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Background and Purpose—Stroke survivors often have permanent deficits that are only partially addressed by physical therapy. This study evaluated the effects of dalfampridine, a potassium channel blocker, on persistent sensorimotor deficits in rats with treatment initiated 4 or 8 weeks after stroke.

Methods—Rats underwent permanent middle cerebral artery occlusion. Sensorimotor function was measured using limb-placing and body-swing symmetry tests, which normally show a partial recovery from initial deficits that plateaus ≈4 weeks after permanent middle cerebral artery occlusion. Dalfampridine was administered starting at 4 or 8 weeks after permanent middle cerebral artery occlusion in 2 blinded, vehicle-controlled studies. Plasma samples were collected and brain tissue was processed for histologic assessment.

Results—Dalfampridine treatment (0.5–2.0 mg/kg) improved forelimb- and hindlimb-placing responses and body-swing symmetry in a reversible and dose-dependent manner. Plasma dalfampridine concentrations correlated with dose. Brain infarct volumes showed no differences between treatment groups.

Conclusions—Dalfampridine improves sensorimotor function in the rat permanent middle cerebral artery occlusion model. Dalfampridine extended-release tablets (prolonged release fampridine outside the United States) are used to improve walking in patients with multiple sclerosis, and these preclinical data provide a strong rationale for examining the potential of dalfampridine to treat chronic stable deficits in stroke patients.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT01605825 **(*Stroke*. 2013;44:00-00.)**

Key Words: 4-aminopyridine ■ chronic ischemia ■ demyelination ■ ischemic stroke ■ recovery

Individuals who survive a stroke typically show some degree of functional recovery during the first few months, but are often left with permanent neurological deficits. These motor, sensory, and cognitive impairments can have significant impact on activities of daily living and quality of life, contributing to a significant healthcare burden for the individual and society. An estimated 15% to 30% of stroke survivors remain permanently disabled.[1] These permanent deficits are addressed only to a limited extent by rehabilitation, and there has been little attention to the potential for pharmacological intervention. This may be based on the generally held concept that there is little that can be done to replace nerve cells and circuits that have been permanently lost. Although the frank loss of neurons and glia in the infarct core is permanent, the long-term neurological impairment may be mediated partially by loss of myelin function in non-necrotic central nervous system tissue.[2,3] Significant white matter involvement occurs in the majority of strokes[4] and has been linked to poorer long-term functional outcomes.[5,6] Additionally, oligodendrocytes have been shown to be particularly sensitive to ischemic insult.[7]

The potassium channel blocker, 4-aminopyridine, known in the United States by its nonproprietary drug name dalfampridine, and in the rest of the world as fampridine, has been studied for many years for its potential to improve conduction in demyelinated axons.[8–10] These effects have also been explored in a variety of models of injury and demyelination,[11–14] and dalfampridine has been explored clinically in a range of neurological conditions, including spinal cord injury and multiple sclerosis.[15–17] An extended-release formulation of dalfampridine is available in the United States and other countries to improve walking in patients with multiple sclerosis.[17,18]

It has also been demonstrated that endogenous plasticity and remodeling occurs in both the ipsi- and contralateral hemispheres after ischemic stroke.[19] Dalfampridine may enhance function by enabling activation of intact pathways that may normally require a greater initial stimulus to propagate an

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impulse. Reducing the loss of potassium could alter the polarization potential of the membranes and lower the activation threshold of existing fibers, allowing action potential conduction at a lower level of stimulation than would normally be required.[20]

The current studies used a rat permanent middle cerebral artery occlusion (pMCAO) model of stroke to evaluate the effects of dalfampridine on sensorimotor function at a time when endogenous recovery has stabilized. This is the first demonstration that dalfampridine can reverse chronic neurological deficits after stroke, possibly through enhanced conduction in areas of myelin damage or other intact pathways. These results, coupled with a clinically available formulation of dalfampridine, lend a strong rationale for the further evaluation of dalfampridine in stroke patients with chronic neurological deficits.

Methods

Permanent Middle Cerebral Artery Occlusion

All animal procedures adhered to the Guide for the Care and Use of Laboratory Animals and used the minimal number of animals to appropriately power the studies. These procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Biotrofix, Inc.

Young adult male Sprague-Dawley rats (300–350 g, 66–77 days old; Charles River Laboratories, Wilmington, MA) were anesthetized with 1% to 3% isoflurane delivered in N_2O:O_2 (2:1) via face mask, and focal cerebral infarcts were made by permanent occlusion of the proximal right middle cerebral artery using a modification of the method of Tamura et al.[21] Briefly, the temporalis muscle was bisected and reflected through an incision made midway between the eye and the ear canal. The proximal middle cerebral artery was exposed through a subtemporal craniectomy without removing the zygomatic arch and without transecting the facial nerve. The artery was then occluded by microbipolar coagulation from just proximal to the olfactory tract to the inferior cerebral vein and was transected. Body temperature was maintained at 37.0±1°C throughout the entire procedure. Day of surgery was designated as day 0.

Randomization and blinding

Animals were randomly assigned to treatment groups (http://www.graphpad.com/quickcalcs/randomize1.cfm) for both the double cross-over and dose-escalation studies postsurgery but before initiation of dosing. In the double cross-over study, no animals were euthanized or replaced, resulting in 15 animals per group at the initiation of dosing on day 30. In the dose-escalation study, animals were replaced if they were euthanized or died before randomization. Animals were excluded before randomization if they did not meet required performance criteria (defined as day 49 forelimb placing scores >2 and <6.5). The remaining animals were randomly assigned to treatment, resulting in 11 animals per group.

Dose solutions were administered by staff with no knowledge of the identity of the test solutions. Functional assessments were performed by observers blinded to the treatment assignment of the animals.

Dose Solution Preparation and Administration

Dalfampridine (Regis Technologies Inc, Morton Grove, IL) was dissolved in water for injection (Cellgro) and sterile filtered. For the double cross-over study with 3 treatment phases and washout periods between dosing, final concentrations of 0.315 mg/mL or 1.0 mg/mL dalfampridine were delivered at 2 mL/kg by oral gavage, resulting in final doses of 0.63 mg/kg and 2 mg/kg, respectively. For the dose-escalation study, solutions of 0.25 mg/mL, 0.5 mg/mL, and 1.0 mg/mL dalfampridine were delivered by oral gavage at 2 mL/kg for final doses of 0.5 mg/kg, 1 mg/kg, or 2.0 mg/kg, respectively. For both studies vehicle control treatment was water (water for injection, Cellgro) delivered at 2 mL/kg by oral gavage.

Treatment Schedule

Double Cross-over Study

This study was divided into 3 treatment phases (I–III), with each randomized cohort of animals (n=15) receiving a different dose level during each of the treatment phases (Figure 1). Starting on day 30 after pMCAO (day 30, start of phase I), the animals received gavage dosing of solutions (2 mL/kg) twice daily ≈12 hours apart (BID), for a total of 5 doses over 3 consecutive days. The same schedule was repeated with different treatments on day 44 and day 58 for phase II and III of the study, respectively. Animals were not treated during the 10 days between phases (washout period). See Figure 1A for a schematic of the study design.

Dose-escalation Study

Starting on day 56 after pMCAO, animals received oral gavage of solutions (2 mL/kg) BID. The vehicle control group (n=11) was treated with water for all doses on days 56 to 65. For the treated group (n=11), 6 doses of dalfampridine at 0.5 mg/kg BID were delivered over days 56 to 59, followed by 6 doses at 1.0 mg/kg BID over days 59 to 62 and 6 doses at 2.0 mg/kg BID over days 62 to 65. Animals in all groups were not treated during days 66 to 70. See Figure 1B for a schematic of the study design.

Behavioral Testing

Blinded assessments of sensorimotor function were performed just before pMCAO surgery, 24 hours after pMCAO surgery, and weekly thereafter until the first phase of dosing, using limb-placing and body-swing behavioral tests. In the double cross-over study, the animals were tested 1 hour after the first and fifth doses of each phase (days 30 and 32 of the first phase, days 44 and 46 of the second phase; and days 58 and 60 of the third phase). Animals were also tested during the washout periods on days 42 and 56. On days when blood was also collected, behavioral testing was completed before blood draw. In the dose-escalation study, animals were tested 1 hour after the sixth dose of each dose level (on days 59, 62, and 65) and 5 days after the end of the treatment period.

Forelimb and Hindlimb Placing

The forelimb placing test scored the rat's ability to place its forelimb on a tabletop in response to whisker, visual, tactile, or proprioceptive stimulation. The hindlimb placing test scored the rat's ability to place its hindlimb on the tabletop in response to tactile and proprioceptive stimulation. Together, these tests reflect function and recovery in the sensorimotor systems.[22] Separate subscores were obtained for each mode of sensory input (half-point designations possible), and added to give total scores (for the forelimb placing test: 0=normal, 12=maximally impaired; for the hindlimb-placing test: 0=normal; 6=maximally impaired). Tests were performed 1 day before surgery (day −1) and then on days 1, 7, 14, 21, and 28 after pMCAO, then periodically depending on the study and dose regimen described above.

Body-Swing Test

Each rat was held along the vertical axis (defined as no more than 10° to either the left or the right side) ≈1 inch from the base of its tail and elevated an inch above a table surface. A swing was recorded whenever the rat moved its head out of the vertical axis to either side. The rat had to return to the vertical position for the next swing to be counted. Thirty (30) total swings were counted. This test reflects symmetry of striatal function,[23] and a normal rat typically has an equal number of swings to either side. After focal ischemia, a rat tends to swing to the contralateral (left) side. The test was performed

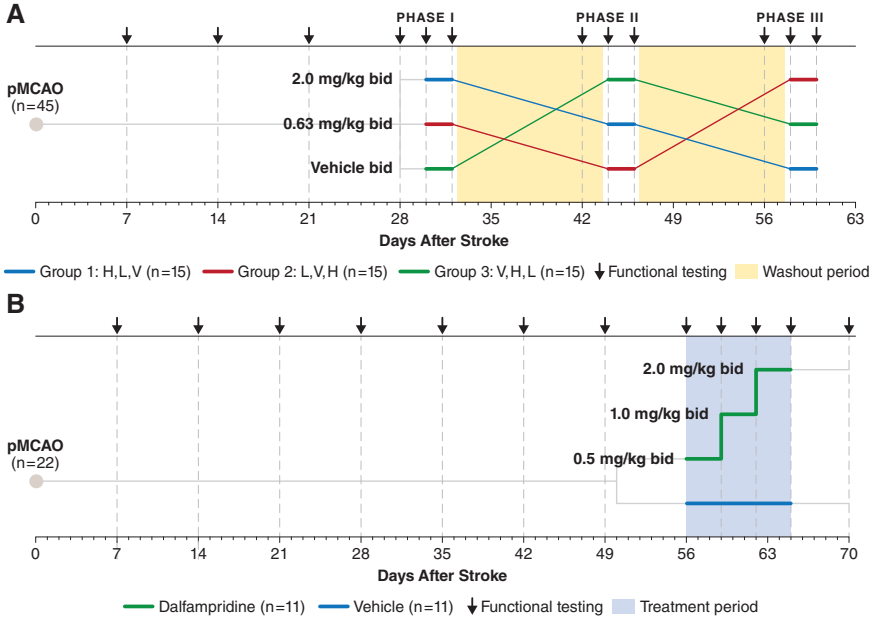

Figure 1. Schematic of the study designs. **A**, Double cross-over study: rats in each group recovered for the first 28 days after permanent middle cerebral artery occlusion (pMCAO) surgery with weekly behavioral assessments. On day 30, they were randomly assigned to the first dose level starting on day 30 and received 5 doses over 3 days, each ≈12 hours apart (phase I). Behavioral assessments were performed after the first and fifth doses in each dosing phase. After a 10-day washout period (shaded area) animals were again evaluated with behavioral tests on day 42 before starting treatment with their phase II dose level on day 44. Treatment continued as described previously with functional testing after the first and fifth doses. Dose-level cross-over was repeated once more, with each group receiving the final dose level it had not been exposed to yet as the treatment in phase III. **B**, Dose-escalation study: rats in each group recovered for the first 56 days after pMCAO with weekly behavioral assessments. On day 56 rats were randomly assigned to dalfampridine or vehicle, based on functional inclusion criteria from day 49 behavioral assessment (Methods). Rats in the dalfampridine-treated group received 0.5 mg/kg twice a day (BID) for a total of 6 doses, followed by 1.0 mg/kg BID for 6 doses, and then 2.0 mg/kg BID for the final 6 doses. Animals receiving vehicle were treated at the same time points as dalfampridine-treated animals. During the treatment period, functional testing was performed after the sixth dose for each dose level and again 5 days after the last treatment.

at the same time as the limb-placing tests in both the double cross-over and dose-escalation study.

Blood Collection and Dalfampridine Plasma Analysis

Approximately 300 µL of blood was collected from the saphenous vein of each animal on days 30, 32, 44, 46, 58, and 60, 90 minutes after dosing for the double cross-over study. The same volume was collected on day 56 just before the first dose and then 90 minutes after the sixth dose at each dose level in the dose-escalation study. Samples were collected in K3 EDTA tubes and centrifuged at 10 000 rpm for 10 minutes at 4°C. Plasma was obtained, frozen, and stored at approximately –80°C. Samples were shipped on dry ice to Covance, Inc (Madison, WI) for determination of dalfampridine concentration, using a validated liquid chromatography with tandem mass spectrometric detection method in positive electrospray mode.

Euthanasia and Tissue Handling

Rats were anesthetized and exsanguinated by transcardial perfusion with normal saline (with heparin, 2 U/mL) followed by perfusion with 4% paraformaldehyde on day 63 for the double cross-over study and on day 70 for the dose-escalation study. Brains were harvested and processed for histological assessment.

Infarct Measurement

For both studies, fixed brains were embedded in paraffin and 5-µm thick coronal sections were cut using a microtome. Sections were stained with hematoxylin and eosin, using standard methods. Seven coronal sections (bregma +4.7, +2.7, +0.7, −1.3, −3.3, −5.3, and −7.3) from each brain were photographed by a digital camera, and the infarct areas determined by National Institutes of Health Image (Image J Bethesda, MD) using the indirect method (area of the intact contralateral [left] hemisphere minus the area of intact regions of the ipsilateral [right] hemisphere) to correct for brain edema. Infarct areas were then summed among slices and multiplied by the distance between sections to give total infarct volume, which was expressed as a percentage of intact contralateral hemispheric volume.

Statistical Methods

Double Cross-over Study

Change from baseline behavior values were calculated for each treatment group in each phase. Baseline is defined as the behavioral value measured while animals receive no treatment before starting a dosing phase (days 28, 42, and 56 for phase I, II and III, respectively). Mean behavioral parameter data for each entire dosing phase were subject to ANOVA. Data were also subjected to mixed-model analyses examining dose, sequence, carryover effect, and phase of the experiment as covariates using SAS pair-wise comparisons between each pair of treatments using a difference in least squares from the mean method. Values of $P<0.05$ were considered statistically significant. See online-only Data-Supplement information for more detail.

Dose-escalation Study

Raw behavioral scores were compared between dalfampridine and vehicle groups at each time point assessed after dosing. Mean behavioral data were compared by *t* test and ANOVA. In this situation ANOVA is appropriate for ordinal data.[24] Furthermore, data were reanalyzed using a Bonferroni step-down procedure with similar results. When analyzed as change from baseline scores (day 56) the lowest dose of the treated animals also reached significance, however, the stricter interpretation of comparison of means is shown.

Infarct volume data were analyzed by ANOVA. All data were expressed as means±SEM.

Results

Limb-Placing and Body-Swing Tests

Double Cross-over Study

Treatment was initiated at 4 weeks after stroke. All groups (1–3) demonstrated a typical recovery response to the pMCAO-induced ischemia with normal scores of 0 just before the surgery (day −1) followed by a complete loss in function (score 12, forelimb; 6, hind limb) measured at 24 hours after the occlusion (Figure 2, day 1). During the next 4-week untreated phase, forelimb and hindlimb scores improved to ≈5.5 and 3, respectively and approached a plateau level of recovery (Figure 2A and 2B). In the body-swing test, animals displayed <5% swings to the right the day after surgery and had recovered to ≈25% right swings by the end of the 4-week untreated period (Figure 2C). Group 1 animals (Figure 2A–2C, blue circle with dashed line) received dalfampridine at 2 mg/kg during the first dosing phase and showed significant improvements in forelimb, hindlimb, and body-swing scores compared with pretreatment baseline scores (day 28 versus day 32; $P<0.05$). Between dosing phases I and II (washout period, days 33–42), the effects on limb placing returned to near baseline levels. During the second dosing phase, animals in group 1 received dalfampridine at 0.63 mg/kg. All behavioral scores were significantly improved compared with scores during the washout just before dosing (day 42 versus day 46; $P<0.05$), though they did not achieve the same degree of improvement as during the first higher-dose phase. During the washout between the second and third phases (days 47–56) the behavioral scores declined to a level similar to baseline scores (day 56). Animals in this group received vehicle during the third dosing phase and saw no change in behavioral scores compared with the day immediately before dosing (on day 56).

Group 2 animals (Figure 2A–2C, open red square, dash-dot line) receiving dalfampridine at 0.63 mg/kg during the first dosing phase showed significantly improved behavioral scores in all measures compared with pretreatment baseline scores (day 28 versus day 32; $P<0.05$). Between dosing phases I and II, while animals were not on the drug, the effects on behavior declined to levels similar to prephase dosing (day 42). During dosing phase II, animals in this group received vehicle, and demonstrated no change in behavioral testing scores. They remained at that baseline level of function during the washout between phases II and III (days 47–56). Animals in this group received dalfampridine at 2 mg/kg during phase III of dosing and all behavioral testing scores were significantly improved compared with prephase baseline scores (day 56 versus day 60; $P<0.05$).

Animals in group 3 (Figure 2A–2C, green diamond, solid line) had results similar to those seen in group 1 and 2 during the different treatment phases. These animals received vehicle during phase I. There was no change in any behavioral score and animals stayed at this level of function through the washout between phases I and II. Dalfampridine treatment at 2 mg/kg during phase II and 0.63 mg/kg during phase

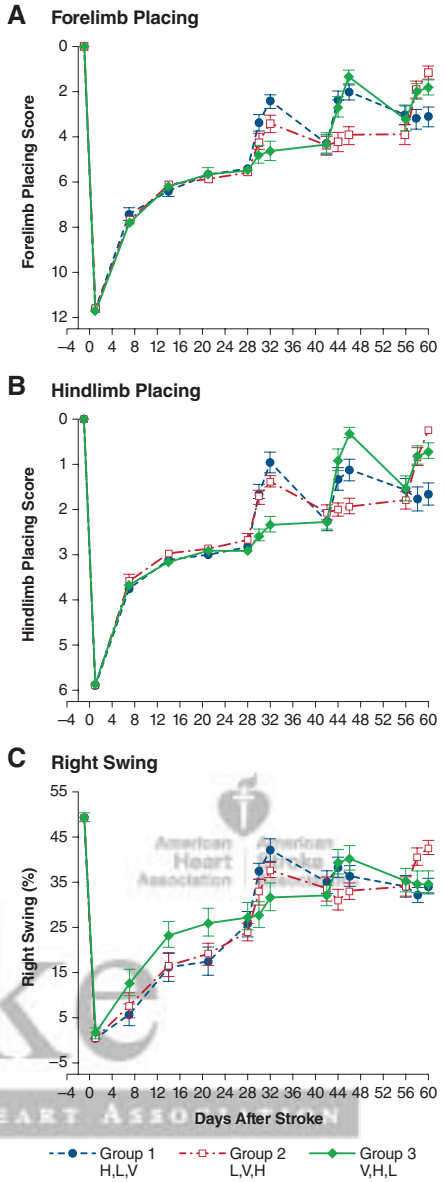

Figure 2. Double cross-over study: limb-placing and body-swing scores. **A–C**, Sensorimotor function was evaluated using forelimb- and hindlimb-placing and body-swing tests. All groups (n=15/group) demonstrated normal scores just before the surgery (day −1) followed by a substantial loss in function within 24 hours (day 1), which recovered partially and approached a plateau around day 30 when treatment was initiated. Treatment phases occurred on days 30 to 32, days 44 to 46, and days 58 to 60. The dose order received by each respective group is indicated by H, L, or V, with H as 2 mg/kg dalfampridine, L as 0.63 mg/kg dalfampridine, and V as vehicle treatment (eg, group 1 [blue circle with dashed line] received treatments in the order of 2 mg/kg dalfampridine, 0.63 mg/kg dalfampridine and vehicle, indicated as H, L, V). All groups responded similarly to the respective treatments regardless of the order in which they were treated, with the greatest improvements seen during the high-dose treatment. In all cases, the highest dose during any dosing phase resulted in significant improvements compared with vehicle and the lower dose; and the lower-dose group was statistically better or trended toward significance compared with vehicle, depending on the statistical model used (ANOVA or mixed-model analysis, see online-only Data-Supplement material). During washout and vehicle-treatment periods functional scores returned to untreated levels. Data are expressed as means±SEM.

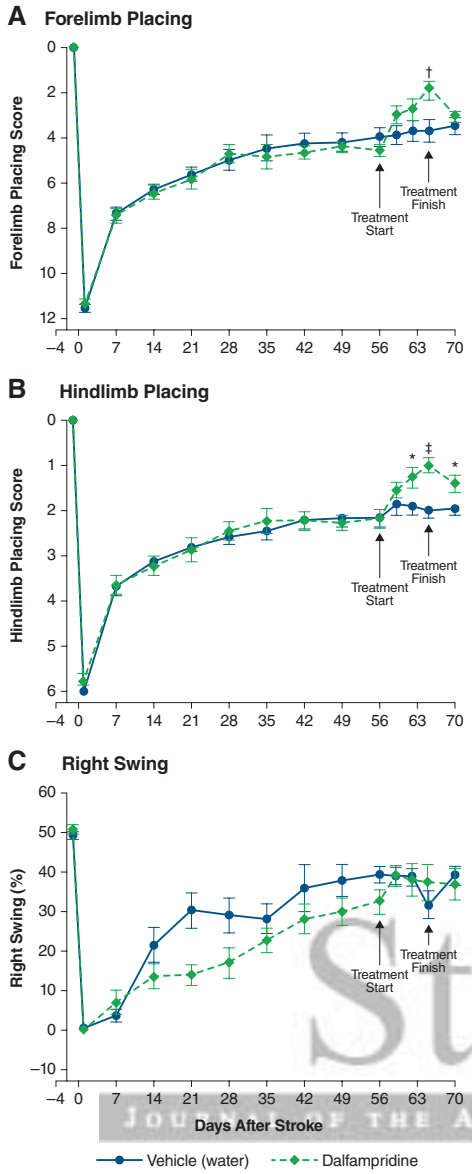

Figure 3. Dose-escalation study: limb-placing and body-swing scores. Sensorimotor function was evaluated using forelimb- and hindlimb-placing and body-swing tests. All groups (n=11/group) demonstrated normal scores just before the surgery (day −1) followed by a substantial loss in function within 24 hours (day 1) that recovered partially and approached a plateau by day 30. Animals remained at this level of function through day 56 when treatment was initiated. Dalfampridine-treated animals (green diamonds, dashed line) first received 0.5 mg/kg dalfampridine, which improved the forelimb- or hindlimb-placing scores, but not significantly (**A** and **B**; day 59). Increasing the dose to 1 mg/kg resulted in a measureable improvement in the hindlimb test (**B**, day 62; $P<0.05$), but not the forelimb test. The final dose escalation to 2 mg/kg dalfampridine demonstrated highly significant improvements in both the forelimb and hindlimb function (**A** and **B**, day 65; $P<0.005$ and $P<0.0005$, respectively). Withdrawal of treatment for 5 days, resulted in a decline in the raw scores, though they were still improved in the hindlimb compared with the vehicle group (**B**, day 70; $P<0.05$). Vehicle-treated animals (blue circles, solid line) remained stable in their deficits during the course of treatment. The body-swing performance has not been extensively characterized at these later time points, and although there was a treatment effect at the first on drug assessment (day 59) compared with the pretreatment score on day 56, no conclusions could be drawn with comparison with vehicle treatment (**C**). Data expressed as means±SEM *$P<0.05$; †$P<0.005$; ‡$P<0.0005$.

III produced significant improvements in limb placing compared with the off-drug assessments just before each phase (day 42 versus day 46 and day 56 versus day 60, respectively; $P<0.05$). Body-swing scores were improved during the high-dose treatment in the phase II (day 42 versus day 46; $P<0.05$), but were unchanged with the low-dose treatment during the third treatment phase. There was a return to baseline behavior during the washout between phases II and III (day 56).

Taken together, all animals responded similarly to the respective treatments regardless of the order in which they were treated. In all cases, the highest dose during any dosing phase resulted in significant improvements ($P<0.05$) compared with both the vehicle and the lower dose. The lower dose was statistically better or trended toward significance compared with vehicle, depending on the statistical model used (ANOVA or mixed-model analysis, see Statistical Methods and online-only Data-Supplement information).

Dose-escalation Study

All animals demonstrated a typical recovery response to the pMCAO-induced ischemia with normal scores of 0 just before the surgery (day −1) followed by a complete loss in function (score 12, forelimb; 6, hindlimb) measured at 24 hours after the occlusion (Figure 3; day 1). During the next 8-week, untreated phase, forelimb and hindlimb scores improved to ≈4.5 and 2.5, respectively, and approached a plateau level of recovery (Figure 3A and 3B). Treatment was initiated on day 56 after pMCAO. The vehicle group demonstrated small and statistically insignificant changes in behavior compared to the last assessment prior to dose initiation. Animals that received 0.5 mg/kg dalfampridine (low dose) had improved limb placing scores, but they were not significant compared with vehicle-treated animals. Increasing the dose to 1 mg/kg resulted in a measureable improvement in the hindlimb test ($P<0.05$; Figure 3B, Day 62) compared with vehicle-treated animals. The final dose escalation to 2 mg/kg dalfampridine was associated with significant improvements in the forelimb and hindlimb functions ($P<0.005$ and $P<0.0005$, respectively; Figure 3A and 3B, Day 65) compared with vehicle-treated animals. When treatment was withdrawn for 5 days, the improvements partially declined in the raw scores, though the hindlimb scores were still greater than the vehicle treated group ($P<0.05$, Figure 3B, Day 70). Only slight improvements were seen in vehicle-treated animals during the entire course of the treatment phase.

The body-swing performance has not been extensively characterized at these later time points, and although there seems to be a treatment effect at the first on-drug assessment compared with the pretreatment score on day 56, no conclusions can be drawn from the data as a whole, in light of the divergence of the body-swing asymmetry observed between the vehicle and dalfampridine groups before treatment initiation (Figure 3C).

Dalfampridine Plasma Levels

In both the double cross-over and dose-escalation studies, samples drawn when the animals were receiving vehicle

Table 1. Mean (SE) Dalfampridine Plasma Levels: Treatment Phase

Double Cross-over Study	Treatment Phase		
Group	Phase I	Phase II	Phase III
Group 1-H, L, V	142.4 (6.7)	64.9 (2.3)	BLOQ
Group 2-L, V, H	78.1 (10.3)	BLOQ	144.1 (7.4)
Group 3-V, H, L	BLOQ	128.6 (5.6)	61.8 (3.3)

Values are expressed as ng/mL. In the double cross-over study dalfampridine plasma levels confirmed exposure in the animals at 90 min after dose administration. For each dosing phase, the means of the samples collected at each of the 2 time points during drug administration correlates with the dose level (V, L, or H representing vehicle, 0.63 mg/kg dalfampridine, or 2.0 mg/kg dalfampridine, respectively). Vehicle-treated animals had undetectable plasma levels of dalfampridine.

BLOQ indicates below lower limit of quantitation (<1.0 ng/mL).

treatment had dalfampridine plasma levels below the lower limit of quantitation for the method. Samples drawn when animals received dalfampridine confirmed exposure at the time of behavioral testing appropriately related to dose level (Tables 1 and 2).

Histology

Mean infarct volumes (% of contralateral hemisphere) were not different between any of the groups in either study (Table 3).

Discussion

There has not been extensive research on therapies to improve deficits that remain during or after the normal period of endogenous recovery in stroke patients, other than various forms of physical rehabilitation. The rat pMCAO model used here showed a recovery pattern that, in many ways, parallels the typical pattern of neurological recovery in humans after stroke. After pMCAO there is a substantial loss of sensorimotor function at day 1 after surgery, as measured with specific tactile, proprioceptive, and sensory tests (forelimb and hindlimb placing and body-swing symmetry). This is followed by a relatively rapid partial recovery period during the first several weeks. A similar, but slower recovery pattern occurs in humans during the first several months after stroke.[25] In this rat model the recovery begins to plateau by 4 weeks after pMCAO, at which time there are still measureable deficits in sensorimotor function. In the double cross-over study,

Table 2. Mean (SE) Dalfampridine Plasma Levels: Dose Level

Dose-escalation Study	Dose Level, mg/kg		
Treatment	0.5	1.0	2.0
Dalfampridine	68.3 (3.3)	114.0 (5.5)	184.7 (13.1)
Vehicle (water)	BLOQ	BLOQ	BLOQ

Values are expressed as ng/mL. In the dose-escalation study, a single blood sample was collected 90 minutes after the last administration of each dose level (0.5 mg/kg, 1.0 mg/kg, and 2.0 mg/kg dalfampridine or vehicle). Plasma levels demonstrated a corresponding increase in dalfampridine concentration with each escalation. Vehicle-treated animals had undetectable plasma levels of dalfampridine.

BLOQ indicates below lower limit of quantitation (<1.0 ng/mL).

Table 3. Mean Infarct Volume

Group	Mean (SE) Infarct Volume, %
Double cross-over study	
Group 1-H, L, V	45.0 (1.8)
Group 2-L, V, H	41.4 (2.3)
Group 3-V, H, L	39.0 (3.3)
Dose-escalation study	
Vehicle (water)	38.5 (2.4)
Dalfampridine	40.0 (2.3)

The infarct area on hematoxylin and eosin stained sections of brain was determined by National Institutes of Health Image (Image J) using the indirect method (area of the intact contralateral [left] hemisphere minus the area of intact regions of the ipsilateral [right] hemisphere). Infarct areas were then summed among sections and multiplied by separation between sections to give total infarct volume, which was expressed as a percentage of intact contralateral hemispheric volume. There was no difference between the groups in either the double cross-over study or the dose-escalation study. Data are expressed as means±SEM.

treatment was initiated at this 4-week time point. Although not statistically significant, there was a slight improvement from baseline (pretreatment) behavioral measures during the drug-free periods between phases I and II and phases II and III. This may be because of slow continued endogenous recovery, training effects of repeated behavioral assessments, and possibly carryover effects of treatment. For these reasons, treatment in the dose-escalation study was initiated on day 56, at a time point even more remote from the initial ischemic event, to allow the animals to reach a more stable level of sensorimotor deficit after endogenous recovery. Additionally at this later time, to limit potential floor and ceiling effects, a performance criterion was applied, requiring day 49 forelimb placing scores to be >2 and <6.5 for randomization into the treatment phase of the study.

In the current studies, dalfampridine was administered twice a day to rats with MCAO. The doses chosen were based on previous animal experiments, which have typically used dosing in the range of 0.5 to 2 mg/kg.[26–28] With a short time to peak and a short half-life of 1 to 1.5 hours (unpublished Acorda good laboratory practices toxicology studies, but similar to guinea pigs and dogs),[29,30] this regimen would not be expected to sustain long-term plasma levels of the compound, but it did allow for repeated daily exposure in the animals and significant plasma levels at the time of behavioral evaluation. Behavioral evaluations were performed at 1 hour after dosing to ensure adequate exposure during the time of assessment and the 3-day sequence for each dosing phase may have helped adapt the animals to the stress of oral gavage before conducting behavioral assessments. Blood was drawn 30 minutes later to confirm a dose-associated level of dalfampridine in the animals, on completion of behavioral assessments (Tables 1 and 2). It must be noted that it is not possible to equate the doses used here or the plasma concentrations obtained with what would be expected in patients treated with a sustained-release formulation of the drug, where the pharmacokinetics are very different. Notably, there is a delay in the peak concentration measured in cerebrospinal fluid compared with that in the

blood, which is approximately an hour in human subjects.[31] This delay also leads to a markedly reduced peak plasma concentration in the cerebrospinal fluid (≈50% in the human after a 2-hour intravenous infusion). Therefore, the concentration of dalfampridine achieved in the central nervous system for a given plasma level is likely to be much less for a transient plasma peak after gavage compared with a similar plasma concentration maintained at steady state. The plasma concentrations that are effective clinically in multiple sclerosis are in the range of 20 to 30 ng/mL, which are lower than the peak levels achieved with even the lowest doses used here in the rat (≈60 ng/mL) though the associated peak CSF levels would be expected to be more similar, based on the observation of delayed transport across the blood–brain barrier in both rats and human subjects[31,32] and the significantly different duration of plasma peak concentration. Higher doses (2.5–6×)[15,33] of the clinical formulation have been studied in human subjects with spinal cord injury, who do not have the lowered seizure threshold observed in people with multiple sclerosis.

Although lowered seizure threshold is an acute comorbidity in the stroke patient population, this is expected to be less of an issue in people who have experienced a stroke >6 months previously and who have not had a seizure in that time. The safety of dalfampridine in this specific patient population (ie, >6 months poststroke with no history of seizure) is under investigation in a clinical trial. Although not specifically monitored, no seizure activity was observed in these studies during treatment periods, when the animals were frequently assessed for behavior and daily observations.

Both the double cross-over and dose-escalation studies demonstrated significant reversible and dose-dependent improvements in forelimb and hindlimb sensorimotor function during times when dalfampridine was at detectable plasma levels in the animals. The body-swing test in the double cross-over study also indicated dose-dependent effect on recovery of postural function. This may be evidence of effects on tracts in the striatum, or perhaps effects on subcortical white matter areas. Although body-swing asymmetry changes were not interpretable in the dose-escalation study, it is important to note that the size of these animals was considerably greater than in the cross-over study (Table 4). This may have played a role in the general motivation and performance ability of the animals during this particular test.

There was a clear and dose-dependent response to treatment in the double cross-over study, within each group and between groups at each phase. All animals received each of the treatments by the end of the study. In addition to being evaluated weekly before treatment, assessments were performed twice during any given dosing phase in the double cross-over study (after the first and fifth doses). Slight improvements between these scores were noted (Figure 2, eg, group 3, between day 30 and day 32, when animals received vehicle treatment). These improvements could have been because of acclimation to the stress of oral gavage, or perhaps are indicative of a learning response as the animals become familiar with and anticipate the tests. This effect was not observed in the dose-escalation study (Figure 3, see vehicle group, day 56 and on) where the animals were tested just once during each of the 3-day dosing periods. Because the baseline was still slightly improving and all possible dosing sequences were not tested, it was not possible to determine whether a previous exposure to dalfampridine predisposes animals to greater or lesser response when dosed with dalfampridine at a later phase. To eliminate this potential carryover effect from dose order variability, the second study was designed as a dose-escalation study without washout periods.

Table 4. Mean Body Weight at End of Study

Double Cross-over Study (Day 60 Post MCAO)	Mean Weight, g	Dose-escalation Study (Day 70 Post MCAO)	Mean Weight, g
Group		Treatment	
Group 1-H, L, V	495.8	Dalfampridine	516.8
Group 2-L, V, H	483.0	Vehicle (water)	532.3
Group 3-V, H, L	485.1		

Rats at the end of the dose-escalation study were larger than the rats at the end of the double cross-over study (488 vs 525 g on average, respectively). MCAO indicates middle cerebral artery occlusion.

In the dose-escalation study animals were dosed in 3 phases, starting with 0.5 mg/kg and escalating to 1 mg/kg and then 2 mg/kg dalfampridine. With each increase in dose level there was a correlating improvement in limb-placing scores. When treatment was withdrawn for 5 days, these improvements declined, though the hindlimb score remained better than in the vehicle-treated group ($P<0.05$; Figure 3B, day 70). It may be that the prolonged and consistent dose period needed additional time to wash out fully compared with the vehicle-treated group. Given the short serum half-life of dalfampridine it seems more likely that there could be a training effect from the repeated testing, which occurred in a relatively short period of time.

Infarct volume analysis of the brain tissue was included in these studies as a typical outcome measure for preclinical stroke studies. Dalfampridine was not delivered as an acute intervention, but rather as a chronic therapy to improve function in demyelinated and therefore dysfunctional areas of the affected brain. As expected, no differences in infarct volume were observed between any groups within a study, and were also similar between studies. Although necrotic tissue loss because of infarction was assessed by infarct volume measurements in the work presented here, we did not quantify myelin loss or evaluate axonal staining. More sensitive microscopic techniques should be used in future studies to further characterize axonal integrity in areas of hypomyelination at late time points after pMCAO.

The effect of dalfampridine on enhancing function poststroke has been characterized with these studies, and there are several possible mechanisms of action that may be involved in eliciting these improvements. Dalfampridine is a potassium channel blocker and has been studied for many years for its potential to improve conduction in demyelinated axons.[8–10] Oligodendrocytes are particularly sensitive to ischemic and oxidative stress,[7,34,35] and there is a correlation between white matter involvement and poor long-term functional outcomes after stroke.[5,6] It may be that dalfampridine is acting on demyelinated but intact pathways that are functionally impaired yet have synaptic connections in areas remote from the lesion, as

has been demonstrated in spinal cord injury studies.[36,37] If this type of situation exists in ischemic brain lesions, then this may be one way for dalfampridine to have a beneficial effect on function poststroke.

In addition to possible effects on hypomyelinated intact fibers, dalfampridine has been shown to increase the electrical response from the ipsilateral hemisphere when the contralateral tracts are disrupted. Brus-Ramer et al[20] demonstrated that there is endogenous excitability between the primary motor cortices of the ipsilateral and contralateral hemispheres. Unilaterally silencing the pyramidal tracts does not completely abolish perilesional networks in the ipsilateral hemisphere, and transmission through these networks can be enhanced with dalfampridine treatment. Functional MRI studies in both animals and humans with unilateral stroke have also shown that there is endogenous activation of the contralateral motor cortex with ipsilateral motor recovery.[38,39] These intact networks contribute to sensorimotor improvements and represent pathways that may be further enhanced with dalfampridine treatment. Still other studies have also suggested that low doses of dalfampridine may modulate synaptic transmission and have effects on muscle tension,[40] and that this effect is beneficial, independent of its effects on demyelinated axons. It should be noted that the full effects of dalfampridine in the central nervous system independent of demyelination have not been fully explored. In the present study we could not conclude which possible mechanism of action, or combination of mechanisms, may be responsible for the functional improvements that were demonstrated while plasma levels of dalfampridine were detectable.

Enhancement of neuronal excitability at any level from dendritic to synaptic may be part of the mechanism of dalfampridine in these studies. A nonspecific increase in reflex excitability might result in improved scores in the impaired limbs. The body-swing test, however, measures the balance of lateralized sensorimotor activity from the intact and damaged cortices. The observed normalization of the body-swing scores is not consistent with simple hyperexcitability as a mechanism for the dalfampridine response. The body-swing test should not be sensitive to dalfampridine if treatment were simply enhancing overall excitability and reflex gain.

Although much of chronic stroke research has focused on rehabilitative interventions, understanding how pathological processes such as demyelination contribute to long-term impaired function has received little attention. The ability to enhance the function of intact pathways or capitalize on some of the endogenous remodeling that naturally occurs after stroke are viable targets for a therapeutic approach using dalfampridine, given its ability to reduce the stimulation required for activation of those networks. The results presented here are consistent with the premise that by restoring the capability of axons to carry electric impulses with dalfampridine, some therapeutic benefit can be gained. Dalfampridine represents a potential therapeutic opportunity for clinical evaluation in poststroke patients, given that an extended-release formulation is now used to improve walking in patients with multiple sclerosis. Current studies are evaluating the ability of dalfampridine to treat long-term deficits in people poststroke.

Disclosures

Acorda Therapeutics, Inc, markets dalfampridine extended-release tablets. Drs Iaci, Parry, Huang, Blight, and Caggiano are employees and stockholders of Acorda Therapeutics. Dr Finklestein is an acting consultant for Acorda Therapeutics. The other authors have no conflict to report.

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SUPPLEMENTAL MATERIAL:

Statistical Methods Summary – double crossover study

Within each phase, the baseline has been defined as the measurement on the first day of that phase, which is Day 28, Day 42 and Day 56. Change from baseline has been taken as the difference from the measurements on other days to the baseline. The average change from baseline for each subject was calculated by summing the two changes from baseline values within each phase (e.g. Day 30 & Day 32 in phase 1) and divided by two.

Change from baseline was calculated at different phases per treatment group (N=15), regardless of which day within the phase the measurement was taken. Within a phase, one-way ANOVA with treatment as the only covariate was used to compare the means under different treatments. The null hypothesis was the means were all the same under different treatments. With a p-value < 0.01 (for body swing) and < 0.0001 (for hind and forelimb), the statistical significance was strongly demonstrated. We could reject the null hypotheses at the 99% confidence level and concluded that the three dose levels show the significantly different treatment effects on the studied muscle functions.

Two sets of mixed models have been used to further investigate other effects on the outcome. In the first set of mixed models, the outcome variable was the average change from the two post baseline measurements within a phase. Fixed effects included the covariates: “dose”, “seq”, “co” and “phase”. “dose” referred to the three treatments, and “seq” referred to the treatment sequence assigned to each group(i.e. “high-low-vehicle”). “co” was the carry-over effect, defined as the

dose from previous phase, in which the carry-over effect for phase 1 was set to 0. There was only one random effect, id, which was the subject id nested in sequence.

In the second set of mixed models, the outcome variable was the raw change (without taking the average of the two post baseline measurements) from the baseline within a phase. Day as a fixed effect was added into the model so that fixed effects included the covariates: “dose”, “seq”, “co”, “phase” and “day”. “day” was the day when the measurement was taken, and it was nested within the phase. The rest of the fixed effects are the same as those in the previous mixed model. There was only one random effect, id, which was the subject id nested in sequence.

In both mixed models, there were two parts of outputs from SAS obtained. The first part is the “Type 3 Tests of Fixed Effects”. With a statistical significant p-value (• 0.05), a conclusion could be made that the effect is significantly predicting the outcome. The second part was “Differences of Least Squares Means”, in which the pair-wise comparison was conducted between each pair of treatments, i.e. high vs low. p-value less than or equal to 0.05 claims a statistical significance in difference of the outcome under different treatments.

For Forelimb function, the first model has shown that phase, dose and carry-over effect were significant effects but not sequence. High dose significantly improved the forelimb function compared to low ($p = 0.0334$) and vehicle ($p = 0.001$); while low didn’t show statistical significant improvement compared to vehicle at level 0.05. In the second mixed model, day was shown as another significant effect and all three treatments were shown as significantly different from each other (i.e. high vs low, high vs vehicle, and low vs vehicle) with $p < 0.0001$.Overall, for forelimb function, both models have shown that phase, dose and carryover effect were

significant effects but not sequence. Both models have demonstrated that high dose would significantly improve the forelimb function compared to lower dose and vehicle. The second mixed model seems more sensitive to detect the treatment effects based on the smaller p-values.

The same analysis procedure was applied for each outcome measurements, including forelimb, hindlimb and body swing.

For hindlimb function, the first model has shown that phase and dose have significant effects but not sequence or carry-over effect. High dose significantly improved the hindlimb function compared to vehicle ($p < 0.0001$), and low dose significantly improved the hindlimb function compared to vehicle as well ($p = 0.0027$), while high dose didn't show the statistical significance at level 0.05, compare to low dose. In the second mixed model, all effects except sequence showed significant effect, and all three treatments were shown as significantly different from each other (i.e. high vs low, high vs vehicle, and low vs vehicle) with $p < 0.0001$. Overall, for hindlimb function, both models have shown that phase and dose were significant effects. Both models have demonstrated that high dose and low dose would significantly improve the hindlimb function compared to vehicle. The second mixed model seems more sensitive to detect the treatment effects based on the smaller p-values.

For body swing function, the first model has shown that only dose was a significant effect. High dose significantly improved the body swing function compared to vehicle ($p = 0.0131$), and low dose significantly improved the body swing function compared to vehicle as well ($p = 0.033$), while high dose didn't show the statistical significance at level 0.05, compare to low dose. In the

second mixed model, phase was shown as another significant effect. High dose significantly improved the body swing function compared to low ($p = 0.006$) and vehicle ($p < 0.0001$); while low didn't show the statistical significant improvement compared to vehicle at level 0.05. Overall, for body swing function, both models have shown that dose had significant effects.