted the number of close proximal movements within our previous studies. Briefly, we drew a horizontal line through the central canal and across the lateral rim of the grey matter. Three vertical lines were then drawn to divide the horizontal line into three equal parts, starting from the central canal to the lateral rim. Only axons crossing the three lines were counted in each section. The results were presented after normalization with the number of counted CST axons at the medulla level. At least three sections were counted for each mouse. To compare the expression of chR2-YFP in the cortex layer V and in the CST axons in the spinal cord, we measured the averaged fluorescent intensities in transverse sections from the animals (three for each group) that were used for the optogenetic stimulation experiment. For layer V cortex, five sections that cover the chR2-YFP expressed areas were selected per animal. For CST axons in the spinal cord, three sections from the cerebral spinal cord C5–C7 were selected per animal. Image was used to calculate the mean fluorescent intensities.

Statistical analysis. The normality and variance similarity were measured by STATA (version 12, College station, TX, USA) before we applied any parametric tests. Error bars in all figures represent mean ± s.e.m. For Figs 1, 3, 4 and 5 and Supplementary Fig. 10, five and three mice were used in each group, respectively. Three sections at the C6–7 levels were quantified per mouse. One-way ANOVA was performed and Bonferroni’s post hoc correction was applied for comparison between individual groups. For Figs 2 and 7, and Supplementary Figs 7 and 8, unpaired Student’s t-test (n = 5 and 5) was applied. For Figs 2 and 3 two samples were used for quantification. Each data was grouped in the homogenised tissue from four animals. For Fig. 6 (n = 11 and 12 for AAV-GFP and AAV-Cre injected group, respectively), Fig. 6c (n = 9 and 10 for without and with tamoxifen injection group, respectively) and Fig. 8e,f, unpaired Student’s t-test (n (n = 6) was applied. Post hoc power analysis, which achieved a power of 0.9167% (Fig. 6) and 88.23% (Fig. 8), respectively, was used to demonstrate a sufficient sample size.

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Selective ablation of corticospinal neurons with sprouted axons to the denervated side of the spinal cord. Mice received cortical injection of AAV-CreERT2 at neonatal stage, tamoxifen (or oil as control) at the age of 6 weeks, and unilateral pyramydotomy at 8–10 weeks. Twenty weeks after injury, tamoxifen (or oil) was injected (i.p.) again to ensure the activation of CreERT2. A laminectomy was performed at C5–7 level. The viruses (1.2 × 1012 copies per ml) generated by a HiRecI carrying the FLEX-DTRE were stereotaxically injected into the denervated side of the spinal cord of the tamoxifen or oil injected animals. After 2 weeks, animals were tested on the irregularly placed horizontal ladder to reassess their performance of the skilled limb movement. Diphertheria toxin was then administrated (100 μg kg−1, i.p.). Animals were tested on the horizontal ladder again 2 weeks after diphertheria toxin administration.
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Acknowledgements

We thank S. Li and C. Wang for excellent technical assistance, H. Nawabi and R. Zhao for advice, D. Ginty, F. Wang, and C. Woolf for critically reading the manuscript. This study was supported by grants from NINDS, Dr Miriam and Sheldon G. Adelson Medical Research Foundation and Hong Kong Spinal Cord Injury Fund Limited, Wongs For Life Massachusetts Walks Again (Z.H.), PVA and the William Randolph Hearst Fund (D.J.), Craig H. Neilsen Foundation (Y.L. and X.W.), and The Leonard and Isabelle Goldenson Fund (X.L.). IDDRC and viral cores supported by the grants NIH P30 HD018655 and P30 EY012196 were used for this study.

Author contributions

D.J., F.S. and Z.H. conceived and performed the experiments, D.J., F.S., X.W. and X.L. analysed data, D.J., Y.L., F.S. and Z.H. prepared the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Jin, D. et al. Restoration of skilled locomotion by sproting corticospinal axons induced by co-deletion of PTEN and SOCS3. Nat. Commun. 6:8074 doi: 10.1038/ncomms9074 (2015)