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Authors

Sellers, Drew
Bergen, Jamie
Johnson, Russell
et al.

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Targeted axonal import (TAxI) peptide delivers functional proteins into spinal cord motor neurons after peripheral administration

Drew L. Sellers[a,b], Jamie M. Bergen[c], Russell N. Johnson[c], Heidi Back[a,b], John M. Ravits[d], Philip J. Horner[a,b,e,1,2], and Suzie H. Pun[c,f,1,2]

[a]Department of Neurological Surgery, University of Washington, Seattle, WA 98104; [b]Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA 98195; [c]Department of Bioengineering, University of Washington, Seattle, WA 98195; [d]Department of Neurosciences, University of California, San Diego, CA 92093; [e]Center for Neuroregenerative Medicine, Houston Methodist Research Institute, Houston, TX 77030; and [f]Molecular Engineering and Sciences Institute, University of Washington, Seattle, WA 98195

A significant unmet need in treating neurodegenerative disease is effective methods for delivery of biologic drugs, such as peptides, proteins, or nucleic acids into the central nervous system (CNS). To date, there are no operative technologies for the delivery of macromolecular drugs to the CNS via peripheral administration routes. Using an in vivo phage-display screen, we identify a peptide, targeted axonal import (TAxI), that enriched recombinant bacteriophage accumulation and delivered protein cargo into spinal cord motor neurons after intramuscular injection. In animals with transected peripheral nerve roots, TAxI delivery into motor neurons after peripheral administration was inhibited, suggesting a retrograde axonal transport mechanism for delivery into the CNS. Notably, TAxI-Cre recombinase fusion proteins induced selective recombination and tdTomato-reporter expression in motor neurons after intramuscular injections. Furthermore, TAxI peptide was shown to label motor neurons in the human tissue. The demonstration of a nonviral-mediated delivery of functional proteins into the spinal cord establishes the clinical potential of this technology for minimally invasive administration of CNS-targeted therapeutics.

phage display | peripheral nerve | drug delivery | motor neuron

Neurodegenerative diseases that affect motor neurons, such as amyotrophic lateral sclerosis (ALS) or spinal muscular atrophy (SMA), have few treatment options due to the formidable challenges associated with drug delivery to the central nervous system (CNS) (1). The inability to cross the blood–brain barrier (BBB) and blood–spinal cord barrier (BSCB) after systemic delivery and insufficient penetration into the parenchyma from the cerebrospinal fluid (CSF) has hampered the use of promising biologic drugs such as recombinant neurotrophic factors and proteins (2, 3). Whereas there have been substantial recent advances in brain-targeted delivery of transcytosing peptides, antibodies, and exosomes to treat diseases like Alzheimer's disease (4–6), few therapeutic options are available for degenerative diseases that affect motor neurons in the spinal cord. Furthermore, many potential drugs are biologics such as proteins, genes, and small interfering RNAs that are not readily transported into the nervous system (7, 8). Therefore, a significant need exists for the development of innovative technologies to delivery biologics into the spinal cord.

Therapeutic molecules have been delivered to the spinal cord by systemic injection, direct injection, intrathecal transplantation of genetically modified cells secreting the molecules of interest, or remote delivery (9). Due to the BBB and BSCB, systemic delivery to the CNS by i.v. or intraarterial injection is limited to selected small molecule drugs. A comparison of adeno-associated virus (AAV) delivery by intraparenchymal versus intrathecal delivery revealed greater overall motor neuron transduction and distribution from intrathecal administration (10). More recently, intrathecal administration of AAV into the cerebral spinal fluid

(CSF) has been shown to be effective in gene transfer to the CNS in nonhuman primates (11, 12) and has also been used in clinical trials to deliver engineered cells acting as protein expression depots (13). Nonetheless, the clinical application of these methods is limited because of the invasive nature of delivery and because delivery into the CNS parenchyma after intra-CSF injection is low due to limited diffusion and penetration (14).

To develop minimally invasive technologies for biologics delivery into the spinal cord, we drew inspiration from viruses that transduce motor neurons. For decades, herpes simplex viruses (HSVs) have been known to enter the CNS by retrograde axonal transport, and HSV has been engineered for remote gene transfer in animal models of disease (15, 16). Engineered viruses and protein chimeras have also been used successfully in animal models to transfer therapeutic genes for conditions such as spinal cord injury, spinal muscular atrophy, chronic pain, and amyotrophic lateral sclerosis (17–21). However, virus-mediated gene therapy has been limited in clinical translation due to issues of immunogenicity, vector safety, and cost of production. Protein delivery methods have been likewise hindered by the high concentrations of protein required for cargo delivery and the lack of methods to dock and shuttle therapeutics into the CNS effectively (22). Still, the efficient retrograde transport of select viral vectors into the CNS led us to hypothesize that we could identify

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Significance

The delivery of drugs into the central nervous system is limited by the blood–brain barrier. Consequently, there exists an unmet need to develop technologies with the ability to deliver biologic drugs (such as peptides, proteins, or nucleic acids) into the brain or spinal cord via peripheral administration routes. In this paper, a screen of recombinant bacteriophage identified a peptide, targeted axonal import (TAxI), that is able to deliver protein cargo into spinal cord motor neurons after intramuscular injection by a retrograde transport mechanism. The demonstration of a nonviral-mediated delivery system demonstrates the clinical potential of this technology to develop powerful therapeutic tools to treat motor neuron diseases.

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a peptide that functions similarly and is able to interact with and be internalized by motor axons for transport into the CNS.

Armed with the precedent that HSV has evolved to deliver complex, biologically-active viral particles to the CNS from a peripheral skin lesion, we developed a strategy to screen bacteriophage display libraries in vivo to identify peptides that mediate M13 bacteriophage transport to the spinal cord. Here, we report the identification of a peptide that is trafficked into the spinal cord after intramuscular (IM) injection. We show that the peptide localizes with motor neurons after administration and can be used to carry proteins into the spinal cord via an intact motor axon that projects to the periphery. We further use this peptide, called targeted axonal import (TAxI) to deliver an active enzyme into spinal cord neurons after peripheral muscle injection in mice. This TAxI peptide shows potential for clinical relevance because it also binds to motor neurons in human spinal cord.

Results

Identification of a Phage Clone (TAxI Phage) That Enters the Spinal Cord via Motor Neurons. We developed a functional phage library selection strategy to identify recombinant bacteriophages that localize to the spinal cord after IM administration (Fig. S1A). To minimize contamination from phages circulating in the blood, which are cleared within 24 h (23, 24), animals were exsanguinated by transcardiac perfusion and spinal cord tissues were collected at 8 or 24 h postinjection. Three reiterative screens
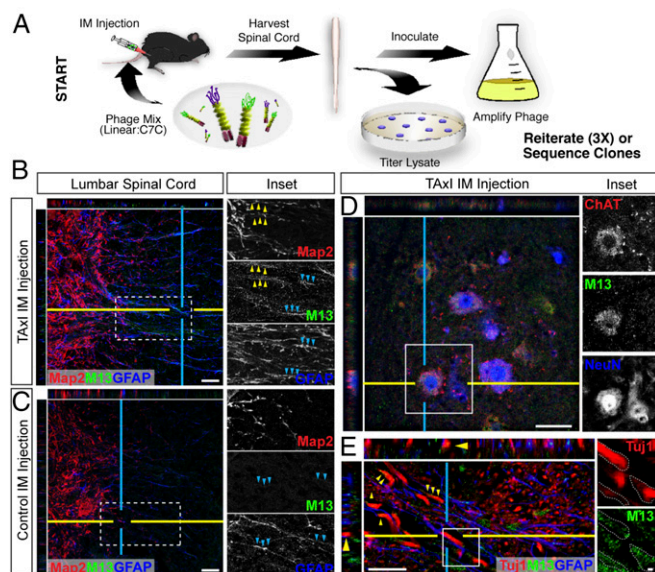

Fig. 1. TAxI phages concentrates within lumbar motor neurons after intramuscular injection. (A) Spinal cord tissue from mice was harvested 8, 24, and 48 h after intramuscular (IM) injection of M13 bacteriophage libraries. Recovered phage was then amplified and injected IM for three additional rounds of selection. (B) At 24 h postinjection, lumbar spinal cord immunostained for Map2 (red), bacteriophage M13 protein (green), and GFAP (blue) demonstrate distinct M13 staining in control versus TAxI-phage–injected animals. Animals injected with control phage had limited M13 staining within white matter GFAP-labeled processes. (C) Conversely, TAxI phage (M13; green) colocalized with Map2 and GFAP processes, particularly around the ventral root entry zone (Insets, yellow and cyan arrows, respectively). TAxI phage (M13) was localized along filamentous structures that extend through ventral but not dorsal root entry zones. (D) TAxI-phage (M13; green) immunofluorescence colocalized with NeuN (blue) in ChAT-labeled (red) motor neurons. (Scale bar, 10 μm.) (E) High-resolution confocal micrographs of spinal cord show TAxI phage (M13; green) colocalization along neuronal axons (Tuj1; red, highlighted by yellow arrows) and glial (GFAP; blue) processes at the ventral root zone (Insets; dashed lines) where axons project out to the periphery. (Scale bars, 10 μm and 1 μm, respectively.)

were conducted (Fig. 1A) resulting in five recurrent clones identified by sequencing. Of these clones, clone 2404 ("TAxI phage", Table S1) produced the highest titers in lumbar spinal cord tissues at 24 h postinjection (Fig. S1 B and C). Furthermore, TAxI phage showed preferential accumulation in lumbar–sacral versus cervical–thoracic spinal cord and thus correlative of the innervation of injected muscles.

To assess the cellular distribution of TAxI phage, spinal cord tissue was isolated 24 h after an IM injection of phages and stained by immunofluorescence. TAxI phage (M13 staining) localized to filamentous projections in the ventral spinal cord as shown by M13-immunofluorescence colocalization with GFAP+ and Map2+ processes (Fig. 1B, highlighted within Insets by cyan and yellow arrows, respectively). However, TAxI phage did not colocalize with glial somas, suggesting that the glial M13 represents a close association with Map2+-dendritic arbors extending from dorsal root ganglia (DRG) and neuronal processes within the ventral and dorsal root entry zones of the spinal cord. In contrast, confocal micrographs of tissue from mice injected with a control phage showed low levels of diffuse bacteriophage M13-coat protein in the ventral white matter of the lumbar spinal cord along GFAP+ astrocytic processes but not Map2+ projections (Fig. 1B, cyan arrows highlight staining within the Insets).

Next we sought to determine if TAxI phage colocalized with neuronal somas and, like HSV, could be entering the spinal cord via motor neurons. TAxI phage showed a distinct colocalization with neurons in the ventral horn that stained positive for choline acetyltransferase (ChAT; motor neuron marker) and neuronal nuclear antigen (NeuN; Fig. 1C). Furthermore, TAxI phage decorated Tuj1+ axons and M13 stain was colocalized along large-bore axons within the white matter of the ventral root entry zone (Fig. 1D, yellow arrows). In contrast, colocalization of M13 on NeuN+ or ChAT+ cells was never observed in control animals (Fig. S2A). Furthermore, we did not observe M13 staining on vascular endothelium in TAxI-phage–injected tissues (Fig. S2B). Combined, these data suggest that TAxI-phage uptake and entry into the spinal cord occurs by transiting the axolemma of somatic motor neurons that project to peripheral neuromuscular junctions.

TAxI Peptide Traffics Protein Cargo into Spinal Cord Motor Neurons. We next sought to determine whether the TAxI-phage peptide insert could have potential as a chemical tag to deliver biologics into the CNS. Cyclized TAxI peptide sequence (SACQSQSQMRCGGG) and a control peptide, TAxI^Q (GGCASGAQARCGGG), were synthesized, biotinylated at the amino termini, and complexed with a biotin-binding protein, NeutrAvidin (NA). Mice were given a unilateral IM injection of Fluorogold (FGold), a technique that is used to label alpha motor neurons by retrograde transport (25). Seven days after FGold administration, mice received bilateral injections of NA, NA:TAxI^Q, or NA:TAxI into the gastrocnemius muscles. NA:TAxI was observed to colocalize with FGold-labeled NeuN+ neurons in the ventral horn, in direct contrast to NA:TAxI^Q immunofluorescence, which did not colocalize with FGold within the lumbar spinal cord (Fig. 2A, Insets). To further verify the phenotype of TaxI-targeted cells we also looked for NA colocalization with calcitonin gene-related peptide (CGRP), which is found in a subset of motor neurons of the ventral horn and sensory neurons in the DRG (26). NA:TAxI colocalized with FGold in neurons that also stained for CGRP (Fig. 2B). These results confirm that the specific sequence of TAxI peptide but not control peptide can facilitate the traffic of protein cargo to motor neurons.

To estimate the efficiency of motor neuron targeting, we counted NA- and FGold-colabeled motor neurons within L3/L4 segments (n = 3) from mice injected with NA:TAxI and NA:TAxI^Q protein complexes. First, we counted the total number of NA-labeled cells in both groups. IM injection of FGold in mice has been previously shown to label ~50 spinal alpha motor neurons by retrograde transport (25). We observed only rare cellular
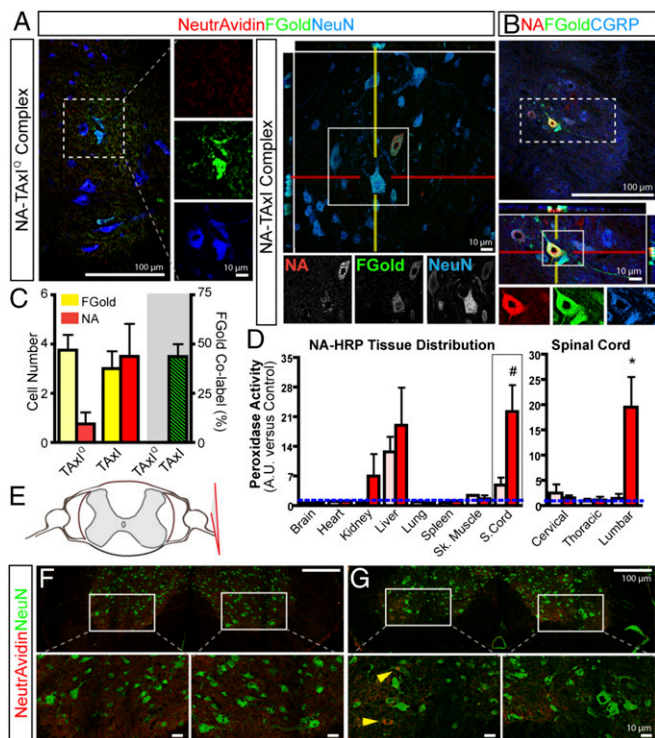
Fig. 2. Gastrocnemius-injected NA:TAxI requires an intact peripheral nerve to transit to spinal cord motor neurons. FGold was injected unilaterally into the gastrocnemius to retrogradely label muscle-specific motor neurons, followed 7 d later by bilateral injections of NeutrAvidin:peptide complexes. (*A*) Spinal cord tissue immunostained for NeutrAvidin (NA; red), FGold (FGold; green), and NeuN (blue) revealed NA:TAxI delivery into FGold-labeled neuronal somas. Conversely, NA:TAxI^Q was not observed within FGold$^+$ somas or spinal neurons. (*B*) NA:TAxI colocalizes with FGold within the cell body of NeuN$^+$ and CGRP$^+$ motor neurons. (*C*) Quantification of NA$^+$, FGold$^+$, and NA$^+$FGold$^+$ neurons within the lumbar spinal cord (L3/L4. Error bars = SEM). (*D*) The biodistribution of TAT or TAxI complexed with NA-HRP was quantified by peroxidase activity in vital organs after IM injection and normalized against naïve control tissues. (*E*) Animals received a unilateral L1–L4 rhizotomy to transect projection axons distal to the DRG to examine whether NA:TAxI delivery occurs via retrograde transport. (*F*) Rhizotimized animals given an ipsilateral NA:TAxI injection did not show neuronal NA accumulation in contralateral or ipsilateral spinal cord neurons (NeuN, green). (*G*) Conversely, animals given bilateral IM injections of NA:TAxI showed reduced but significant NA delivery (red) to NeuN$^+$ neurons (green) in the contralateral spinal cord (yellow arrows), which indicates that TAxI delivery occurs via retrograde transport along axons in the intact flank ($n = 3$).

colabeling in NA:TAxI^Q-injected animals. No cells could be identified with either FGold labeling or the common motor neuron characteristics of a large soma and prominent nucleolus. In contrast, animals injected with NA-TAxI complexes had greater than four times the total number of cells labeled (Fig. 2 *A* and *B*). Importantly, NA:TAxI-labeled motor neurons showed a high incidence of colabeling in motor neurons traced by FGold. Remarkably, TAxI delivered NA to ∼50% of all retrogradely labeled motor neurons within a targeted motor unit, showing uptake in three to four motor neurons per lumbar section, compared with none in NA:TAxI^Q control (Fig. 2*C*). Surprisingly, TAxI peptide was able to double the motor neuron targeting ability of a recently described lentiviral chimera, which was able to modify gene expression in one to two motor neurons in a lumbar section after injection into the gastrocnemius (27). Therefore, these results suggest that TAxI peptide is an efficient means to facilitate delivery of biologics into the CNS.

To quantify TAxI-mediated delivery into the spinal cord, a NeutrAvidin horseradish peroxidase (NA-HRP) conjugate was complexed with either biotinylated TAxI or transactivator of transcription (TAT) peptide. The TAT peptide is derived from the transactivator of transcription protein of HIV and was selected as a positive control because it has been used to facilitate intracellular delivery of proteins (28). Mice received bilateral injections of NA-HRP:TAxI or NA-HRP:TAT (10 μg) into the gastrocnemius, and major organs were harvested 24 h after injection to quantify HRP activity. TAxI increased NA-HRP distribution to the spinal cord by 4.5-fold over TAT (Fig. 2*D*, $^\#P$ = 0.058). More specifically, TAxI peptide increased HRP delivery to the lumbar aspects of the cord by >18-fold compared with TAT (*P < 0.02). Both NA-HRP:TAxI and NA-HRP:TAT showed similar biodistribution to other major organs, and, whereas each targeting peptide conjugate showed HRP accumulation in kidney and liver tissue, the off-target trend was not significantly different between TAT and TAxI peptides. Thus, these data collectively demonstrate the utility of TAxI peptide to transport protein cargo to spinal cord motor neurons by IM injection.

TAxI Peptide Is Mobilized to the Spinal Cord by Retrograde Axonal Transport. We next sought to confirm the mechanism of TAxI-mediated delivery to the spinal cord. Because viral pathogens have been hypothesized to be transported to the CNS via infection of circulating monocytes (29), we first confirmed that CD11b$^+$-purified CD115$^+$ murine monocytes do not bind to or internalize TAxI-protein complexes (Fig. S3). In addition, we did not observe NA:TaxI in brain sections. Therefore, NA-TAxI distribution to the CNS could not be attributed to monocyte-mediated delivery.

The association of NA:TAxI with neuronal processes suggested that the TAxI peptide transits to the CNS along the axons of motor neurons. To test this hypothesis, NA:TAxI was injected into the gastrocnemius of mice that had undergone a unilateral L1–L4 rhizotomy to transect the nerve just distal to the DRG. This lesion severs efferent and afferent input from the periphery (Fig. 2*E*). At 4 d postrhizotomy, NA:TAxI was injected either ipsilaterally or bilaterally in the gastrocnemius. Animals with a single ipsilateral injection did not have any NeutrAvidin staining in the spinal cord (Fig. 2*F*). However, animals given bilateral NA:TAxI injections showed NeutrAvidin accumulation in NeuN$^+$ neurons in the spinal cord contralateral to the nerve lesion (Fig. 2*G*, yellow arrows). The amount of NA:TAxI staining within the contralateral cord was reduced, however, compared with injections in intact animals. This is likely due to the bilateral gliotic response induced by the surgical rhizotomy. Together, these data demonstrate that intramuscular injection of TAxI conjugates require an intact motor neuron axon for efficient delivery of protein into the spinal cord. Because skeletal muscles contain sensory nerve endings as well as motor endplates, it is possible that TAxI conjugates might also accumulate in neurons of the DRG. In the present study, we did not examine whether NA:TAxI accumulated within the DRG, but the observed NA:TAxI colocalization with CGRP$^+$ sensory neurons (Fig. 2*D*) in the gray matter warrants future studies to examine effects within the sensory ganglia.

TAxI-Mediated Delivery of Active Cre-Recombinase to Spinal Cord Neurons After Peripheral Administration. A challenge in delivery of proteins with intracellular targets is avoiding cargo degradation after internalization. To test for TAxI-mediated delivery of an active enzyme to the spinal cord, we generated a series of Cre-recombinase chimeras: wild-type Cre (Cre), Cre fused to TAT peptide (TAT-Cre), and Cre fused to the TAxI peptide (TAxI-Cre). Fusion proteins bearing the HIV TAT protein have been shown previously to cross cell membranes (28), but cell-penetrating peptides have not been shown to transduce CNS neurons

in vivo. We chose mTmG mice as a test platform. Cells from the mTmG mouse switch color from membrane-bound tomato to membrane-bound GFP when active cre-recombinase is localized to the nucleus. Spinal cords from mTmG mice that received an IM injection of TAxI-Cre or control injection were imaged by multiphoton microscopy to detect Cre-mediated conversion at 7 d postinjection. The highest conversion of mTomato-to-mGFP expression in neuron-like cells (complex morphology with elongated processes) was observed in mice injected with TAxI-Cre chimeras. TAT-Cre–injected animals did not show mGFP conversion in neuronal-like cells; instead, TAT-Cre cells only displayed a simple round punctate morphology devoid of the neural characteristics observed in TAxI-Cre–injected animals (Fig. S4). To further validate the delivery of a functional protein to neurons, we injected recombinant Cre proteins into the gastrocnemius of a second reporter line, the Ai14 mouse. Ai14 mice do not have a constitutive reporter but delivery of biologically active Cre-recombinase results in cytoplasmic tdTomato expression after the excision of a floxed stop codon (30). In contrast to mice receiving IM injections of wild-type Cre protein, the lumbar spinal cord of mice receiving IM injections of TAxI-Cre contained many tdTomato$^+$ cells with complex morphologies and processes (Fig. 3 A and B). Importantly, tdTomato$^+$ cells also expressed NeuN and contained prominent nucleoli and a large

soma, characteristics typical of mature neurons within the gray matter (Fig. 3D).

Surprisingly, we did observe tdTomato-expressing cells in the brainstem as well as cervical and thoracic regions of the spinal cord after IM injection (Fig. 3C). Consequently, we sought to determine if widespread targeting of motor neurons occurred or whether motor neuron conversion was limited to the corresponding locations of muscle injection. Confocal imaging revealed that the majority of the tdTomato-labeled cells in the cervical and thoracic regions colocalized with the glial marker GFAP but not with the neural marker NeuN (Fig. 3 E and F). However, supportive of a retrograde neuronal targeting mechanism, tdTomato colocalized with NeuN and ChAT in the lumbar spinal cord (Fig. 3D). We also did not detect tdTomato colocalization in vascular endothelium (CD31, Fig. 3G), suggesting that vascular delivery to the CNS does not play a significant role. As a final control, TAxI-Cre was injected into spinal cord parenchyma directly where we observed numerous NeuN$^+$ and GFAP$^+$ cells that expressed tdTomato (Fig. S5). Thus, only IM-injected TAxI results in intracellular protein delivery to muscle-specific motor neurons. Future work will be needed to determine if cre-recombinase is communicated broadly to glia via exocytosis, exosomal release, or an alternative novel pathway (31).

TAxI Peptide Labels Neurons in Human Spinal Cord. The ability to deliver proteins into the spinal cord provides tremendous potential for the development of new CNS therapeutics. Consequently, we probed fresh frozen sections of human spinal cord to examine whether TAxI peptide can bind to human neural elements. Consistent with the mouse data, biotinylated-TAxI complexed with streptavidin-coated quantum dots (Qdots) colocalized with NeuN$^+$ and ChAT$^+$ motor neurons in the ventral horn (Fig. 4A). High-resolution confocal micrographs showed prominent TAxI-Qdot complexes in nuclei of ChAT$^+$/NeuN$^+$ neurons (Fig. 4B) and relatively few TAxI-Qdot–labeled neurons in the dorsal gray matter (Fig. 4C). Moreover, tissue preblocked with 10× excess TAxI peptide showed a significant reduction in cells labeled by TAxI-Qdot (Fig. 4D). Although these data do not explore the rigor of in vivo delivery, they support further development and testing of TAxI as a delivery tool for muscle-specific targeting of motor neurons or even broad targeting of glia (intrathecal delivery), in nonrodent mammals.

Discussion

This work presents several notable advances. First, in vivo library screening was successfully used to identify a peptide sequence with the ability to mediate transport into the CNS after a peripheral injection into the gastrocnemius muscle. Whereas the majority of in vivo phage screens have selected for binding to organ-prevalent endothelial targets (32), the studies presented here show a phage-derived peptide that is able to mediate protein delivery to spinal cord motor neurons by a retrograde transport mechanism. The phage-derived TAxI peptide showed an efficiency on par with viruses that infect the CNS via retrograde trasport showing delivery to 50% of neurons in a motor unit after a single injection into the gastrocnemius.

Second, after engineering a recombinant TAxI chimera, efficeint delivery of a biologicially active TAxI-Cre recombinase was demonstrated when reporter protein expression was induced in motor neurons that project to the gastrocnemius. Similar methods have tried to use tetanus toxin fragment C as a fusion protein with glial cell line-derived neutrotrophic factor (GDNF), insulin-like growth factor (IGF) and superoxide dismutase (SOD) (33–35) in an attempt to deliver therapeutic proteins into the spinal cord via routes of peripheral adminstration. However, in some of these examples, a significant biological effect was not observed, which could be attributed to poor intracellular trafficking (e.g., inefficient vesicular release) or subtherapeutic doses (34). In the
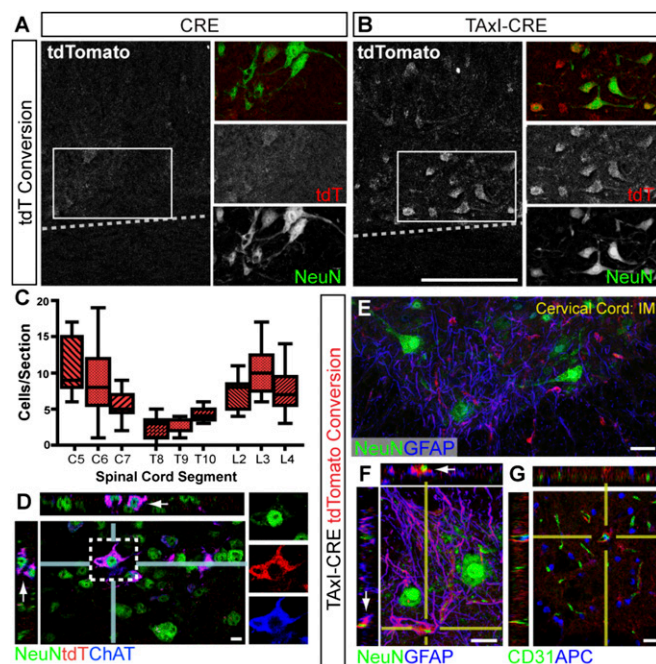

Fig. 3. TAxI delivers functional Cre to spinal motor neurons after IM injection. (A and B) Ai14 reporter mice injected with recombinant Cre proteins (Cre and TAxI-Cre; 25 μg) into the gastrocnemius muscle were examined for reporter-protein expression (tdTomato) within the lumbar gray matter (above the dotted line). (Scale bar, 50 μm.) (C) Recombinant Cre delivery in Ai14 mice was quantified by conversion to tdTomato$^+$ cells in cervical, thoracic, and lumbar spinal cord (n = 3, error bars = SEM). (D) Orthogonal z-planes from confocal micrographs show tdTomato (tdT, red) colocalization with NeuN$^+$ChAT$^+$ (labeled in green and red, respectively, with arrows) motor neurons from TAxI-Cre–injected animals; Insets show individual channels for NeuN, ChAT, and tdTomato. (E) Immunofluorescence staining of the cervical spinal cord reveals coexpression of tdTomato (red) with glial fibrillary acid protein (GFAP, blue) in the white matter. (F–G) Regions of the lumbar cord showed tdTomato (red) colocalized with GFAP (blue) in ventral root entry zones proximal to NeuN$^+$ neurons (green; arrows) but not oligodendrocytes (APC, blue) or vascular endothelium (CD31, green). (Scale bar, 10 μm.)
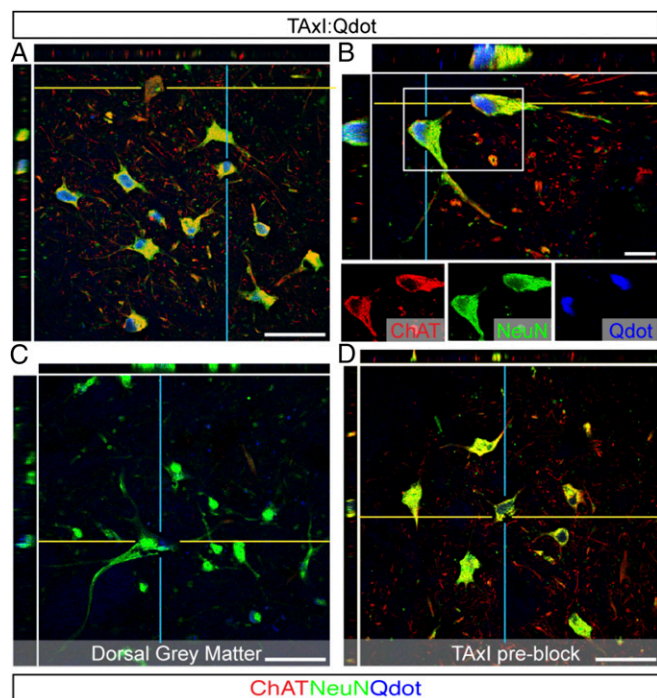
Fig. 4. TAxI streptavidin labels neurons in the adult human spinal cord. Steptavidin conjugated with quantum dots (Qdots) was complexed with biotinylated TAxI and incubated on fresh-frozen sections of human spinal cord tissue. (*A*) Confocal micrographs of ventral horn motor neurons stained by immunofluorescence for NeuN (green) and ChAT (red) showed TAxI-Qdots (blue) localization in the nucleus. High magnification images of the (*B*) ventral horn and (*C*) dorsal gray matter of human spinal cord tissue show TAxI-Qdots stain ventral motor neurons predominantly. (*D*) Spinal cord tissue preblocked with 10-fold excess of TAxI peptide before immunofluorescence for NeuN (green) and ChAT (red) competitively inhibits TAxI-Qdots (blue) labeling.

present study, TAxI peptide was able to deliver both biologically active Cre-recombinase and horseradish peroxidase into the spinal cord after a peripheral IM injection. In addition, delivery of Cre-recombinase was demonstrated in two unique strains of reporter mice in which the successful induction of reporter protein expression suggests effective vesicular escape and nuclear delivery of Cre to affect recombination in the nucleus. Consequently, these data suggest that TAxI chimeras might not suffer the same shortcomings observed with tetanus toxin fusion proteins to ensure affective delivery of protein therapeutics to spinal motor neurons (Fig. 3). Furthermore, engineered TAxI peptides offer additional advantages of smaller size, compared with protein targeting ligands, which can result in improved tissue diffusion, and the possibility of synthetic rather than recombinant production. Peptides can also be easily incorporated in a variety of delivery vehicles and cargos. In addition to the fusion proteins described in this work, peptides can be readily conjugated to synthetic delivery systems and inserted in viral vectors to mediate delivery to spinal cord motor neurons.

Whereas Cre has been delivered in vivo by viral gene delivery using adenovirus, lentivirus, and adeno-associated virus (36–38), the data we describe herein is the first nonviral delivery paradigm to our knowledge to deliver a biologically active Cre in vivo. Whereas viral delivery can provide high levels of stable expression, TAxI-mediated delivery of proteins offers better control over dosing and would be better tolerated in repeat dosing regimens. Hence, TAxI-based peptide:protein chimeras offer a potential replacement of the viral methods used currently for protein delivery to the spinal cord. In turn, cell penetrating peptides such as TAT have been used to deliver recombinant Cre

in cultured cells or explants cultures (37, 39, 40). However, efficient TAT-mediated delivery of Cre requires conjugation to nuclear localization peptides and membrane destabilizing peptides to facilitate intracellular vesicular release and enhance natural energy-dependent nuclear transport mechanism of Cre (40, 41). In this work, we showed that TAxI peptide delivered wild-type Cre more efficiently to neurons in the CNS compared with a TAT-Cre construct after a peripheral injection (Fig. S4), which leads to the hypothesis that TAxI chimeras could mediate effective delivery of therapeutic proteins via a mechanism that preserves function after uptake into the spinal cord. In addition, the cre conversion observed in spinal cord glia after an intra-parenchymal injection, as well as the observed glial conversion in the cervical cord suggest that the TAxI peptide can be used for the transfer of protein from neurons to CNS glia after intramuscular injection. We hypothesize motor neuron to astrocyte shuttling could occur via systems much like the astrocyte-neuron lactate shuttle (ANLS), glutamate uptake, or amyloid scavenging systems (42). This capability may have important therapeutic implications for neurological disorders where neuron-to-glia communication and homeostasis are implicated, but further research is required (43).

Finally, our data suggest that the TAxI peptide is able to deliver protein-based cargos through nondegrading pathways that do not require passage across the blood–brain barrier. We demonstrate that effective protein delivery is sequence-specific because neither TAT peptide nor mutated TAxI sequences (i.e., TAxI^Q) facilitate substantial protein delivery to the spinal cord (Fig. 2 and Fig. S4). Furthermore, our experiments indicate that TaxI may not only target motor neuron delivery by intramuscular injection but also widespread neuronal targeting through intra-parenchymal injection. The direct delivery to spinal cord motor neurons is particularly unique and linked to the site of muscle injection. We show that the combination of TAxI peptide and IM delivery can be used to target select groups of motor neurons in the CNS by retrograde axonal transport through an intact nerve, because a L1–L4 rhizotomy ablates delivery to spinal cord neurons. In contrast, direct spinal cord injection resulted in efficient widespread delivery to both neuronal and glial cells throughout the cord; still, i.v. injections (i.v.) of TAxI-Cre failed to induce reporter protein expression (tdTomato) within the neuraxis (Fig. S5). Thus, intramuscular injection is key to restrict protein delivery to muscle-specific efferent neurons and provides tremendous therapeutic potential for targeting specific motor units in neurodegenerative disease or injury to facilitate regeneration. Furthermore, these studies suggest that other muscle groups could be used to deliver and/or target alternate CNS sites; however, further investigation is needed.

Perhaps the most exciting aspect of our data is the demonstration of targeted delivery of a biologically active protein to the CNS and the demonstrated binding to neurons in human spinal cord. Impressively, a single injection of TAxI-Cre into the gastrocnemius led to efficient transfer of functional protein to motor neurons in the spinal cord. Recently, Hirano et al. showed remarkably similar motor neuron targeting via a lentivirus engineered with a glycoprotein type B fusion protein (27). Whereas the lentiviral method was able to transduce one to two motor neurons in a lumbar section, TAxI peptide mediated NeutrAvidin delivery to 50% of motor units within a muscle group. Combined, these data support the potential utility of TaxI-mediated protein delivery to therapies to modify the function and/or regeneration of neurons affected by motor neuron diseases. Further optimization of TAxI peptide and methods for its delivery in human disease models could develop potentially powerful therapeutic tools for select or broad targeting and treatment of the degenerative or diseased nervous system.

Experimental Procedures

Animals. The Institutional Animal Care and Use Committee at the University of Washington authorized all animal work in accordance with animal use and regulations. C57bl/6,*B6;129S6-Gt(ROSA)26Sortm14(CAG-tdTomato) Hze/J* mice and *Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J* were purchased from The Jackson Laboratory. Because only naïve wild-type animals were used throughout the study, we did not use a method for randomization of animals chosen for an individual experiment. Animal number was chosen to ensure biological replicates.

In Vivo Phage Display Selection of CNS-Transported Phages. For library screening, a 50:50 mixture of linear and circularized bacteriophage libraries [Ph.D.-12 and C7C phage library (New England Biolabs, NEB), 1.5×10^{13} pfu/mL in 5% (wt/vol) glucose] was injected bilaterally into the gastrocnemius muscle of C57bl/6 mice (5 µl each side, $n = 2$ per group). Spinal cord and cardiac tissue were harvested after transcardiac perfusion with 20 mL saline at 8, 24, and 48 h. The phage titer was determined following standard protocol (NEB) and subsequent rounds of phage injection and tissue harvest were performed as described. For phage delivery of purified phage clones, 1×10^9 pfu/mL of phages were injected IM.

Intramuscular Injections and Rhizotomy Surgery. Mice were anesthetized by an i.p. injection of Avertin (500 mg/kg body weight). Hindlimb fur was shaved and the skin disinfected with alcohol and betadine swabs. A small opening in the skin, less than 1 mm in length was cut with microscissors and a 30-gauge needle connected to a Hamilton syringe inserted ~1.5 mm into the muscle. Unilateral injections of 3 µL of a 4% (wt/vol) FGold solution in sterile saline were made into each muscle at a rate of 1.5 µL/min ($n = 2$ per group). After injection, the needle was left in place for 1 min and then slowly withdrawn. In cryosectioned tissue, FGold was stained with a 1:500 dilution of a rabbit anti-FGold antibody (Millipore, AB153). To transect peripheral nerve input to the lumbar spinal cord, the spinal columns of animals, under aseptic conditions, were exposed from L3–L4 and a dorsal rhozitomy was performed by cutting the peripheral nerve root distal to the DRG. After completion, the muscle was sutured in layers and the skin closed with wound clips, and the animals were placed on heating pads to recover.

Statistical Analysis. A nonpaired Student's t test evaluated differences in phage distribution and streptavidin:peptide binding in vitro to evaluate significance versus controls. NeutrAvidin biodistribution was analyzed by ANOVA with a Dunnett's multiple comparison post hoc analysis. For all statistical analyses, testing was two-sided and significance was accepted at a P value of 0.05 and lower.

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