UNIVERSITIY OF CALIFORNIA SAN DIEGO

Identifying New Drug-Disease Connections for Spinal Cord Injury

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by

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The Thesis of Theresa Marie Riley is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

CNS	Central Nervous System
CST	Corticospinal Tract
GSEA	Gene Set Enrichment Analysis
NEP	Neutral Endopeptidase Inhibitor; Neprilysin
NPC	Neural Progenitor Cells
NSC	Neural Stem Cells
SCI	Spinal Cord Injury

DEDICATION

I dedicate this thesis to my family and friends, in recognition of their enduring love and support.

ABSTRACT OF THE THESIS

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Spinal cord injury (SCI) is a widespread and drastic condition that causes permanent paralysis below the level of the lesion, as well as a myriad of associated problems. In the past, efforts to promote regeneration have remained largely unsuccessful. Recent work from the Tuszynski lab reported that grafts of spinal cord neural progenitor cells (NPCs) or neural stem cells (NSCs) grafted into sites of SCI support extensive corticospinal tract (CST) regeneration into the lesion site [1; 2; 3]. This finding was significant because the CST is the most important motor system in humans and efforts to maximize regeneration of this system are key to

developing translational human therapies. The regenerating transcriptome of the CST was identified, showing neurons underdoing active regeneration display a sustained upregulation of gene expression over time [4].

We mapped our regenerating transcriptome signature onto the Broad Connectivity Map (CMAP). Our CST regeneration signature was queried against 1.3 million signatures, where a positive match showed a similar transcriptome profile to our CST signature. *In silico* analysis identified Thiorphan as a candidate drug that most closely resembles the transcriptome of our regenerating CST signature. *In vitro*, Thiorphan caused an increase in neurite outgrowth compared to control. When tested in a cervical contusion lesion, Thiorphan treatment alone showed an increase in functional recovery after SCI compared to lesion-only controls. When used in combination with NPC graft, no additive benefit was seen. In conclusion, there may be a new drug-disease connection between Thiorphan and spinal cord injury.

INTRODUCTION

In the United States and across the world, spinal cord injury (SCI) remains a drastic, incurable condition that causes a wide variety of associated problems. The National Spinal Cord Injury Statistical Center indicated that there are around 17,000 new cases of SCI per year in the United States alone (NSCISC, 2019). The rate at which acute SCI occurs has remained constant since the 1900s, but due to population increases, the number of cases has likewise increased [5]. There remains a pressing need to find an effective treatment for SCI.

Spinal Cord Injury

There are different types of SCI, each with various consequences on motor and sensory abilities. Tetraplegia results from a cervical level SCI, and causes impairment of motor function as high as the neck [6]. Paraplegia, on the other hand, results from thoracic level SCI that impairs the movement of the lower half of the body, including the legs [7]. An incomplete SCI refers to an injury where the patient still has some degree of movement and/or sensation in the affected limbs, whereas a complete injury results in no feeling or movement in the affected limbs [8]. The National Spinal Cord Injury Statistical Center indicated that incomplete tetraplegia is the most common level of injury, comprising nearly 50% of the population of SCI patients (NSCISC, 2019). Thus, there is an increased need to develop useful therapies for tetraplegia, as it occurs at a higher incidence rate, and it is more severe than paraplegia.

To develop treatments for SCI, studies must first be conducted in model organisms. SCI is often studied in rodents, particularly rats, as this model has great translational significance when compared to other possible models [9]. In particular, cervical level SCI has well-established models in rodents, which result in deficits in forelimb function that model the deficits

experienced by humans following cervical-level injury [10]. These deficits can be easily measured using behavioral tests such as the staircase test [11] and the catwalk test [12], which measure skilled forelimb function like grabbing and reaching, and weight support of the affected limbs, respectively. Measuring these parameters can provide valuable information on the impairment and recovery following cervical level SCI in the rodent model, indicating the efficacy of a potential treatment.

Therapies for Spinal Cord Injury

The main challenge in finding a treatment is that following SCI, central nervous system (CNS) neurons do not regenerate, which is why SCI results in permanent motor and sensory deficits [13]. Recent work from the Tuszynski lab has shown that when spinalized neural stem cell grafts are placed into the lesion cavity, remarkable regeneration of axons is observed, wherein they grow back into the lesion site [1; 2; 14].

There are many different intrinsic and extrinsic factors that impact whether CNS neurons are able to regenerate, including changes in neuron morphology following injury, immune response to neuron injury signaling, and synthesis of materials to rebuild the injured neurons [13]. Many of these factors have been experimentally manipulated to determine their effect on inducing a regenerative state in CNS neurons [1; 15; 16]. While these methods have successfully induced several CNS tracts to regenerate in rodent models, the corticospinal tract (CST) still remains resistant to regeneration, and yet it is one of the most important systems for voluntary motor control in humans [1].

Neural Progenitor Cell Grafts

Enhancing the regeneration of the CST should result in an improvement in motor function and neural recovery following SCI. To improve CST regeneration, several studies conducted in rodents and primates have experimented with implanting neural progenitor cell (NPC) grafts at the site of the spinal cord lesion to restore neuronal connectivity across the lesion site [1; 3; 17]. These NPC grafts consist of neural stem cells, which are selected to ensure they are homologous to the area of implantation, specifically that spinalized stem cells are grafted into spinal cord tissue [1]. Additionally, growth factors and scaffolding are included in the graft to ensure complete filling of the lesion site [1; 3].

When a lesion site has a NPC graft implanted, the neural stem cells create a supportive environment for injured host CST neurons to regenerate, allowing the injured axons to grow into the site of the spinal cord lesion and form synapses with grafted neurons [1]. These synapses demonstrated functional activity, meaning that they could convey signals across an area where signal transmission had previously been disrupted [1; 3; 18]. NPC grafts were demonstrated to significantly improve motor function in rodent models [1]. Further study in primates supported their clinical translatability to humans, displaying improved behavioral outcomes [17].

While the NPC grafts have been demonstrated to improve connectivity and some motor function in both primates and rodents, none of the subjects achieved complete motor function recovery [1; 3; 17]. We next aimed to identify the molecular mechanisms driving this growth state, in efforts to manipulate the CST towards greater regeneration and functional recovery. RNA sequencing was used to identify the translational profile of the regenerating CST over a time period from the pre-growth state, early regenerative growth state, and late regenerative growth state [4]. The regenerating transcriptome of corticospinal neurons showed during active

regeneration into an NPC graft, that these adult neurons regress towards an embryonic transcriptional state, activating classical embryonic pathways [4]. CST neurons also upregulated known modulators of growth such as PTEN, mTOR, GAP43, CREB, Fos and PKC, where this sustained pattern of gene expression was absent in non-regenerating CST neurons [4].

Once identified, this regenerating transcriptome could be used as a transcriptional signature to identify drugs or small molecules that exhibit a similar signature. Here we present a pipeline for the discovery of new drug-disease connections for spinal cord injury.

METHODS

Animals

A total of 110 6 to 8-week-old female Fischer F344 rats (150-200g) were used for these experiments. Animals were housed in groups of 2-3 per cage and kept on a standard 12:12 light-dark cycle. They had standard cage bedding and a cardboard roll for enrichment. Food restriction was implemented one day before staircase training. Animals were fed between half a pellet and one pellet per day of continuous food restriction while being trained, and they were given *ad libitum* food on the fifth day of behavior training each week. Animal weights were monitored to ensure no animals lost more than 15% of their body weight from food restriction. Animals were trained on behavior testing tasks for up to 4 weeks prior to surgery. The guidelines for laboratory animal care and safety according to the US National Institutes of Health were followed. The Institutional Animal Care and Use Committee of the Department of Veterans Affairs San Diego Healthcare System approved all animal surgeries. Animal surgeries were performed under anesthesia, using ketamine dosed at 2mL/kg, and continuous gaseous isoflurane administration.

Behavior Testing

Staircase Forelimb Reach

For 4 weeks, rats were acclimated to the staircase task [11]. Food-deprived rats underwent habituation for one week, where they were placed inside the Montoya staircase (Lafayette Instrument Company, Lafayette, Indiana) for two consecutive 15-minute periods and allowed to consume sugar pellets *ad libitum* from the 14 overfilled wells, with 7 wells per side. After one week of habituation, the wells were filled with only 2 sugar pellets each, and the animals were once again trained for two consecutive 15-minute periods. On the fifth and final day of training each week, the number of displaced and eaten pellets, as well as the level reached, was recorded

for each animal after only one 15-minute training session. After 4 weeks of training, animals could successfully reach at least the 5th level and consume at least 10 pellets with their right paw. The final scores recorded were used as the baseline. The following week, animals underwent SCI lesion surgery. One week after the lesion, animals underwent one 15-minute round of staircase testing. After grafts and drug pumps were implanted, animals underwent weekly testing on staircase until the end of behavior data collection.

CatWalk Gait Analysis

For one week immediately prior to the SCI lesion surgery, animals were acclimated to the CatWalk test [12]. Animals were trained to walk across the glass floor of the CatWalk XT apparatus (Noldus, Leesburg, Virginia) without pausing, and return to their home cage at the end of the walkway. Animals were initially acclimated by allowing them to walk across the floor alongside their cage-mates, until they grew used to the apparatus. On the fifth and final day of acclimation, when animals could consistently walk across the floor without pausing, 3 runs that met software-specific inclusion criteria were recorded for each animal. Quantitative data is measured for each run, including step patterns, coordination, and individual paw data like contact area, footprint intensity, and paw positions [12]. One week after the SCI lesion surgery, the animals were re-tested on the CatWalk apparatus. Due to the severity of the injury, it was not always possible to obtain 3 runs that matched inclusion criteria, whether due to animals' inability to cross the walkway in under 6 seconds, or stopping before the end of the walkway. For these animals, the best available runs were used for analysis. After the first post-lesion test, animals were consistently able to complete 3 compliant runs.

Spinal Cord Lesions

Animals underwent a bilateral contusion at the spinal cord cervical level 5. The spinal cord was exposed at the C5 level, and centered beneath a computer-controlled impactor. The impactor struck the dorsal portion of the spinal cord, creating the bilateral contusion injury and leaving the dura intact. Impact force scores and displacements were recorded for every animal. Animals were allowed to recover for two weeks following the injury, and underwent behavior testing one week after the lesion to obtain "post-lesion" scores.

Cell Engraftment

NPC cell grafts were implanted into the lesion site 2 weeks after the spinal cord lesion. Embryonic day 14 spinal cords were dissected from eGFP rats as previously described [1], and digested in 0.25% trypsin at 37 Celsius for 10 minutes. The cells were washed with 10% FBS in neurobasal medium 3 times and triturated in 5mL of neurobasal medium with B27. Cells were filtered through 70 μm mesh, then spun for 5 minutes at 200g. Cells were then resuspended in 2mL of neurobasal medium with B27, then kept on ice until implantation. Immediately before grafting, cells were once again spun at 200g for 3 minutes. The supernatant was aspirated, and cells were resuspended in fibrin matrix consisting of 25mG/mL of fibrinogen and 25 U/mL thrombin (Sigma F6755 and T5772). This graft mixture was injected into the intact spinal cord lesion site at C5.

Pump Implantation

At the same time of NPC graft implantation, animals underwent implantation of the subcutaneous pump and intracortical infusion device. A portion of the skull was removed, and

the left motor cortex was identified through use of stereotactic indicators. The brain infusion cannula (Azlet, Cupertino, California) was implanted, and the implant was adhered to the skull. The cannula was attached via vinyl tubing to the osmotic minipump, which was pre-filled with either 100mM Thiorphan in 50% DMSO solution, or 50% DMSO solution alone. The osmotic minipump was inserted subcutaneously in the animal's back, placed so as to not interfere with animal movement.

Cell Culture

Adult cortices were isolated from mice and transferred to a gentle MACS C tube (Miltenyi, 130-096-334) containing the combined enzyme mixes 1 and 2 prepared from the adult brain dissociation kit (Miltenyi, 130-107-677). Automated mechanical tissue dissociation debris and red blood cell removal was performed using the adult brain dissociation kit, in combination with the gentleMACS octo dissociator with heaters (Miltenyi, 130-096-427), according to the manufacturer's instructions. The cell suspension was cleared using MACS smart strainers (70mM) and subsequently proceeded to neuron isolation.

In order to purify neurons, non-neuronal cells were depleted using the Neuron isolation kit (Miltenyi, 130-098-752) in combination with the QuadroMACS separator and LS columns (Miltenyi, 130-042-401) according to the manufacturer's instructions. Eluded neurons were plated onto 10ug/ml laminin plates and cultured for 5 days in vitro. Cells were then fixed with 4% paraformaldehyde for 20 minutes at room temp, and then permeabilized and blocked simultaneously with 0.25% Triton X 100 and 5% FBS in 1XPBS.

Immunofluorescent staining and quantification

Fixed cultures were stained overnight at 4°C with mouse anti-bIII tubulin antibody G7121 (Promega) at 1:2000 dilution, chicken anti-MAP2 antibody CPCA-MAP2 (EnCor Biotechnology) at 1:5000 dilution. Secondary antibody labeling was performed with donkey anti-mouse IgG-Alexa 488 (Invitrogen) at 1:1000 dilution, and donkey anti-chicken IgG-Alexa 647 (Jackson Immuno) at 1:1000 dilution. Images of individual neurons were taken on Leica SP8 confocal microscope. Neurite outgrowth was measured using ImageJ NeuronJ plugin.

RESULTS

Enhancing the growth of the adult corticospinal tract is of utmost importance for human translation. Although NPC grafts support extensive growth of this tract after SCI, there still remains room for improvement. We explored alternative therapies to a) enhance the number of CST axons growing into the graft, and b) the distance of growth into the graft. My hypothesis is that by cataloguing transcriptional responses to chemical and genetic perturbations, we can augment CST regeneration into stem cell grafts after SCI. We used the regenerating corticospinal transcriptome profile to identify drugs or small molecules that show a similar pattern of gene expression.

Drug-Disease Connections

The Broad Connectivity Database compiles the RNA expression patterns that a given small molecule or drug causes in a treated cell line and indexes these entries so they can be easily searched [19]. Thus, previously overlooked connections can be identified between drugs and disease states based on the RNA expression patterns that they cause [19]. To investigate potential candidates that mimic our corticospinal regeneration state, we used the RNA sequencing signature generated during active CST regeneration into an NPC graft. This pattern of RNA regulation was used as a search term on the Broad Connectivity Database to find drugs that may induce the same RNA pattern of expression. Many potential candidate drugs were found, where from our top 100 drugs, only 15 drugs showed a consistently high enrichment score. Out of our top 15 drugs, only 12 had significant p values, suggesting they would most likely mimic our regenerating transcriptional state. Gene set enrichment analysis (GSEA) further showed a strong alignment with our top candidates (Fig 1a).

We next aimed to test our top candidate drugs *in vitro*. For this, we developed an adult cortical culture screen, where post-natal day 60 neurons were isolated and plated, and drugs were administered to these cultures for 5 days in vitro. This constitutes the best available medium-throughout screen for injured adult CST neurons, which have traditionally been exceptionally difficult to culture. We tested three compounds from our top 15 candidates, indicated by the labeled arrows (Fig 1a). We included a positive regulator of growth, Inosine, that has been previously shown to stimulate extensive axon growth in CST axons [20]. We also included a predicted negative regulator of growth, Adiphenine, that had a -0.975 enrichment score. This strongly negative enrichment score indicates a significantly dissimilar expression profile from our regenerating CST, where enrichment scores range between +1 and -1.

The compound with the highest enrichment score and GSEA alignment, Thiorphan, showed the most robust total neurite outgrowth with the longest neurite length in our in vitro screens (Fig 2b). Inosine similarly showed augmented neurite outgrowth and increased neurite length above control, where Milrinone with a 0.5 enrichment score showed no significant increase in neurite outgrowth (Fig 2b). Adiphenine, the predicted negative regulator for growth, attenuated neurite outgrowth with shorter neurite length compared to control (Fig 2b). Overall, our *in silico* analysis showed marked consistency to our *in vitro* screens, where previous vetted regulators of growth increased neurite outgrowth in our adult cortical neuron screens (Fig 2b).

We next tested our top candidate, Thiorphan in an in vivo model of SCI for its therapeutic efficacy.

Thiorphan

Thiorphan is an enkephalinase inhibitor, specifically inhibiting enzymes that degrade neuropeptides. Neuropeptides have been shown to play a role in memory and learning [21], neuroprotection, and survival. They have complex interactions with the cholinergic, glutamatergic, dopaminergic and GABA-ergic pathways. Neuropeptides are first presented in a pre-peptide form. To achieve maturity, pre-peptides are cleaved into active neuropeptides, where these peptides are rapidly degraded by proteolytic enzymes such as neprilysin.

Thiorphan is a neprilysin, or neutral endopeptidase (NEP) inhibitor, and is the active metabolite in the anti-diarrheal drug racecadotril used in human infants [22; 23]. Thiorphan does not cross the blood-brain barrier when administered orally [23], so intracerebral injection is required to cause an effect in the CNS. Thiorphan appeared to prevent neuron death in the brains of neonatal mice, as its administration into the grey matter of the cortex reduced the size of lesions [22]. This neuroprotective effect was established to be a result of its inhibition of NEP [22]. To our knowledge, the use of Thiorphan as a neuroprotective substance in the spinal cord has not yet been investigated [24].

In Vivo Testing of Thiorphan in Models of SCI

To identify if Thiorphan would remain bioactive for a period of 4 weeks, we sought to test its activity using a neprilysin assay. A cannula was implanted into the left motor cortex, 1mm deep with stereotaxic coordinates targeting the forelimb area. The pump containing the drug or diluent was implanted subcutaneously on the animal's back where no interference in motor activity could occur. We analyzed levels of neprilysin following Thiorphan administration

for 1 to 4 weeks. Neprilysin levels are inversely related to Thiorphan activity, so a neprilysin assay was used to infer relative Thiorphan activity (Fig. 2a).

Thiorphan activity varied widely across brain regions and timepoints. The left cortex displayed relatively high levels of Thiorphan activity across all three timepoints, consistent with the fact that the drug was administered directly to this region (Fig. 2a). As administration time increased, Thiorphan activity was detected more in adjacent brain areas, namely the right cortex and cerebellum (Fig. 2b). The right cortex, contralateral to the left cortex, generally displayed higher Thiorphan activity than the more distal cerebellum. Thiorphan activity appeared to reach a plateau in the left cortex by 4 weeks (Fig. 2a). Notably, across all timepoints and brain regions, the amount of neprilysin was never reduced more than 50 percent compared to control (Fig. 2a). To confirm the safety of intracerebral infusion of Thiorphan as required by our experimental paradigm, cortical tissue was Nissl stained around the cannula implant region to detect any changes in ventricle enlargement or neuron cell loss caused by the cannula implant. No adverse effects were seen in cortical tissue comparing DMSO diluent control, 100mM Thiorphan delivery, or 200 mM Thiorphan delivery (Fig 2b). This suggests the delivery method chosen would prove to deliver Thiorphan into the target area, while being tolerated by the animals.

Behavioral Outcomes Following SCI and Treatment

To determine if our candidate drug would have any effect on motor recovery, subjects were pre-trained in forelimb reach task using the Montoya staircase and catwalk apparatus for four weeks. Subjects received a cervical level 5 contusion, a clinically relevant model. Two weeks later, they either received a graft implant into the lesion cavity or Thiorphan alone, both treatments, or neither. Experimental groups included lesion alone (N=9), lesion and Thiorphan

(N=9), graft alone (N=10), or a combination of graft and Thiorphan (N=10). Animals were tested once a week to assess behavioral improvements over time.

In the Montoya staircase task, animals had an average baseline score of 9 pellets eaten on the right side (Fig. 3A). Immediately after the lesion, animals dropped to an average of approximately 1 pellet eaten (Fig. 3A). One week after treatment administration the groups diverged, with lesion-only animals continuing to eat an average of 1 pellet on the right side, and treated animals rising to roughly 3 pellets eaten on the right side (Fig. 3A). No significant difference was seen between any of the treated groups (Fig. 3A). In following weeks, the treated groups continued to improve compared to the lesion only group, reaching an average of 5 pellets eaten compared to only 2 pellets (Fig. 3A).

Additionally, accuracy scores were calculated by dividing the number of eaten pellets by the number of pellets eaten plus the number of pellets displaced. Accuracy scores dropped from roughly 90% prior to the lesion to around 20% after the lesion (Fig. 3A). Again, treated groups immediately diverged from untreated groups one week after lesion, with improvements to roughly 40% accuracy, where the lesion-only group remained at roughly 10% accuracy (Fig. 3A). Again, none of the treated groups were significantly different from one another in terms of accuracy (Fig. 3A).

Parameters analyzed from the Catwalk apparatus included contact area of the right forepaw, right forelimb to left hindlimb coordination, and number of steps. After lesion, the contact area of the right forepaw significantly decreased for all animals, indicating less weight support on the forelimbs (Fig. 3B). Improvements in post-treatment scores overlapped for all groups, such that there was no significant difference between any of the groups for contact area (Fig. 3B). On the coupling measure, animals' coupling scores increased after lesion, indicating

less coordination (Fig. 3B). Recovery of coordination with treatment was not significantly different across groups (Fig. 3B). Number of steps increased significantly for all animals following the lesion, and decreased as treatment time went on (Fig. 3B). The difference in number of steps was not significantly different between any of the groups (Fig. 3B).

DISCUSSION

To investigate the effects of intracerebral infusion of the neuroprotective Thiorphan, an NEP inhibitor, and elucidate its pattern of activity in the rat brain, we continuously administered Thiorphan through direct brain infusion for up to 4 weeks, and demonstrated its activity increased over time. Our experiments showed Thiorphan decreased neprilysin levels by up to 50% in three selected areas of the rat brain compared to a control. Additionally, our data showed Thiorphan activity appeared to plateau in the left cortex after 4 weeks of administration. These findings are significant because they provide information about the pattern of Thiorphan activity in the rat brain, which enabled us to proceed with an *in vivo* study on the effects of Thiorphan on recovery from spinal cord injury.

To our knowledge, only one study has investigated the neuroprotective effect of Thiorphan, and it confirmed that Thiorphan protected against neuron death in the neonatal mouse brain [22]. Most other studies have investigated Thiorphan predominantly for its effects as an anti-diarrheal agent, or as a potential treatment for cardiovascular disease [24]. Its use in the nervous system, aside from the Medja study, was mostly limited to inconclusive studies on its use as an analgesic, and no other studies investigated its neuroprotective effects [24].

Our primary reason for investigating Thiorphan resulted from a relatively novel method of drawing drug-disease connections based on RNA expression patterns using the Broad Connectivity Database [19]. The database was used to find drugs that caused a similar RNA expression pattern to that of a regenerating spinal cord neuron. While several candidate drugs caused patterns that closely resembled the criterion pattern, Thiorphan was demonstrated to cause the greatest increase in neuron growth in culture. Multiple studies on Thiorphan confirm it works as an NEP inhibitor in various body systems, including the brain [24]. Our experiment

confirmed Thiorphan activity in the brain caused inhibition of NEP, and administration to one particular area of the brain resulted in its dispersal to different regions of the brain.
The results from this experiment on the activity pattern of Thiorphan were used to refine our areas of observation for the entire study, as its dispersal from the site of infusion implies that other, previously unforeseen areas of the CNS will also receive a dosage of Thiorphan.

Our preliminary results indicate Thiorphan does have an effect on neurite growth, resulting in improved behavioral outcomes. Thiorphan appeared improve animals' ability to retrieve sugar pellets at a level similar to the NPC graft. This improvement resulting from the drug treatment alone suggests that Thiorphan may have a positive effect on CST axon regeneration, but without histological analysis, we cannot reach any conclusions.

Interestingly, there is no added effect of Thiorphan and graft together, suggesting these two treatments do not act synergistically. However, several avenues need to be investigated before a conclusion can be drawn. First, Thiorphan was administered at 100mM dosage *in vivo*. This might not be an optimal dose, and time of treatment administration (how long after injury) as well as duration of administration needs to be tested. Further, histological data is not available at this time, as subjects are still undergoing testing, Histological data will help identify mechanistic contributions of Thiorphan on CST growth. Notably, identifying if Thiorphan induces sprouting above the lesion from intact axons may explain why Thiorphan alone can elicit improved functional outcomes compared to lesion alone. These findings will help identify Thiorphan's efficacy for further studies aimed at human intervention.

In summary, if this work is successful, it will identify a new drug-disease connection between Thiorphan and spinal cord injury, and may take a rapid path towards human intervention.

FIGURES

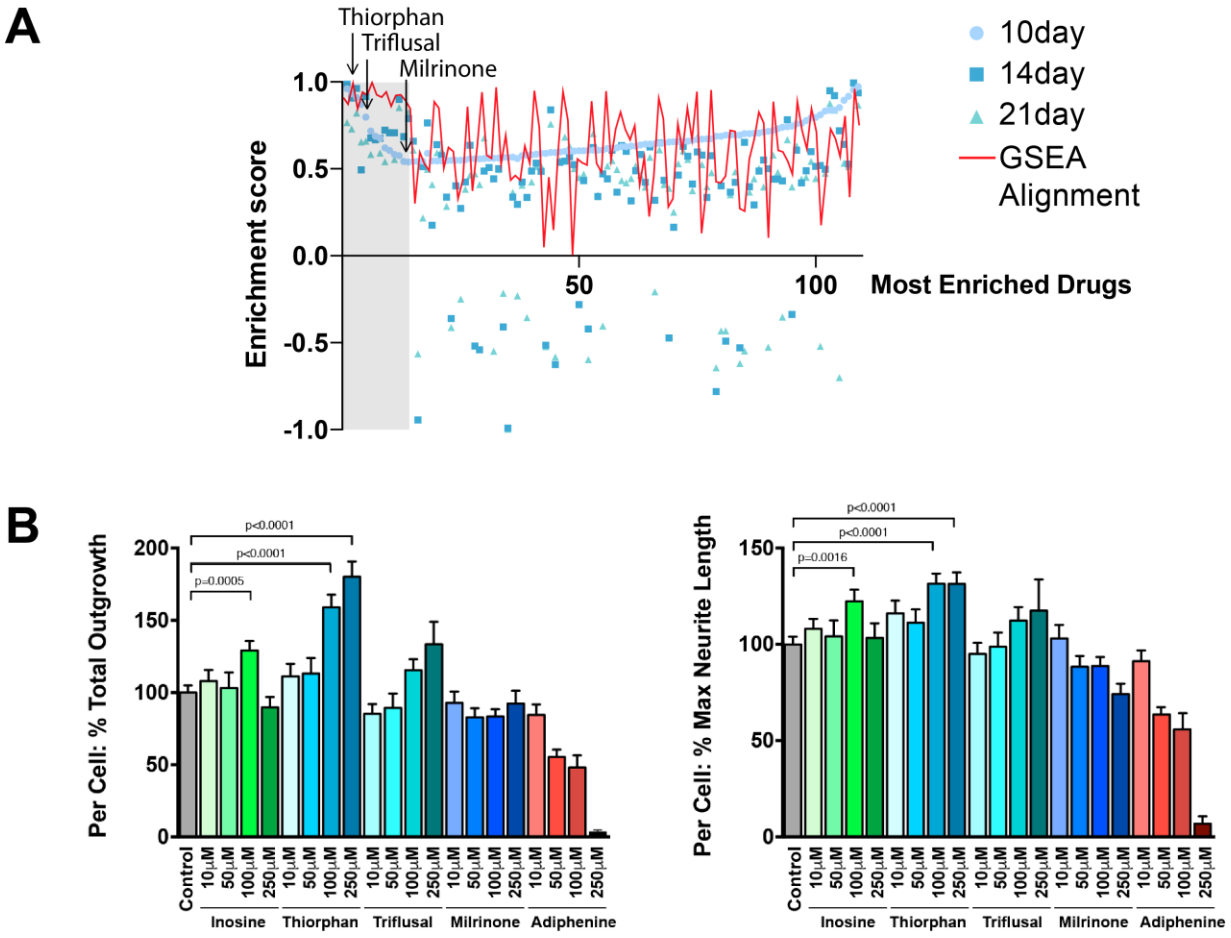

Figure 1. *In Silico* and *In Vitro* Analysis of the Regenerating Corticospinal Tract

A. In silico analysis of our regenerating CST identified novel small molecules. The regenerative transcriptome was used to search the Broad Connectivity Database, and many drugs closely matched this signature, including the top 100 closest matches displayed here. GSEA of the top 100 candidates revealed a subset of 15 drugs (grey shaded bar) whose transcriptional profiles included genes belonging to stem cell gene sets, similar to the regenerative transcriptome. **B**. Testing the top 3 compounds *in vitro* identified the top candidate for *in vivo*. The most closely matching drugs from the *in silico* screen were applied in 4 different concentrations to adult primary cortical neurons in culture. Thiorphan showed the greatest increase in total outgrowth and maximum neurite length compared to controls and the other top candidates.

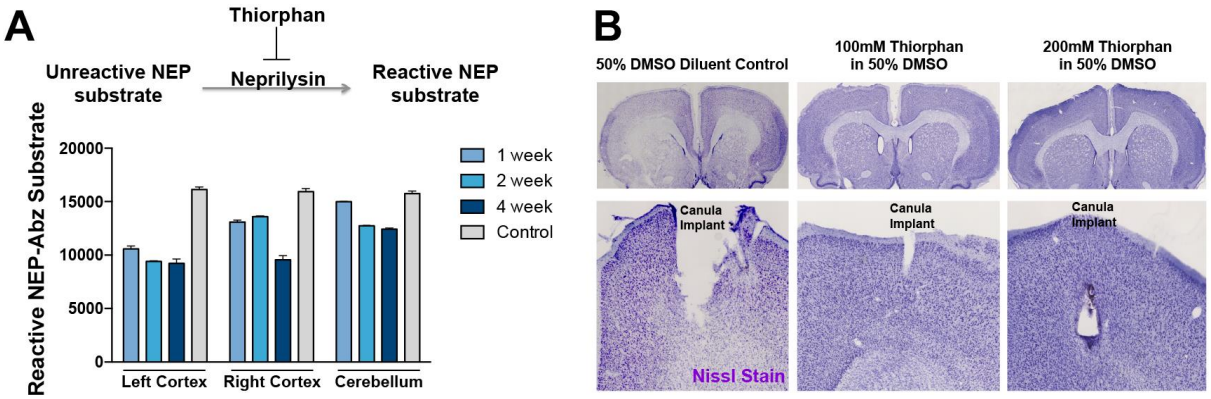

Figure 2. Thiorphan Activity in the Rat Brain.

A. Neprilysin assay in vivo shows Thiorphan activity over time, and that the drug remains bioactive while continuous administration occurs. The left cortex saw the highest Thiorphan activity after 1 week, but by 4 weeks, the left and right cortices saw similar levels of Thiorphan activity. **B**. Histology of cannula implant shows minimal damage to cortical tissue where no adverse effects of the drug can be seen.

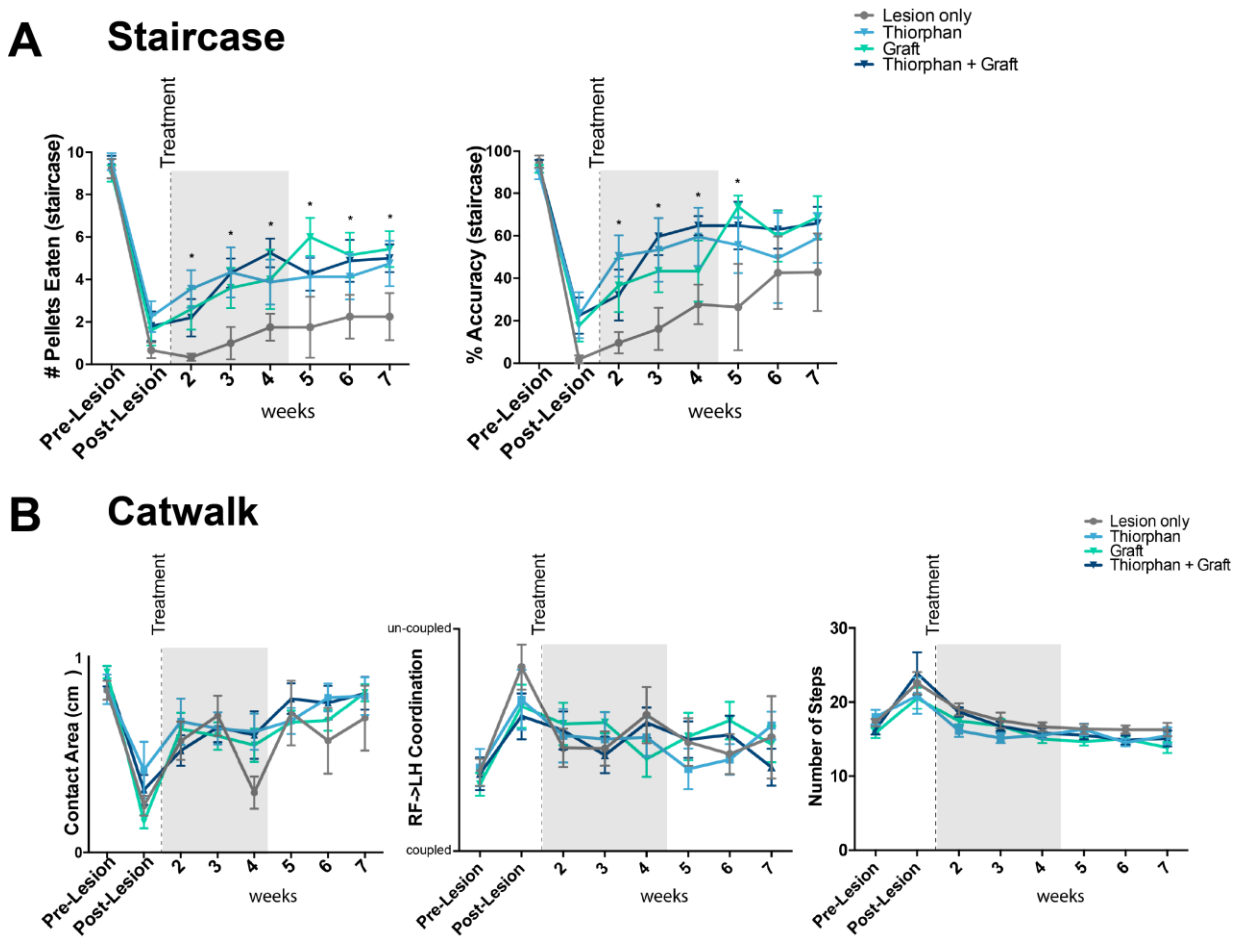

Figure 3. Behavioral Outcome Measures after SCI.

A. The staircase task was used to assess and quantitatively measure functional improvement in forelimb function after SCI. Animals were tested weekly after treatment administration or no treatment control. Behavioral improvement was seen in subjects that received either Thiorphan alone (N=9) or graft alone (N=10), as well as a combination in Thiorphan + graft (N=10) compared to lesion only controls (N=9). The grey bar represents the time (4 weeks) of Thiorphan or diluent-only administration.

B. Catwalk analysis showed a reduction in contact area of the right forepaw after lesion in all groups. However, this reduction was not significant between treatment paradigms or lesion only control. Right Forelimb (RF) and Left Hindlimb (LH) coordination did not show any improvement above lesion only control animals, and neither did the number of steps taken during testing.

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