Effect and Reporting Bias of RhoA/ROCK-Blockade Intervention on Locomotor Recovery After Spinal Cord Injury
A Systematic Review and Meta-analysis

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IMPORTANCE Blockade of small GTPase-RhoA signaling pathway is considered a candidate translational strategy to improve functional outcome after spinal cord injury (SCI) in humans. Pooling preclinical evidence by orthodox meta-analysis is confounded by missing data (publication bias).

OBJECTIVE To conduct a systematic review and meta-analysis of RhoA/Rho-associated coiled-coil containing protein kinase (ROCK) blocking approaches to (1) analyze the impact of bias that may lead to inflated effect sizes and (2) determine the normalized effect size of functional locomotor recovery after experimental thoracic SCI.

EVIDENCE REVIEW We conducted a systematic search of PubMed, EMBASE, and Web of Science and hand searched related references. Studies were selected if they reported the effect of RhoA/ROCK inhibitors (C3-exoenzyme, fasudil, Y-27632, ibuprofen, siRhoA, and p21) in experimental spinal cord hemisection, contusion, or transection on locomotor recovery measured by the Basso, Beattie, and Bresnahan score or the Basso Mouse Scale for Locomotion. Two investigators independently assessed the identified studies. Details of individual study characteristics from each publication were extracted and effect sizes pooled using a random effects model. We assessed risk for bias using a 9-point-item quality checklist and calculated publication bias with Egger regression and the trim and fill method. A stratified meta-analysis was used to assess the impact of study characteristics on locomotor recovery.

FINDINGS Thirty studies (725 animals) were identified. RhoA/ROCK inhibition was found to improve locomotor outcome by 21% (95% CI, 16.0-26.6). Assessment of publication bias by the trim and fill method suggested that 30% of experiments remain unpublished. Inclusion of these theoretical missing studies suggested a 27% overestimation of efficacy, reducing the overall efficacy to a 15% improvement in locomotor recovery. Low study quality was associated with larger estimates of neurobehavioral outcome.

CONCLUSIONS AND RELEVANCE Taking into account publication bias, RhoA/ROCK inhibition improves functional outcome in experimental SCI by 15%. This is a plausible strategy for the pharmacological augmentation of neurorehabilitation after human SCI. These findings support the necessity of a systematic analysis to identify preclinical bias before embarking on a clinical trial.

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Spinal cord injury (SCI) affects hundreds of thousands of new patients worldwide every year, and it is believed that 1.5 to 5.2 million patients are experiencing the consequences of SCI.1 Despite increasing optimism2 based on an exponentially increased molecular and cellular knowledge, no intervention has yet been reported to be effective in human SCI. The lack of therapies for patients with SCI points to the need for developing validated therapeutic interventions after SCI and encourages translational effort.

Compared with other central nervous system injury paradigms, including ischemic injury, translation of findings from preclinical in vivo models of SCI to human clinical trials is relatively novel3 and to be successful will require systematic and sustained efforts to establish a bidirectional translational dialogue.4-6 Clinical-trial design is complicated by the range of injury severity, and reducing this heterogeneity with a focus on incompletely paralyzed patients may allow for smaller, but more effective, clinical trials.3,7 Preclinical studies are likely to be challenged by inflated effect sizes8 (Delta inflation), which render an objective prioritization of interventions to be taken forward to clinical trials difficult. Systematic review series have provided a relevant first approach to structure evidence based on published in vivo experiments.9 The vulnerable nature of the fragile and resource-intensive translational path is not unique to SCI; failure of apparently well-evidenced well-executed preclinical, neuroprotective stroke approaches in later clinical trials has raised concerns10 and led to a number of initiatives aiming to identify and reduce bias to optimize the predictive value of preclinical research.8,10,12

One of the major barriers for an improvement of functional outcome after SCI is limited axonal regeneration and plasticity based on insufficient regrowth of axonal fibers at the lesion site.13 Molecular axonal outgrowth inhibitors embedded within the forming scar tissue and myelin lead to axon growth cone collapse and failure of axonal regeneration14 on binding to cognate receptors.15

The signals elicited by most of the growth inhibitory molecules converge on the intracellular activation of the Rho (Ras homology gene family member)-GTPase/Rho-kinase (Rho-associated coiled-coil containing protein kinase [ROCK]) pathway16 (Figure 1). In combination with elicited neuroprotective effects,14,16 blockade of the RhoA/ROCK pathway is thought to foster functional neurological recovery after SCI.

Conventional methods of summarizing research findings, such as narrative reviews, are potentially confounded by selective or incomplete citation bias, and even systematic reviews are potentially confounded by lack of access to unpublished data or if the contributing studies are at risk for bias owing to poor study design.12 It would be troubling if erroneous conclusions drawn from such research summaries were used in the prioritization and validation of approaches to clinical trials.

In this analysis, we studied the in vivo evidence for efficacy of RhoA/ROCK-pathway inhibition after SCI using systematic review and meta-analysis with the DerSimonian and Laird random effects model. Subsequently, we used stratified meta-analysis to investigate the role of study design characteristics that might be associated with increased risk for bias. We then assessed the presence and impact of publication bias using funnel plotting, Egger regression, and the trim and fill method. We believe that these adjusted estimates of effect size and assessment of the risk for bias should improve the predictive value of preclinical data to better inform approaches to human studies.

### Methods

The study protocol was finalized in advance of any data collection (http://www.camarades.info/index_files/Protocols.html). The method and statistical approach is described in greater detail elsewhere.44

#### Search Strategy

We identified studies that reported the neurobiological effects of modulation of the RhoA/ROCK pathway after SCI from PubMed, EMBASE, and ISI Web of Science (search conducted June 11, 2013) using the search terms: (C3 OR C3-transferase OR BA-207 OR Y27632 OR Y-27632 OR Nonsteroidal Anti-Inflammatory OR NSAID OR Ibuprofen OR Rho Kinase OR Rho-Kinase OR Rho OR ROCK OR RhoA OR Fasudil OR HA-1077 OR AT-B77 OR Cethrin OR BA-210 OR Dimethylfasudil OR H-1152) AND (spinal cord injury OR hemisection OR contusion injury OR dorsal column injury OR complete transection OR corticospinal tract injury). Search results were limited to animals. Reference lists of identified publications and previously published reviews of SCI treatment options were hand searched for additional studies. Two investigators (R.W. and E.S.S.) independently assessed the search results.

#### Inclusion and Exclusion Criteria

We included controlled studies reporting the effect of RhoA/ROCK-pathway blockade on locomotor recovery in experimental spinal cord hemisection, contusion, or transection. We focused on the most commonly used standardized outcome measures (the Basso, Beattie, and Bresnahan [BBB] score45 or the Basso Mouse Scale [BMS] for Locomotion46) and excluded studies reporting other outcome measures. We also excluded studies that did not report the number of animals in each group, as well as the mean effect size and its standard deviation or standard error of the mean. Studies were excluded if they combined multiple treatments (eg, stem cells and Rho-GT-Pase inhibitory agents) or if models of nontraumatic SCI were used.

#### Data Extraction

We extracted details of individual study characteristics from each publication, and where a single publication reported more than 1 experiment, these data were extracted and treated as independent experiments. Where neurobehavioral tests were performed serially, we only extracted data for the final time point. In these studies reporting locomotor outcomes, we also extracted data quantifying corticospinal tract nerve fibers. We assessed the risk for bias using a modified 9-point-item checklist, adapted from a statement of good laboratory practice in stroke modeling,8 and the CAMARADES (Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies) quality checklist.47 This quality checklist overlaps substantially with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guideline for reporting animal research48 and comprises items of pretested relevance with re-
The RhoA/ROCK pathway is activated after SCI by neurons (A) and glial cells (B). A, The RhoA/ROCK pathway is a converging cascade that mediates signaling from myelin and scar-derived growth inhibitory proteins. Activated RhoA-guanosine triphosphate (GTP) binds to Rho-binding domain of ROCK, thereby leading to its activation. ROCK activation induces neuronal growth cone collapse/neurite retraction bulbs by 2 different ways: one acting on microtubuli, the other affecting the actin cytoskeleton. Activation of collapsing response mediator protein 2 (CRMP2) propagates microtubuli destabilization. Microtubuli stabilization has been shown to prevent growth cone collapse and to enable axonal outgrowth in vivo. ROCK also activates myosin light chain (MLC) and LIM kinase (LIMK), which control actin-myosin interactions, leading to cell contraction, stress fiber formation, and ultimately also growth cone collapse. Finally, one of the recently identified targets of ROCK is phosphatase and tensin homologue (PTEN), being directly upregulated by ROCK. In central nervous system lesions, PTEN signaling abrogates axonal outgrowth, and blocking PTEN activity strongly promotes axon regeneration/plasticity, in part through mTOR-dependent upregulation. Together, it appears that the RhoA/ROCK pathway is a key target in the injured spinal cord to foster neurite outgrowth/plasticity. This neurite growth-promoting effect was validated in other central nervous system injury paradigms. Cross-paradigm verification is considered a prerequisite for a robust translational potential. Furthermore neuroprotective (eg, anti-excitotoxic, improved perfusion, and reduced edema formation), immunomodulatory (reduced leukocyte infiltration), antineurodegenerative, proneurorestorative, together with neuropathic pain–alleviating, properties are beneficial for tissue damage and foster repair after SCI. RhoA/ROCK inhibition can be achieved at different levels in several ways. First, through ADP ribosylation, the exoenzyme C3-ADP-ribosyltransferase (Cethrin/BA-210) prevents the conversion to active GTP-bound Rho, which stabilizes the inactive RhoA-guanine nucleotide dissociation inhibitor complex in the cytosol. Second, certain nonsteroidal anti-inflammatory drugs, including ibuprofen, reduce but do not eliminate, RhoA signaling activation, leading to activation of the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ), which has been shown in an article by Kopp et al. Agents that bind and activate the transcription factor PPARγ have been reported to minimize or prevent deleterious cascades after SCI (reviewed in an article by McTigue). Third, small interfering RNA strategies directed at RhoA will block de novo synthesis of RhoA, reducing the amount available for activation. Finally, ROCK inhibition is established by 2 different small-molecule ROCK inhibitors (Y-27632 and fasudil [HA-1077, AT877]) and by the peptidic receptor ROCK inhibitor p21CIP1/WAF1. ROCK I/II activates PTEN in mammalian cells, which has to be investigated for neurons.
Figure 2. Study Selection

2817 Papers identified by search strategy  
1306 From PubMed/MEDLINE  
760 From EMBASE  
751 From ISI Web of Science

755 Excluded duplicates

2062 Potentially relevant papers

2040 Papers excluded based on abstract

8 Papers excluded  
7 With inappropriate outcome scale  
1 With statistical inconsistencies

22 Papers selected for full-text reading

14 Papers included in final analysis

Interventional preclinical trials applying Rho/Rho-associated coiled-coil containing protein kinase (ROCK) pathway inhibitory strategies to investigate the effect on neurological locomotor outcome.

Analysis

We calculated a normalized effect size for each experimental comparison as the proportional improvement in the treated group compared with the control group, along with a standard error of this estimate. Where a single control group served multiple treatment groups, the size of the control group was adjusted to account for this. We assessed for possible publication bias using funnel plot,\(^5\) Egger regression,\(^5\) and the trim and fill method.\(^5\),\(^5\)

We applied the DerSimonian and Laird random effect meta-analysis to calculate an overall treatment effect. We used stratified meta-analysis with partitioning of heterogeneity to investigate the impact of different drugs, level of SCI lesion, anesthetic used, different animals, type of SCI, sex, the method of induction of injury, and study quality checklist items, both individually and the aggregate number of checklist items scored. The significant difference between \( n \) groups was assessed by partitioning heterogeneity and by using the \( \chi^2 \) distribution with \( n-1 \) degrees of freedom. To allow for multiple comparisons, we adjusted our significance level to \( P < .002 \) using Bonferroni correction. Figures were drawn using SigmaPlot (Systat Software Inc).

Results

Study Selection

Our systematic search identified 2817 studies, of which 14 met our prespecified inclusion criteria and were included into the final analysis (Figure 2). Seven studies identified assessed functional neurological outcome but did not use the BBB or BMS scales and were therefore excluded. Thirty experiments using a total of 725 animals (control animals, \( n = 353 \), and treated animals, \( n = 372 \); see eTable and eReferences in the Supplement for study design details) reported neurobehavioral outcomes (Figure 2).

Description of Studies

Eight different strategies were used to target the RhoA/ROCK pathway, 5 using RhoA-GTPase inhibitors (BA-210 [4 experiments], C3-ADP-ribosyltransferase [4 experiments], siRhoA [2 experiments] and ibuprofen [5 experiments]) and 3 using ROCK inhibitors (fasudil [7 experiments], p21 [1 experiment], and Y-27632 [5 experiments]). Twenty-four of 30 experiments (80%) used intrathecal drug administration, and the time of drug administration ranged from 30 minutes before the induction of SCI to 4 weeks after, although most administered the drug at the same time as SCI induction (median, 0 minutes; interquartile range, 0-0). Ten experiments used mice and 20 used rats; most of the animals were female. Fifteen experiments applied contusion injury, 14 performed a hemisection, and 1 study investigated transection injury. All studies lesioned the thoracic spinal cord between T3 and T10. Five experiments assessed functional recovery using the BBS scale and the remainder used the BBB scale.

Neurobehavioral Outcome

Overall, RhoA/ROCK-pathway inhibition improved locomotor function in models of traumatic SCI by 21% (95% CI, 16.0-26.6; \( \chi^2_{29} = 83.3 \)) (Figure 3). Using stratified meta-analysis, we identified 4 aspects of the study design that accounted for a significant proportion of between-study heterogeneity:

1. Drug-specific blockade of the RhoA/ROCK pathway. Among the differential approaches to block RhoA activation (Figure 1), C3-ADP-ribosyltransferase was the most effective intervention, consisting of 8 studies investigating C3 peptides (\( \chi^2 = 19.3, P < .002 \)) followed by Rho-kinase (ROCK) inhibitors (Figure 4A). Among the RhoA-inhibitory strategies, siRhoA appeared a little less effective compared with C3-ADP-ribosyltransferase. Among the ROCK inhibitors, Y-27632 appeared superior to fasudil or p21 (Figure 4A).
A. Stratified meta-analysis for the specific drug used revealed the RhoA-inhibiting C3-ADP-ribosyltransferase being more effective than RhoA/Rho-associated coiled-coil containing protein kinase (ROCK) inhibitors acting downstream. B. Despite a possible variability in lesion extent or depth due to different techniques of injury induction, improvements in effect size were greatest in animals with hemisectioned spinal cords. C. The use of fentanyl/fluanisone in combination with diazepam was associated with higher estimates of effects compared with other reported anesthetics. D. The sex of the animal used was identified as another aspect of the study design characteristics that accounted for a significant proportion of between-study heterogeneity. E. Effect size correlates inversely with study quality (ie, studies with a better improvement in functional outcome are associated with low study quality). The shaded bars represent the 95% CI limits of the global estimate. The vertical error bars represent the 95% CIs for the individual estimates. The width of each bar reflects the log of the number of animals contributing to that comparison. Each stratification accounts for a significant proportion of between-study heterogeneity (P <.002).
2. The type of injury: Improvements in neurobehavioral function were most pronounced in animals with cord hemisection ($\chi^2 = 27.2; P < .002$) compared with contusion or transection (Figure 4B).

3. Dependence of the anesthesia used during the induction of injury: The use of hypnorm (fentanyl/fluanisone) in combination with diazepam was associated with higher estimates of effects compared with volatile or other anesthetics ($\chi^2 = 29.5; P < .002$) (Figure 4C).

4. The sex of the animals: Studies not reporting the sex of the animals used reported the highest estimates of effect. In those reporting the sex of the animals, efficacy was lower in males than females ($\chi^2 = 15.5; P < .002$) (Figure 4D).

**Study Quality**

The median number of quality-checklist items scored was 4.5 out of a possible 9 (interquartile range, 3-5). Nine of 14 publications (64%) reported the blinded assessment of outcome, 4 (29%) reported randomization, and none reported performing a sample-size calculation. There was an inverse relationship between the study-quality score and effect size, with studies scoring 1 or 2 points associated with the largest estimates of effect ($\chi^2 = 28.6; P < .002$) (Figure 4E).

**Publication Bias**

We observed funnel plot asymmetry, with an excess of experiments of large effect size and low precision (Figure 5A), and this was confirmed using Egger regression (Figure 5B). Trim and fill analysis imputed 9 missing studies, with an adjusted effect size of 15.6% (95% CI, 10.1-21.2), 6% lower than the unadjusted estimate (Figure 5C).

**Discussion**

We assessed the effect of RhoA/ROCK-pathway inhibition on locomotor recovery after experimental SCI. Thirty studies using 725 ani-
mals met our inclusion criteria and were analyzed. We found evidence of publication bias and estimated 9 theoretical missing studies with a relative overstatement of efficacy of around 30%.

Complementary to other strategies to validate preclinical evidence aimed at minimal variability to enable the most accurate reproduction (eg, FORESCI initiative^53), an alternative approach is the deliberate incorporation of heterogeneity in multiple modeling characteristics to (1) validate the robustness of the effect under different modeling conditions and (2) quantify the impact of individual experimental modeling aspects on outcome (stratification). Targeting the thoracic spinal cord assessing motor outcome is relevant to human disease.^3

The effects of interventions at different points along the RhoA/ROCK pathway suggest that blockade of RhoA activation by C3-ADP-ribosyltransferase is significantly more effective than fasudil or ROCK inhibitors acting further downstream.

Furthermore, siRho approaches are less effective, possibly because these rely on inhibition of RhoA synthesis and do not affect existing intracellular RhoA levels. Additionally, effects of RhoA/ROCK inhibitors on immune cells have been described, whereas they vary between proinflammatory^54 and anti-inflammatory^55 consequences.

Efficacy was significantly higher with cord hemisection compared with more widespread and severe contusion or transection injuries. The observed effect was robust, although variability in lesion extent or depth must be assumed owing to multiple surgeons, techniques of injury, and differences of distinct surgery days (intra-surgeon differences).^56 It is possible that spared tissue bridges in the ventral spinal cord provide opportunities for remodeling, regeneration, and repair, which do not hold for other models, and that these opportunities can be realized if the inhibitory effects of the RhoA/ROCK pathway can be suppressed. Finally, higher efficacy in female animals might relate to a higher degree of endogenous nerve protection owing to the reported properties of either progesterone or estradiol.^57

The range of circumstances under which efficacy is observed is limited, particularly with regard to the demonstration of efficacy at clinically relevant timeframes. Most preclinical studies are not powered to detect small effects,^58 yet these small effects may be clinically highly relevant; a treatment that improved outcome following SCI by only 5% would have a profound impact on public health. For this reason, we assume that large, adequately powered phase 3 preclinical studies at low risk for bias might provide a key step in the chain of translational neuroscience, and that these might best be organized through multicenter animal studies (http://www.multi-part.org).^47,^59

Notably, we detected a low prevalence of measures to reduce bias, and that studies with the largest improvement in neurobehavioral outcome were characterized by reporting only 1 or 2 points on the checklist. These results indicate implications for the improvement of preclinical animal experiments (design and conduction to reduce the risk for bias).

Previous cumulative meta-analyses of in vivo data^60 suggest that the estimate of efficacy becomes stable after data from around 1500 animals have been included; the current analysis included data from only 725 animals, limiting the confidence we might have in the estimates of efficacy. Furthermore, our analysis depended on the validity of the scales used, and these have been questioned.^61,^62 However, these scales are in widespread use and are considered the most informative, commonly used neurobehavioral element to assess outcome in modeling SCI. For this reason, it is unlikely that a preclinical intervention would be approved for translation if it did not result in improvements in the BBB/BMS score.

The summary estimates provided here include all data from all identified studies, not just the maximum value for efficacy from each study. In many cases, the observed low efficacy is an expected consequence of the circumstances of testing rather than representing the maximum achievable efficacy for that drug. For instance, all points of a dose-response relationship are included, even those where the dose is very low. The route of administration may also be important; C3-ADP-ribosyltransferase is poorly cell permeable. Following intrathecal delivery, one group found a very small improvement of only 0.4 points on the BBB scale (18.5 vs 18.9 for the untreated group),^63 whereas another group that used topical application at the lesion site by dispersion in a fibrin clot found a 91% improvement in outcome. To this extent, at least, our findings underestimate the maximum efficacy that might be achieved.

Conclusions

Publication bias is known to be a major problem in the reporting of clinical trials, but its impact in preclinical SCI research has not previously been quantified. Here, we showed that publication bias is prevalent in reports of laboratory-based research in animal models of SCI investigating the effect of RhoA/ROCK blockade on locomotor recovery. We estimate that one-third of all experiments remain unpublished. Given the finding of publication bias in vivo experiments in stroke,^26 it is unlikely that the publication bias reported here is limited to the effect of RhoA/ROCK blockade efficacy on locomotor recovery and is likely to be prevalent in experimental SCI models.

In spite of these caveats, the in vivo data are consistent with RhoA/ROCK inhibition improving locomotor outcome after experimental SCI by a magnitude that, were it replicated in human clinical trials, would represent an important advance in the management of acute SCI.

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