

● PERSPECTIVE

Delayed peripheral treatment with neurotrophin-3 improves sensorimotor recovery after central nervous system injury

Neurotrophin-3 (NT3) is a growth factor found in many body tissues including the heart, intestines, skin, nervous system and in skeletal muscles including muscle spindles (Murase et al., 1994). NT3 is required for the survival, correct connectivity and function of sensory (“proprioceptive”) afferents that innervate muscle spindles; these neurons express receptors for NT3 including tropomyosin receptor kinase C. These proprioceptive afferents are important for normal movement (Boyce and Mendell, 2014) and signals from muscle spindles are important for recovery of limb movement (e.g., after spinal cord lateral hemisection) (Takeoka et al., 2014). The level of NT3 declines in most tissues during postnatal development; its level is low in adult and elderly humans and other mammals (Murase et al., 1994). Elevation of NT3 has been shown to improve outcome in various animal models of neurological disease and injury. For example, many groups have shown that delivery of NT3 directly into the central nervous system promotes recovery after spinal cord injury but this often involved invasive routes or gene therapy (Boyce and Mendell, 2014; Petrosyan et al., 2015; Wang et al., 2018).

Our lab began evaluating NT3 in a rat model of focal stroke about 10 years ago. The work was inspired by experiments from Prof. David Shine’s lab (Zhou et al., 2003) involving a model of injury created by transection of one corticospinal tract in the medullary pyramids (pyramidotomy; **Figure 1A**). Viewed from the perspective of a spinal cord, unilateral pyramidotomy has much in common with unilateral cortical stroke: one corticospinal tract is spared entirely while the other is partially or completely lesioned, resulting in a (predominantly) contralateral deficit in sensorimotor function (**Figure 1B**). Prof. Shine’s team showed that a spared corticospinal tract could be induced to sprout across the midline of the spinal cord into the affected side when NT3 was expressed in lumbar motor neurons (Zhou et al., 2003). Specifically, 2 weeks after unilateral pyramidotomy, to enhance expression of NT3 by lumbar motor neurons on the affected side, they applied an adenoviral vector encoding the rat preproNT3 gene (which encodes a precursor to the mature form of NT3), to a freshly-cut end of the sciatic nerve. They injected an anterograde tracer into the less-affected motor cortex (10 days after pyramidotomy) and tissues were analyzed 3 weeks after treatment. They showed that corticospinal axons crossed into the affected lumbar hemicord in rats treated with NT3 (relative to a negative control transgene lacZ). However, because the sciatic nerve had been cut, the authors did not explore whether the corticospinal axon sprouting led to improvements in hindlimb movements.

When the first of Prof. Shine’s experiments on this matter was published,

Dr. Jeff Petruska and other colleagues were studying the transport of various adeno-associated viral vectors (AAVs) from hindlimb muscles to lumbar motor neurons and dorsal root ganglia (DRG). They showed that AAV serotype 1 underwent retrograde transport more effectively than AAV2 or AAV5 and that injection of AAV1 encoding human preproNT3 (AAV1-hNT3) into hind limb muscles could modify lumbar motor neuron properties and responses (Petruska et al., 2010).

We wondered whether it might be possible to induce sprouting of the spared corticospinal tract in a rat model of unilateral cortical stroke by injecting AAV1-hNT3 into forelimb muscles. We hypothesised that AAV would be transported in nerves retrogradely to cervical motor neurons (i.e., without the need for axotomy) with subsequent expression of human NT3 in the cord. We ran a blinded, block-randomized study to determine whether AAV1-hNT3 (relative to AAV1-GFP) improved sensorimotor outcome after delivery into affected biceps brachii and triceps brachii 24 hours after focal ischemic cortical stroke. After unblinding the treatment groups, it was apparent that NT3 modestly improved the accuracy of limb placement during walking on a horizontal ladder with irregularly spaced rungs. Moreover, anterograde tracing showed that the less-affected corticospinal tract sprouted across the midline and into the affected cervical hemicord (Duricki et al., 2016). However, to our surprise there was little evidence for expression of the transgenes in the cervical spinal cord: 1) The human NT3 transcript can be distinguished from the rat NT3 transcript by qRT-PCR but we found little evidence for human NT3 mRNA in the spinal cord or DRG (Duricki et al., 2016; Kathe et al., 2016); 2) We did find a few motor neurons and DRG neurons positive for green fluorescent protein but the number was considerably smaller than others had shown convincingly.

Not being sure what to make of this, we decided to repeat the experiments, except using elderly rats (because stroke principally affects the elderly) and with a slightly higher dose of AAV1 (7×10^{10} viral genomes rather than 3×10^{10} viral genomes). Injections of AAV1 encoding hNT3 into affected forelimb muscles 24 hours after stroke again induced sprouting of the spared corticospinal tracts into the affected hemicord and improved sensorimotor recovery in elderly rats (on the horizontal ladder and also using a test of forelimb sensory function) (Duricki et al., 2016). As in our previous experiment, there was underwhelming evidence of expression of the human NT3 or green fluorescent protein transgene in many neurons of the cervical spinal cord or DRG. We are still not sure why in our experiments retrograde transport of the AAV was poor. However, in both experiments, the level of NT3 in the injected muscles was very high and we found evidence for transport of NT3 protein to DRG (either by transport of the growth factor in axons or via the bloodstream). We concluded tentatively that synthesis of human NT3 in the spinal cord and DRG of rats might not be necessary for sensorimotor recovery after stroke, after all but that peripheral elevation of NT3 might suffice.

Accordingly, we set out to test a new hypothesis: that infusion of NT3 protein into affected forelimb muscles would be sufficient to promote sensorimotor recovery after unilateral cortical stroke. We induced unilateral cortical ischemia in adult rats and, 24 hours later, implanted a catheter into the triceps brachii muscle on the affected side (**Figure 1C**). A subcutaneously implanted osmotic minipump infused NT3 protein (gift of Genentech) or vehicle intra-

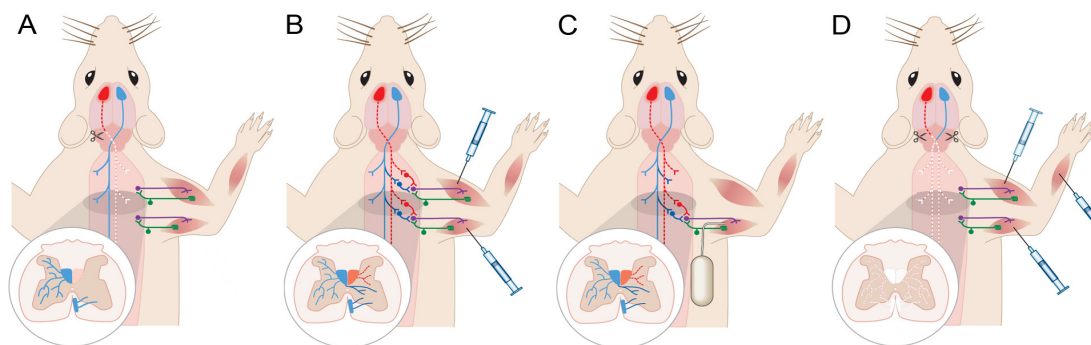


Figure 1 Twenty-four hour-delayed, peripheral treatment with neurotrophin-3 induces neuroplasticity and enhances sensorimotor recovery after corticospinal tract axotomy or cortical stroke in rats.

(A) Unilateral pyramidotomy involves transection of the corticospinal tract in the medullary pyramids (injury location denoted by scissors) and results in near-total loss of corticospinal axons on one side (red and white dots) but a preservation of the corticospinal tract from the other hemisphere (blue). (B) Unilateral ischemia in motor cortex induced by endothelin-1 (red) led to a partial (20%) reduction in corticospinal axons to the contralateral spinal cord (red dots). Injection of AAV1 encoding NT3 into biceps brachii and triceps brachii resulted in sprouting of the spared corticospinal tracts into the denervated hemicord and sensorimotor recovery in adult and elderly rats (Duricki et al., 2016). (C) After unilateral cortical ischemia, infusion of NT3 protein into triceps brachii resulted in sprouting of the spared corticospinal tracts into the denervated hemicord and sensorimotor recovery in adult rats (Duricki et al., 2019). (D) Bilateral pyramidotomy involves section of both corticospinal tracts in the medullary pyramids. Other sensorimotor pathways (not shown) are spared but little or no corticospinal inputs to the spinal cord remain. Injection of AAV1 encoding NT3 into multiple forelimb flexors reduced spasm, normalised H reflexes to a hand muscle and improved sensorimotor recovery (Kathe et al., 2016). Figures reproduced from Duricki et al. (2016, 2019) and Kathe et al. (2016) under the Creative Commons Attribution License. NT3: Neurotrophin-3; AAV: adeno-associated viral vector.

muscularly for 1 month. Anterograde tracing revealed that the intact corticospinal tracts sprouted into the affected cervical hemisection and neurophysiology showed that there was increased output from the intact corticospinal tracts to a nerve in the affected forelimb (Duricki et al., 2019). This proved that retrograde transport of a viral vector to DRG or spinal cord encoding NT3 was not necessary for modest recovery of sensorimotor function [although this may be additionally beneficial (Petrosyan et al., 2015; Wang et al., 2018)]. In all these studies, MRI showed that NT3 did not induce neuroprotection, consistent with the delayed time-to-treat (Duricki et al., 2016, 2019). Thus, this series of block-randomised and fully-blinded *in vivo* studies showed that peripheral elevation of NT3 can improve outcome after central nervous system injury.

How might peripheral infusion of NT3 protein cause corticospinal axonal sprouting? It is possible that NT3 acts directly in the central nervous system entering *via* the bloodstream (Duricki et al., 2019). However, another possibility is that sustained infusion of NT3 causes another molecule to be synthesised and secreted in the spinal cord, for example, from sensory afferents or motor neurons. We have discovered that DRG neurons modify their gene expression profile after bilateral pyramidotomy (Kathe and Moon, 2018) and that NT3 normalises some of these gene expression changes. For example, one of these genes, *Vgf*, codes for a secreted molecule: it would be interesting to determine whether this protein induces corticospinal axon sprouting. NT3 can be transported from muscle to sensory ganglia via the bloodstream and in nerves by retrograde transport (Duricki et al., 2016; Kathe et al., 2016) and our data show that it can modify gene expression in DRG. In other words, corticospinal tract neuroplasticity may be secondary to changes in DRG evoked by muscle-derived NT3, reminiscent of the remodelling of descending pathways after spinal cord hemisection evoked by a signal from muscle spindles (Takeoka et al., 2014).

How did peripheral infusion of NT3 induce sensorimotor recovery? One possibility is that sensorimotor recovery is a consequence of the spared (and sprouted) corticospinal tract obtaining some control over the spinal cord controlling the affected limb. However, an additional possibility is that NT3 normalises proprioceptive reflexes that control muscles on the affected side. After stroke, pyramidotomy or spinal cord injury, proprioceptive afferents below the injury can sprout and proprioceptive reflexes are modified (Kathe et al., 2016) which contributes to abnormal movement. We have shown that intramuscular injection of AAV1-hNT3 normalised a reflex to a paw muscle (involving low threshold afferents including proprioceptive afferents) after bilateral pyramidotomy in adult rats. This was accompanied with reduced spasm and improved walking on a horizontal ladder (Kathe et al., 2016). We propose that, primarily, NT3 normalizes various sensory reflexes and that, secondarily, this leads to plasticity in other pathways which, together, improve movement.

Together, our results are exciting and important for the following four reasons: 1) Various phase I and II clinical trials for other conditions show that subcutaneously injected NT3 is safe and well-tolerated in humans (Duricki et al., 2019): this paves the way for NT3 as a therapy for stroke. Various MHRA-, EMA- and FDA-approved medicines exist which involve subcutaneous infusion of other substances in wearable formats (e.g., miniature pumps for insulin) with thin catheters that are implanted without anaesthesia and could be used for NT3 infusion during stroke rehabilitation. Moreover, subcutaneous infusion of fluids (hypodermoclysis) is safe in stroke patients. With regard to gene therapy delivery of NT3, a phase I/IIA clinical trial using intramuscular injections of AAV1-NT3 for Charcot-Marie-Tooth type 1A neuropathy is currently recruiting subjects (NCT03520751) and Sarepta Therapeutics have acquired the rights to this program from Nationwide Children's Hospital. These studies will confirm whether long-term elevation of NT3 is safe and well tolerated. 2) We elected to initiate NT3 treatment 24 hours after stroke because this time point would be clinically feasible in the majority of new human stroke survivors. Our five pre-clinical studies show that this protocol enhances neurorestoration (as expected, this being too late for significant neuroprotection) (Duricki et al., 2016, 2019; Kathe et al., 2016). 3) Low risk of side effects: Unlike other neurotrophins, NT3 does not cause pain in humans (Duricki et al., 2019) consistent with the expression of its receptors in proprioceptive afferents from muscle and not in nociceptive neurons. There is some *in vitro* data indicating that NT3 can enhance survival or metastasis of existing cancers (Bouzas-Rodriguez et al., 2010) although there is no evidence that NT3 causes cancer *de novo*. As a precaution, one might exclude cancer patients from trials of NT3 for stroke.

In summary, NT3 improves outcome in rats after unilateral stroke or after bilateral pyramidotomy when given in a clinically-feasible time frame and by a clinically-feasible route. We will continue to optimize the timing, duration and method of delivery in order to determine the most promising strategy for clinical trials in people with neurological injury.

This work was funded by the Brain Research Trust, the Rosetrees Trust and the International Spinal Research Trust; a grant from the European Research Council under the European Union's Seventh Framework Programme (FP/2007-

2013)/ERC Grant Agreement n. 309731 and by a Research Councils UK Academic Fellowship and by the Medical Research Council (MRC) and the British Pharmacological Society (BPS)'s Integrative Pharmacology Fund; supported by the Dowager Countess Eleanor Peel Trust and a Capacity Building Award in Integrative Mammalian Biology funded by the Biotechnology and Biological Sciences Research Council, BPS, Higher Education Funding Council for England, Knowledge Transfer Partnerships, MRC and Scottish Funding Council.

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Received: January 11, 2019
Accepted: March 26, 2019

doi: 10.4103/1673-5374.257518

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Plagiarism check: Checked twice by iThenticate.

Peer review: Externally peer reviewed.

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C-Editors: Zhao M, Li JY; T-Editor: Jia Y