Memantine Enhances Recovery From Stroke

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Background and Purpose—Stroke treatment is constrained by limited treatment windows and the clinical inefficacy of agents that showed preclinical promise. Yet animal and clinical data suggest considerable poststroke plasticity, which could allow treatment with recovery-modulating agents. Memantine is a well-tolerated N-methyl-D-aspartate glutamate receptor antagonist in common use for Alzheimer disease.

Methods—Memantine, 30 mg/kg per day, or vehicle, was delivered chronically in drinking water beginning >2 hours after photothrombotic stroke.

Results—Although there was no difference in infarct size, behavior, or optical intrinsic signal maps in the first 7 days after stroke, mice treated chronically with memantine showed significant improvements in motor control, measured by cylinder test and grid-walking performance, compared with vehicle-treated animals. Optical intrinsic signal revealed an increased area of forepaw sensory maps at 28 days after stroke. There was decreased reactive astrogliosis and increased vascular density around the infarcted cortex. Peri-infarct Western blots revealed increased brain-derived neurotrophic factor and phosphorylated-tropomyosin-related kinase-B receptor expression.

Conclusions—Our results suggest that memantine improves stroke outcomes in an apparently non-neuroprotective manner involving increased brain-derived neurotrophic factor signaling, reduced reactive astrogliosis, and improved vascularization, associated with improved recovery of sensory and motor cortical function. The clinical availability and tolerability of memantine make it an attractive candidate for clinical translation. (Stroke. 2014;45:2093-2100.)

Key Words: brain-derived neurotrophic factor ★ memantine

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troke is the fourth leading cause of death and a major cause of morbidity worldwide.1 Despite an enormous amount of research, treatment options are limited. Tissue plasminogen activator is restricted to the few hours after stroke; beyond this window, treatment is limited to supportive care, secondary prevention, and rehabilitation.2,3 Neuroprotection, the prevention of cell death beyond the boundaries of the infarct core, has been disappointing in human studies. Yet despite the loss of neuronal tissue, considerable plasticity is retained after stroke. Manipulation of the recovery process has emerged as an alternative and potentially more tractable target in stroke research.4,5 Memantine is a noncompetitive, use-dependent N-methyl-D-aspartate antagonist, which is used to treat Alzheimer disease.5,7 Memantine is neuroprotective, reducing infarct size when given acutely.6,11 However, memantine has mechanisms that may be relevant beyond the acute setting.15,16 We treated mice chronically with memantine, using doses designed to mimic usual serum concentrations in humans,17 delivered to avoid neuroprotection and isolate recovery effects.

Materials and Methods

Protocols were approved by the Animal Research Committee of University of California, Los Angeles.

Focal Ischemia

Male C57BL/6J mice (n=82; 28–32 g) underwent photothrombosis (2-mm diameter irradiation, positioned 1.5 mm lateral from Bregma, 5 minutes after 200 μL of a 10 mg/mL Rose Bengal solution was injected intraperitoneally) or sham treatment as described.4,18 Animals were then allocated to one type of follow-up experiment (behavior, sensory mapping, histology, or Western blot; see below) after treatment with memantine or vehicle.

Memantine Treatment

Mice were randomly assigned to treatment for 28 days with memantine (Sigma; 30 mg/kg per day in 2% sucrose solution)17 or 2% sucrose vehicle delivered continuously in drinking water, beginning 2 hours after photothrombosis. This dosing regimen results in serum concentrations of ≈1 μmol/L in C57Bl/6J mice, comparable with therapeutic concentration in humans.17,19

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2093
Behavioral Testing
Cylinder test and grid-walking test were performed 7 days before and 7, 14, 21, and 28 days after stroke (8 animals/group; Figure 1A). Video analysis was performed by blinded raters.

Cylinder Test
Animals were video-recorded in a clear acrylic cylinder for 10 minutes to determine forelimb preference. Limb contacts were counted and the index for preference was obtained: index=(left−right)/(left+right+simultaneous).20–22 Grid-walking test was performed as described.4,5 Mice walked on an elevated wire grid for 5 minutes while being video-recorded. Total normal steps and foot faults were counted for each limb, and the ratio between foot faults and total steps calculated.

Sensory Mapping
Optical intrinsic signal (OIS) imaging (5 animals/group) was performed 7 days before and 7, 14, 21, and 28 days after focal ischemia, as described.18 Briefly, electric stimulation (50 Hz, 0.001 seconds pulse width, 0.14–0.22 mA) was delivered to the forepaw and hindpaw through pairs of subdermal needle electrodes, while 617-nm reflectance was recorded through the exposed skull. Images were normalized to averaged prestimulus frames. Functional maps were quantified as the area whose pixels showed a stimulus response of >50% of maximum response. Image analysis was performed using either plugins or custom routines written for ImageJ.23

Histology
Full histological methods appear in the online-only Data Supplement. Briefly, mice were perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in phosphate-buffered saline, 7 or 28 days after photothrombosis. Forty-micrometer frozen sections were prepared as described.24 Every third section was collected to quantify infarct volumes using Nissl stain (4 animals, 26 sections each per group). Brightfield immunohistochemistry (4 animals, 26 sections each per group) was performed using biotinylated secondary antibodies, biotin–avidin–peroxidase complex, and diaminobenzidine as the developing agent.25 Primary antibodies were: rabbit anti-glia fibrillary acidic protein (GFAP), rat anti–platelet endothelial cell adhesion molecule-1 (PECAM-1), mouse anti-neuronal antigen (NeuN). Stained sections were examined and photographed using brightfield microscopy. Immunohistochemical images from animals with stroke were analyzed from the border of the glial scar to a lateral distance of 2100 μm, divided into 6 regions for analysis. Sham-treated animals were analyzed over identical cortical regions. Because poststroke scarring causes changes in cortical thickness, all measures were normalized to the area of the region analyzed.

Western Blot
Brains were placed in a mouse matrix in which a 2-mm coronal section was cut, -1 mm to +1 mm from bregma. This section included the whole ischemic area and cognate regions from the contralateral hemisphere (4–6 animals/group; Figure 1A and 1B). Hemispheres were separately frozen in dry ice. Hemispheres were subjected to Western blots using standard methods.26 Primary antibodies used for Western blots were anti-vascular endothelial growth factor (VEGF), anti-glia-derived neurotrophic factor (GDNF), anti–brain-derived neurotrophic factor (BDNF), anti–tyrosine kinase B (TrkB), and antiphospho-TrkB. Quantitative densitometric analyses were performed using the Gel Analyzer Plugin (ImageJ).

Figure 1. A, Timeline. Separate groups of animals were used for each kind of experiment, after identical stroke, recovery, and treatment procedures. Image shows optical intrinsic signal (OIS) functional mapping preparation, including photothrombosis and boundaries of cortex taken for Western blot. B, Poststroke memantine (MEM) treatment shows no structural or cellular evidence of neuroprotection. Representative 40-μm Nissl-stained sections collected 7 days after infarct for vehicle- and MEM-treated animals. Inset: No significant difference in mean (±SEM) infarct area in mm3; 4 animals, ≥6 sections per group. C, Representative mouse anti-neuronal antigen (NeuN)-stained sections from vehicle- and MEM-treated animals. A1–6: regions of interest (ROI); *infarct core. D, Percent area occupied by NeuN-positive cells in 350-μm-wide ROIs, increasing distances from infarct core. There was no significant difference in area between groups (4 animals, ≥6 sections per group). FP indicates forepaw; and HP, hindpaw.
Statistical Analysis
Experiments were performed in accordance with the Animal Research-Reporting In Vivo Experiments (ARRIVE) and National Institute of Neurological Disease and Stroke guidelines. Group sizes were determined from a combination of power analysis (online-only Data Supplement) and our previous work in similar models. Animals were randomized to treatment versus sham groups, and experimenters and raters were blinded to group identity. Comparisons were made with 1- or 2-way ANOVA with post hoc Tukey test for pairwise comparisons. A P value of <0.05 was considered statistically significant. Data are expressed as mean±SEM.

Results

Identical Infarct Size and Neuronal Density in Memantine- and Vehicle-Treated Animals
Consistent with previous work, an infarct centered 1.5 mm lateral to Bregma affected primary motor cortex, as well as forepaw and hindpaw sensory cortex, with hindpaw sensory cortex being more severely affected. There was no difference in infarct size between memantine- and vehicle-treated animals (Figure 1B), consistent with other work in which memantine was given >2 hours after ischemia. NeuN staining revealed no difference in neuronal density between treated and untreated animals (Figure 1C and 1D). Although histological measures alone do not rule out all types of neuroprotection, we inferred that memantine was not exerting significant neuroprotective effects using our treatment regimen.

Improved Forepaw Behavioral Recovery in Memantine-Treated Animals
Despite identical infarct volumes, there was significant improvement in behavioral measures of recovery in memantine-treated compared with vehicle-treated animals. Although both memantine- and vehicle-treated animals showed a significant increase in forelimb use asymmetry after stroke, memantine-treated animals showed a progressive recovery of the impaired limb on cylinder test, which was significant at 28 days after stroke (Figure 2A). For grid-walking test, there was a significant increase in forepaw and hindpaw foot faults after stroke and a subsequent slow recovery of function. Memantine-treated animals showed a greater reduction in forepaw (but not hindpaw) foot faults, which became significant at 28 days after stroke (Figure 2B and 2C). Taken together, these data show that memantine treatment was associated with improved recovery of forepaw, but not hindpaw, function after stroke.

Improved Recovery of Forepaw Sensory Maps With Memantine Treatment
We used OIS to measure the sensory physiology of the peri-infarct cortex during recovery. Forepaw and hindpaw stimulation produced distinct regions of activation in primary somatosensory cortex (Figures 1A and 3A), which were essentially abolished after stroke. Supporting a lack of meaningful neuroprotection, there was no significant difference in area of forepaw or hindpaw activation between memantine- and vehicle-treated animals at 7 days after stroke. Activation area for both forepaw and hindpaw sensory maps slowly increased during stroke recovery, but remained substantially below prestroke conditions for all animals (Figure 3A and 3B). Forepaw maps showed a significant increase in activation area in memantine-treated compared with vehicle-treated animals at 28 days after stroke. There was no significant difference in hindpaw maps with memantine treatment. Our functional activation data thus showed a pattern similar to behavioral data, with improved recovery in forepaw but not hindpaw with memantine treatment.

Although the behavioral and functional activation data were collected in separate animals, we examined whether there was a correlation between the 2 measures. There was a significant negative correlation between OIS response area and forepaw use asymmetry (Pearson correlation coefficient (r)=−0.9413; r²=0.8860; P=0.0169 (2-tailed), 95% confidence intervals...
−0.9962 to −0.3476), supporting an association between functional hemodynamic recovery and behavioral improvement.

**Decreased Astrogliosis and Increased Vascular Density in Peri-Infarct Cortex of Memantine-Treated Animals**

Stroke centered over forepaw motor cortex resulted in a full-cortical-thickness lesion, which at 28 days consisted of a core region of necrotic tissue (stroke+vehicle: 1.13±0.12 mm³; stroke+memantine: 1.22±0.10 mm³), encircled by a border of compact glial scar and a surrounding zone of reactive astrogliosis that exhibited a gradient of elevated GFAP expression (highest near the lesion; indistinguishable from control hemisphere >1770 µm from the lesion border; Figure 4B and 4C). Vascular density, measured by PECAM-1 staining of the endothelium, was maximally decreased in the immediate vicinity of the infarct lesion and became indistinguishable from control hemisphere at distances >2 mm from the lesion border (Figure 5B and 5C). GFAP and PECAM-1 immunoreactivity were significantly altered in memantine-treated animals. Total GFAP-expressing-cell area was significantly reduced in each region of interest, becoming indistinguishable from control hemisphere at 1050 µm from the lesion border (Figure 4D). PECAM-1 expression was significantly increased in memantine-treated versus vehicle-treated animals within the first 3 zones of tissue (0–1050 µm) adjacent to the lesion border (Figure 5D).

**Figure 3.** Significant recovery of forepaw sensory maps with memantine (MEM) treatment. A, Forepaw (FP) and hindpaw (HP) sensory maps in representative vehicle (Veh) and MEM-treated animals. B, Area of activation for FP and HP. There was a significant increase in FP, but not HP, activation area at 28 days after MEM treatment compared with Veh-treated animals (P<0.05, 2-way repeated-measures ANOVA; post hoc Tukey Test; 5 animals per group).

**Figure 4.** Decreased reactive astrocytosis in memantine (MEM)-treated animals. A–C, Representative glial fibrillary acidic protein (GFAP) immunohistochemistry from sham-, vehicle-, and MEM-treated groups at 28 days after phototothrombosis. Area 1 to area 6, Regions of interest; *infarct core; arrows: glial scar. D, There was a significant reduction in percent area occupied by GFAP-positive cells in MEM- compared with vehicle-treated animals (significant differences indicated by asterisks). Inset, No significant difference in the area of the glial scar between MEM- and vehicle-treated animals (1-way ANOVA with post hoc Tukey Test *P<0.05, **P<0.01, ***P<0.001; 4 animals; ≥6 sections each per group).
Increased BDNF Pathway Signaling in Peri-Infarct Cortex of Memantine-Treated Animals

BDNF expression is increased during stroke recovery, and our in glial reactivity and vascular morphology results motivated an examination of GDNF and VEGF. We found no difference in GDNF or VEGF expression by Western blot. However, there was a significant increase in peri-infarct BDNF and phosphorylated-tropomyosin–related kinase-B receptor expression in memantine-treated compared with vehicle-treated animals (Figure 6).

Discussion

We have shown that chronic treatment with a clinically tolerated medication, dosed to deliver concentrations comparable with human use, improves stroke outcomes. The improvements occurred despite the fact that memantine was delivered orally after the stroke, and the lack of any significant difference in infarct size, OIS maps, or behavioral testing in the first 7 days after photothrombosis suggests that neuroprotection did not play a significant role in this improvement. The translational
significance of our findings is 2-fold. First, it suggests a treatment for stroke recovery that is clinically feasible. Second, it suggests that stroke recovery can be improved without the stringent time-dependency of neuroprotective strategies.

Recovery-Promoting Versus Neuroprotective Effects of Orally Dosed Memantine

Memantine is neuroprotective when given before,12 and sometimes within the first 2 hours of stroke.8–10,12,14 Although we cannot completely rule out all aspects of neuroprotection (eg, changes in synaptic physiology or dendritic structure/function),30 we are fairly confident that neuroprotection did not play a significant role in our results, as we observed no significant difference between memantine- and vehicle-treated animals in cell number, behavior, or OIS maps during the first 7 days after stroke. This is important as it allows us to specifically assess memantine effects on poststroke recovery.

Improved Sensorimotor Recovery in Memantine-Treated Animals

Cylinder and grid-walking tests document stroke recovery4,5 and OIS imaging has been used to demonstrate sensory map plasticity after stroke.18,34 We observed improvements in both behavior and OIS after memantine compared with vehicle treatment, with a significant correlation between the 2 measures (albeit collected from separate groups of animals). Interestingly, both tests showed improvement in forepaw, but not hindpaw, behavior and sensory maps. This is likely due to greater destruction of hindpaw cortex by our stroke technique (Figures 1 and 3). Alternatively, memantine is an activity-dependent blocker of N-methyl-D-aspartate receptors35; differential use of the forepaw compared with hindpaw (eg, for exploratory activity) might account for a greater effect on forepaw sensory maps and behavioral function than hindpaw. There are also intrinsic differences in forepaw and hindpaw excitability and plasticity which might explain a difference between the 2 cortices.36

Increased Vascular Density

OIS maps are generated primarily by increases in blood volume or oxygenation specific to the activated region of cortex,37 and functional stroke recovery is correlated with recovery of vascular density in peri-infarct regions.38 We found that PECAM-1 staining, which outlines vascular endothelium, was increased adjacent to the lesion border in memantine-treated compared with vehicle-treated animals. This area corresponds to regions activated on OIS mapping (Figures 1 and 3). Vascular integrity is a prerequisite for survival of peri-infarct tissue and subsequent functional recovery.24,38 Given the use-dependent nature of both poststroke angiogenesis38 and the neurovascular coupling relationship, it is likely that OIS map plasticity, increased vascular density, and behavioral recovery were mutually dependent processes in memantine-treated animals. Regarding mechanism, BDNF signaling is involved in angiogenesis40,41 and BDNF polymorphisms, associated with poor stroke outcomes in humans, show reduced angiogenesis in animal models.32 Our peri-infarct BDNF increases may be relevant in this regard.

Decreased GFAP Expression

GFAP expression is a hallmark of reactive astrogliosis.31 Our observation of a decrease in GFAP immunoreactivity in peri-infarct cortex after memantine treatment is consistent with other reports associating improved stroke recovery with reduced astrocytic reactivity.43,44 Moreover, in a similar animal model, BDNF treatment was associated with a significant decrease in astrogliosis, which is consistent with our results.43 It remains unclear whether decreased astrogliosis is a cause or consequence of the recovery process.

Increased BDNF Signaling

Increases in BDNF expression have been reported in peri-infarct cortex after stroke; attenuation of BDNF activity worsens outcomes.46–48 In human studies, BDNF polymorphisms affect stroke outcomes.49 We observed an increase in BDNF and phosphorylated-tropomyosin-related kinase-B receptor expression in memantine- compared with vehicle-treated animals after stroke. This increase was specific to the infarcted hemisphere, suggesting that signaling related to peri-infarct recovery and plasticity is important.

Memantine may supplement an endogenous tendency toward BDNF increase after stroke: treatment at levels comparable with ours and has been shown to increase BDNF mRNA expression across the brain.15,50 The mechanism of memantine-induced BDNF increase is not clear; BDNF increase has been associated with both activation31,52 and suppression53 of N-methyl-D-aspartate receptor activity. Moreover, such effects might be independent of memantine’s N-methyl-D-aspartate antagonism.54

Conclusions

We have shown that chronic memantine treatment improves stroke outcomes in an apparently non-neuroprotective manner, concomitant with sensory map recovery, decreased reactive astrogliosis, increased vascular density, and increased BDNF/tropomyosin-related-kinase-B receptor expression. Memantine has been used for many years in treatment of Alzheimer and other neurological diseases, and has proved well tolerated in an elderly, medically complex population. Recently, memantine has been used to successfully treat aphasia,55 showing its promise in poststroke populations. Additional evaluation of memantine in the clinical setting may be warranted.

Author Contributions


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Disclosures

None.

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The version of the article, “Memantine Enhances Recovery From Stroke” by López-Valdés et al (Stroke. 2014;45:2093–2100) that published online ahead-of-print on June 17, 2014, contained an error in Figure 6B.

The image of the Western blot was mistakenly inverted right-to-left during figure editing. This resulted in the Western blot image conveying the opposite information to the Western blot quantification in Figure 6A. In addition, lane descriptors for the Western blot were missing. The revised figure corrects these errors. The authors regret any confusion caused, and thank the astute reader who picked up the errors.

This has been corrected in the online version of the article, which is available at http://stroke.ahajournals.org/content/45/7/2093.
SUPPLEMENTAL MATERIAL

**Power analysis.** For behavior experiments we need 8 animals per group to achieve > 80% power (88% calculated), considering the following parameters: $\alpha = 0.05$; effect size = 0.5; 5 repetitive measures; 4 groups, and a correlation between measures = 0.5. For OIS experiments we need 5 animals per group to achieve > 80% power (87% calculated), considering the following parameters: $\alpha = 0.05$; effect size 1; 5 repetitive measures; 2 groups, and correlation between measures = 0.5. OIS parameters were determined from our prior work, in which we demonstrate both significant behavior and OIS effects\(^1\). It should be noted that our prior work examined the effects of stroke itself and not differences in stroke recovery; thus we used much more conservative effect sizes for the present analysis. For immunohistochemistry experiments, with an effect size of 0.37 and $\alpha$ of 0.05, we need 4 animals per group, and 6 measures per animal to achieve 80% power. For Western blot experiments, with an effect size of 0.48 and $\alpha$ (two-sided) of 0.05, we need 4 animals per group to achieve 80% power. Power calculations were performed with G Power Software (version 3.1.5).

**Histology image analysis.** Stained sections were examined and photographed using brightfield microscopy (5X magnification) with the same microscope settings (Zeiss, Oberkochen, Germany) and on the same day. Image analysis was conducted using ImageJ 1.40g\(^2\). Prior to any quantification, contrast in all the original images was automatically and uniformly adjusted. The contralateral hemicortex served as a control. Immunohistochemical images from animals with stroke were analyzed from the end of the scar to a lateral distance of 2100 $\mu$m, divided into 6 regions (350 $\mu$m width, height was thickness of cortex; Figures 1C, 4, 5). Sham treated animals were analyzed over identical cortical regions. Because post-stroke scarring causes changes in cortical thickness, all measures were normalized to the area of the region analyzed. For NeuN quantification the color images were binarized and a minimal threshold size of 7 $\mu$m\(^2\) was established for all images. The percent area occupied by NeuN immunoreactivity in each individual area was measured using ImageJ (Analyze Particles Plugin). For GFAP quantification (Figure 4), the original color images were decomposed into their color channels (blue, green and red) and the green image was chosen for posterior analysis as it exhibited the greatest contrast. An arbitrary threshold of intensity of 100 was established for all images and the percent area occupied by GFAP immunoreactivity in each area was measured automatically. For PECAM-1 quantification (Figure 5) the background for each color was subtracted and the resulted images were binarized. For vascular quantification, in order to evaluate tubelike structures, a minimal threshold of 10 $\mu$m\(^2\) and maximum circularly of 0.5 was established for all images (ImageJ Analyze Particles Plugin). The percent of each area occupied by PECAM-1 immunoreactivity and the size of individual pixel cluster were measured using ImageJ and then averaged. For scar
quantification, the scar was manually traced in GFAP images and area measured using ImageJ. For all quantifications, the pixels were converted to μm (pixel size=0.738 μm).

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