Background and Purpose—Rehabilitation is the only treatment option for chronic stroke deficits, but unfortunately, it often provides incomplete recovery. In this study, a novel combination of growth factor administration and rehabilitation therapy was used to facilitate functional recovery in a rat model of cortical stroke.

Methods—Ischemia was induced via injection of endothelin-1 into the sensorimotor cortex. This was followed by either a 2-week infusion of epidermal growth factor and erythropoietin or artificial cerebrospinal fluid into the ipsilateral lateral ventricle. Two weeks after ischemia, animals began an 8-week enriched rehabilitation program. Functional recovery was assessed after ischemia using the Montoya staircase-reaching task, beam-traversing, and cylinder test of forelimb asymmetry.

Results—The combination of growth factor infusion and rehabilitation led to a significant acceleration in recovery in the staircase task. When compared with controls, animals receiving the combination treatment attained significant recovery of function at 4 weeks after stroke, whereas those receiving rehabilitation alone did not recover until 10 weeks. Significant recovery was also observed on the beam-traversing and cylinder tasks.

Conclusions—Combining behavioral rehabilitation with growth factor infusion accelerates motor recovery. These data suggest a promising new avenue of combination therapies that may have the potential to reduce the rehabilitation time necessary to recover from sensorimotor deficits arising from stroke. (Stroke. 2014;45:1856-1858.)

Key Words: endothelin-1 ■ epidermal growth factor ■ erythropoietin ■ rehabilitation ■ stroke

Accelerating recovery in individuals who have experienced a stroke is crucial for limiting the effect of this condition. To enhance the rate of poststroke functional recovery, an optimal approach may be to combine rehabilitation with treatments that enhance neuroplasticity. Previous work has shown that serial application of epidermal growth factor (EGF) and erythropoietin enhances tissue regeneration and proliferation of neural precursor cells.1 As a prelude to future mechanistic studies, the present study sought to determine whether an enhanced neuroplastic environment created by growth factor infusions and concurrent physical rehabilitation would augment behavioral recovery. We hypothesized that combining EGF and erythropoietin with rehabilitation after forelimb sensorimotor cortex stroke would result in accelerated functional improvements when compared with either rehabilitation or EGF and erythropoietin alone.

Methods

Subjects
Sixty-seven male Sprague–Dawley rats were matched for poststroke grasping/retrieving performance on the staircase test and randomized into 4 groups: rehabilitation+EGF/erythropoietin (n=13), rehabilitation+artificial cerebrospinal fluid (aCSF; n=12), no rehabilitation+EGF/erythropoietin (n=12), and no rehabilitation+aCSF (n=13). All procedures were approved by the Memorial University Animal Care Committee and comply with regulations set by the Canadian Council of Animal Care (see detailed Methods in the online-only Data Supplement).

Behavioral Testing
Animals were trained on the staircase (pellet grasping/retrieving), beam (paw placement/balance), and cylinder (asymmetrical limb use for postural support) tasks 2 weeks before ischemia, and baseline abilities were assessed 3 days before stroke. Subsequent testing was performed 5 days after stroke and at poststroke weeks 4, 7, and 10 (Figure 1). Animals that did not meet preset training performance criteria (n=5), or were functionally unimpaired after stroke (n=12), were excluded. Behavioral data are presented as a percentage of prestroke performance.

Surgical Procedures
After baseline behavioral testing, rats were anesthetized with isoflurane and delivered 2 stereotaxic injections of endothelin-1 (2 µL/
injection; 400 pmol/μL) into the sensorimotor cortex. Three days after ischemia, an infusion cannula was inserted into the lateral ventricle to deliver either EGF (10 μg/mL) or aCSF vehicle via osmotic minipump (1.0 μL/h; 7 days). Seven days after EGF pump implantation, the pump was replaced with either erythropoietin (1365 IU/mL) or aCSF. This serial administration of EGF/erythropoietin has been previously found effective in promoting repair of damaged cortical tissue. All osmotic minipumps were removed 7 days later.

Enriched Rehabilitation Protocol

Animals receiving rehabilitation were housed in large wire-mesh cages (5–6 per cage) containing a variety of interactive objects, whereas those in the nonrehabilitation group were pair-housed in standard cage conditions. Rehabilitation animals also received voluntary access to a training chamber (6 hours per day, 5 days per week, and 8 weeks). This apparatus encouraged repeated reaching attempts similar to the movements specific to the staircase-reaching task. After the conclusion of rehabilitation and testing, brains were collected for analysis of stroke damage using cresyl violet staining.

Statistical Analysis

Behavioral data were analyzed using 2-way repeated-measures ANOVAs with Ryan-Einot-Gabriel-Welsch F post hoc and independent samples t tests (Bonferroni correction) for multiple comparisons. Cortical damage was analyzed using the Kruskal–Wallis and Mann–Whitney U nonparametric tests. Significance was set at P≤0.05 for all analyses, and values are expressed as mean±SEM.

Results

In the staircase task, a significant rehabilitation×growth factor interaction existed among groups after both 4 (P<0.04) and 10 weeks (P<0.01) after stroke (Figure 2A). Post hoc analysis showed that the rehabilitation+EGF/erythropoietin group performed significantly better than the nonrehabilitation+aCSF and nonrehabilitation+EGF/erythropoietin groups at both 4 and 10 weeks (P<0.05). To assess magnitude of improvement, group performance at each time point was subtracted from the corresponding poststroke test point. This revealed that 4 weeks after stroke, the rehabilitation+EGF/erythropoietin condition improved significantly more on the staircase test than those in the nonrehabilitation+EGF/erythropoietin and nonrehabilitation+aCSF groups (P<0.05; Figure 2B). Animals in the rehabilitation+aCSF group did not show a significant improvement compared with the nonrehabilitation+EGF/erythropoietin group until 10 weeks after stroke (P<0.05; Figure 2C). Both the beam and cylinder tasks showed significant time×rehabilitation interactions (P<0.02; Figures I and II in the online-only Data Supplement). No significant differences in cortical damage were detected between groups (P>0.05).

Discussion

This study demonstrates that functional recovery is significantly accelerated with the combination of EGF/erythropoietin and rehabilitation. By week 4 after stroke, animals receiving the combined treatment improved to a significantly greater extent on the staircase task (grasping/retrieving) than animals in the growth factor alone or in control groups. In contrast, the
group that received rehabilitation with a vehicle infusion also received significantly more than the control conditions, but recovery emerged only at 10 weeks after stroke. Interestingly, neither the beam walking (paw placement/balance) nor cylinder (asymmetrical limb use for postural support) tasks revealed the same acceleration effect observed in the staircase-reaching task (although rehabilitation alone was still efficacious). This suggests that beneficial effects of the combined treatment may be restricted to fine motor skill, as reflected by the staircase test. Clinically, it has been noted that recovery is enhanced for tasks that are specifically targeted by a given rehabilitation program and this is in line with our reaching data. 2

Our data suggest that engaging multiple recovery processes may be critical for enhancing functional outcome, in contrast to monotherapies targeting single mechanisms. Previous studies demonstrated that the individual components of our enriched rehabilitation paradigm (environmental enrichment and taskspecific reach training) are not effective for improving recovery of skilled reaching deficits 3; however, the combination of these components has a significant beneficial effect on recovery. 4 This is underscored by recent work that demonstrates enhanced activation of layer II and III neurons in peri-infarct cortex in response to enriched rehabilitation but not its individual components. 5 Similar results have been found on the efficacy of EGF and erythropoietin in combination, but not individually, in enhancing neural precursor cells. 1 Results such as these emphasize the need to develop interventions targeting multiple, complementary mechanisms, given the repeated failure of single interventions to translate into successful clinical application. 5

Task-specific rehabilitation may be a critically important supplement to realizing the beneficial effects of exogenous growth factor infusion into the brain. With daily reaching rehabilitation, neuroplastic reorganization of the motor cortex takes place, which is thought to be responsible for functional recovery. 7 When rehabilitation is combined with EGF/erythropoietin infusion, we speculate that cortical maps involved in task-specific motor patterns may enlarge at an accelerated rate relative to motor maps involved in other stroke-disrupted movements. This increased neuroplastic response could be induced by EGF/erythropoietin through processes, such as neural precursor proliferation, neurovascular remodeling, and synaptic plasticity. 6,9 These possibilities, as well as other potential mechanisms underlying the beneficial effects of this combination therapy and precise timeline of recovery, await further investigation but offer promise as an adjunct to conventional rehabilitation.

In summary, combination drug/rehabilitation paradigms represent a promising approach to stroke recovery that has been largely unexplored. In the present study, we demonstrated that combining EGF and erythropoietin infusion with rehabilitation significantly accelerates the rate of recovery of fine motor ability after stroke. This finding is particularly noteworthy because the benefits of the combination therapy were compared with the current most effective or best practice intervention (ie, enriched rehabilitation) for enhancing recovery in preclinical stroke models, instead of an untreated control. Interestingly, although we observed some beneficial effects of rehabilitation alone across all of our behavioral tests, similar effects were not found in our drug-only condition. This emphasizes the importance of testing novel drug targets in conjunction with (and against) other therapies that are known to be efficacious because synergistic effects may exist that could otherwise go undetected.

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Disclosures
None.

References
Epidermal Growth Factor and Erythropoietin Infusion Accelerate Functional Recovery in Combination With Rehabilitation
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Supplemental Methods

Subjects
Sprague-Dawley rats were obtained from Charles River Laboratories, Montreal, Canada; weighing 300-325g upon arrival. Animals were pair-housed in Plexiglas® cages on a 12:12 hour reverse light/dark cycle with food and water ad libitum except during behavioural training and testing periods, when animals were restricted to 90% of their free-feeding weight. All procedures were performed during the dark cycle.

Staircase Task
Forelimb reaching ability was assessed using the staircase task, as described previously.1 Animals were trained over ~20 trials (2/day; 15 min/trial; 4 hours apart) to a minimal criterion of 12±2 of the 21 possible pellets with the dominant paw (although mean performance was 17.8±1.23 pellets). One day prior to testing, animals were acclimatized to the staircase apparatus for two, 15-minute periods. Staircase testing was conducted over 2 days (2 trials/day; 15 min/trial; 4 hours apart) and the number of pellets consumed was recorded and averaged across all trials. Animals were excluded from the study if they failed to reach criterion at baseline testing (n=5) or if they retrieved >60% of their baseline performance (i.e. minor stroke) at post-stroke testing (n=12).

Beam Test
Animals were trained to cross a 1.0m tapered beam to gain access to a darkened chamber located at the narrow end of the beam.2 On each test day, animals were required to traverse the beam four times and data from all trials were averaged. A fault was scored if the pad of the foot slipped from the top of the beam and successful steps were calculated as: (1 – [foot faults/total steps]) * 100.

Cylinder Test
Forelimb use for postural support was assessed with the cylinder task.3 Animals were placed in a Plexiglas® cylinder (20cm diameter) on a glass tabletop and filmed from below. Each trial continued until the animal completed a minimum of 20 independent rears and wall contacts. The number of contacts with each paw was analyzed and use of the limb contralateral to the stroke was calculated as: ([contralateral contacts + ½ bilateral contacts]/total contacts) * 100.4

Focal Ischemia
Following baseline behavioural testing, rats were anesthetized with isoflurane (3% induction, 1.5% maintenance in 100% O₂), secured in a stereotaxic frame and received a 0.2mL subcutaneous injection of 1% lidocaine (AstraZeneca, Mississauga, Canada) under the scalp. The scalp was incised and three holes were drilled in the skull over the forelimb sensorimotor
cortex, contralateral to the dominant paw as determined by the limb used to obtain the highest average number of pellets obtained in the pre-stroke staircase test. Three drill holes were made at the following coordinates (relative to bregma): (1) 0.0mm anteroposterior (AP), ±2.5mm mediolateral (ML); (2) +2.3mm AP, ±2.5mm ML and (3) -0.5mm AP, ±1.5mm ML. Dorsoventral (DV) coordinates were taken from the surface of the skull and 2µL/site of endothelin-1 (ET-1; 400 pmol/µL; Calbiochem, La Jolla, USA) was injected into the brain at drill sites (1) and (2) at -2.5mm DV. Rectal temperature was maintained at a minimum of 36.5°C with a homeothermic blanket (Harvard Apparatus, Saint-Laurent, Canada) throughout the surgery. Following ET-1 injection, the incision site was sutured, topical 1.0% Xylocaine gel and antibiotic ointments were applied and animals were monitored until recovered from surgery.

**Osmotic Mini-Pump Implantation**

Three days following ischemia, animals were re-anesthetized and their scalps incised as above. A 5mm infusion cannula was inserted into the lateral ventricle through a previously drilled hole (3) -0.5mm AP, ±1.5mm ML) and secured in place with cyanoacrylate glue (Loctite, Mississauga, Canada). This cannula was attached to an osmotic mini-pump (1.0µL/hr, 7 days; Alzet, Cupertino, USA) containing either EGF (10µg/mL) or aCSF vehicle via surgical tubing. The osmotic mini-pump was placed subcutaneously between the scapulae and incisions sutured. Seven days following EGF pump implantation, a small incision was made slightly anterior to the position of the osmotic pump and the pump was replaced with either EPO (1365IU/mL) or aCSF. The incision site was sutured and all osmotic mini-pumps were removed seven days later.

**Enriched Rehabilitation Protocol**

Two weeks following induction of focal ischemia, animals were randomized to either the enriched rehabilitation or standard group. Animals in the rehabilitation group were housed in large wire-mesh cages (length, 105cm; width, 67cm; height, 75cm) in groups of five or six while those in the non-rehabilitation group were pair-housed in standard cage conditions. Enriched environment cages contained a variety of objects (platforms, tubes, balls, etc.) that were changed twice per week and placed in different locations in order to increase novelty.

Animals housed in the enriched environment also received rehabilitation consisting of voluntary access to a reach training chamber (6 hours/day, 5 days/week, 8 weeks) that has been previously described. This reaching chamber is similar to the Montoya staircase but uses a trough to hold a large number of reward pellets instead of a tiered staircase. Fourteen grams of pellets (0.45mg; TestDiet, Richmond, USA) were loaded in the rehabilitation box, which allowed free access to the food reward only by using the impaired forelimb. Both mid-way (3 hours) and at the end of each day of rehabilitation the pellets were refilled and the amount consumed was recorded. This apparatus encourages repeated reaching attempts similar to the movements specific to the staircase-reaching task.

**Histology**

Following behavioural testing, animals were deeply anesthetized (5% isoflurane) and transcardially perfused with ice-cold 0.9% heparinized saline, followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were removed and post-fixed in 4% PFA overnight at 4°C, then transferred into 20% sucrose-PBS until saturated. The brains were then frozen in isopentane on dry ice, sectioned on a cryostat at 15µm and stained with cresyl violet to
assess cortical damage. The section of maximal cortical damage was identified and severity of cortical damage was calculated using ImageJ (NIH) as follows: 1-(area of undamaged ipsilesional cortex/area of undamaged contralesional cortex) * 100. This generated a value for the percentage of damaged cortex in the lesioned hemisphere. Each animal was assigned a score on a 5-point scale that corresponded to the amount of damaged cortical tissue: 0, no ischemic damage; 1, 1-25% damage; 2, 26-50% damage; 3, 51-75% damage; 4, >75% damage.7

**Statistical Analysis**

Statistical analyses were conducted using SPSS software (version 20 Professional for Mac OS X, IBM, Armonk, New York). Cortical damage was analyzed using the Kruskal-Wallis and Mann-Whitney U non-parametric tests. Behavioural data were analyzed using a two-way (Rehabilitation X Growth Factor) repeated measures analysis of variance (ANOVA) with Ryan-Einot-Gabriel-Welsch F (REGW-F) post-hoc and independent samples t-tests (Bonferonni correction) for multiple comparisons. Significance was set at p≤0.05 for all analyses and values are expressed as means ± SEM.

**Supplemental Results**

**Reaching Rehabilitation**

An independent samples t-test revealed no significant differences between the rehabilitation groups in terms of the daily amount of pellets consumed during reaching rehabilitation (P>0.05). Animals in rehabilitation consumed on average 14.85±0.74 g of 0.45mg pellets throughout each 6-hour rehabilitation session. All animals successfully used the reaching chamber in order to retrieve the food reward.

**Skilled Reaching Performance**

A two-way repeated measures ANOVA revealed a significant Time X Rehabilitation X Growth Factor interaction (P<0.01). Two-way ANOVAs at each time point indicated that a significant Rehabilitation X Growth Factor interaction existed among groups after both 4 (P<0.04) and 10 weeks (P<0.01) post-stroke (Figure 2A). Post-hoc analysis showed that the rehab + EGF/EPO group performed significantly better than the non-rehab + aCSF and non-rehab + EGF/EPO groups at both 4 and 10 weeks (P<0.05).

In order to assess magnitude of improvement, group performance at each time point was subtracted from the corresponding post-stroke test-point. Two-way repeated measures ANOVA of this data indicated a significant Time x Rehabilitation x Growth Factor interaction (P<0.01) with similar Rehabilitation X Growth Factor interactions existing at 4 and 10 weeks, as observed when comparing average group performances (P<0.05). Post-hoc analysis indicated that 4 weeks post-stroke the rehab + EGF/EPO condition had improved significantly more on the staircase test than those in the non-rehab + EGF/EPO and non-rehab + aCSF groups (P<0.05) (Figure 2B). Animals in the rehab + aCSF group did not show a significant improvement over the non-rehab + EGF/EPO group until 10 weeks post-stroke (P<0.05) (Figure 2C).
**Beam Test**
Data from the contralateral fore- and hindlimb were averaged to provide a measure of beam-walking success. Two-way repeated measures ANOVA indicated a significant Time X Rehabilitation interaction ($P<0.01$) with animals in the rehab conditions performing significantly better than those in non-rehab conditions (Figure IB). Rehabilitation significantly improved performance compared to non-rehab conditions after 10 weeks post-stroke ($P<0.01$) (Figure IC).

**Cylinder Test of Forelimb Asymmetry**
Two-way repeated measures ANOVA indicated significant Time X Growth Factor ($P<0.02$) and Time X Rehabilitation ($P<0.02$) interactions (Figures IIB and IIC, respectively). Animals in the rehab conditions increased the use of their impaired forelimb significantly more during the cylinder test than those in the non-rehab groups at 4 weeks post-stroke ($P<0.03$) (Figure IID).

**Stroke Damage Score**
A Kruskal-Wallis non-parametric test was used to assess individual group differences on damage score ($P>0.05$). Two Mann-Whitney U tests were used to assess the main effects of Rehabilitation ($P>0.05$) and Growth Factor ($P>0.05$) on damage score, neither of which resulted in differences among conditions, thus indicating that ischemic damage was similar among conditions (average score, 2.28±0.09; Figure III).
Supplemental References


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Figure 1. Post-ischemic performance on beam-traversing task (mean±SEM). (A) Performance of all experimental conditions across time (presented as % of baseline performance; not significant (NS)). (B) Performance of rehab vs. non-rehab conditions across time. Significant differences between conditions exist 10-weeks post-stroke, where animals in the Rehab condition demonstrated significant improvements over animals in the standard conditions (*p<0.01). (C) Improvement of the Rehabilitation and No-Rehabilitation groups from “post” to 10-week time point. Animals in rehab groups recovered significantly more than animals in groups that did not receive rehab (*p<0.01).
Figure II. Post-ischemic performance on cylinder test of forelimb asymmetry (mean±SEM). (A) Performance of all groups on the cylinder test of forelimb asymmetry across all test periods (NS, p>0.05). (B) There was a significant effect of Growth Factor across Time. However, no differences at individual time points could be isolated. (C) There was a significant effect of Rehab across Time, but again these differences could not be isolated with post-hoc comparisons. (D) After 4-weeks post-stroke, animals in rehab groups had recovered to a significantly greater extent than animals not receiving rehabilitation (*p<0.05).
Figure III. Representative image showing average area of damage throughout the brain, relative to Bregma (mm). Damage decreased rapidly beyond the coordinates shown in this diagram. No significant differences in infarct size were observed between groups.