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## Memantine enhances recovery from stroke

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### Abstract

**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 post-stroke plasticity, which could allow for treatment with recovery-modulating agents. Memantine (MEM) is a well-tolerated N-methyl-D-aspartate (NMDA) glutamate receptor antagonist in common use for Alzheimer's disease.

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

**Results**—Though there was no difference in infarct size, behavior, or optical intrinsic signal (OIS) maps in the first seven days after stroke, mice treated chronically with MEM showed significant improvements in motor control, measured by cylinder test and grid walking performance, compared to vehicle treated animals. OIS 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 (BDNF) and phosphorylated-Tropomyosin-related-kinase-B receptor (p-TrkB) expression.

**Conclusions**—Our results suggest that MEM improves stroke outcome in an apparently non-neuroprotective manner involving increased BDNF signaling, reduced reactive astrogliosis and improved vascularization, associated with improved recovery of sensory and motor cortical function. The clinical availability and tolerability of MEM make it an attractive candidate for clinical translation.

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## Keywords

memantine; photothrombosis; stroke recovery; BDNF

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## Introduction

Stroke is the fourth leading cause of death, and a major cause of morbidity worldwide<sup>1</sup>. 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<sup>2,3</sup>. 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<sup>4,5</sup>.

Memantine (MEM) is a non-competitive, use-dependent NMDA antagonist, which is used to treat Alzheimer's disease<sup>6,7</sup>. MEM is neuroprotective, reducing infarct size when given acutely<sup>8-14</sup>. However, MEM has mechanisms that may be relevant beyond the acute setting<sup>15,16</sup>. We treated mice chronically with MEM, using doses designed to mimic usual serum concentrations in humans<sup>17</sup>, delivered to avoid neuroprotection and isolate recovery effects.

## Materials and Methods

Protocols were approved by the Animal Research Committee of UCLA.

### Focal Ischemia

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

### Memantine Treatment

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

### 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 by blinded raters. *Cylinder test*. Animals were video-recorded in a clear acrylic cylinder for 10min in order to determine forelimb preference. Limb contacts were counted and the index for preference was obtained:

$\text{Index} = (\text{Left} - \text{Right}) / (\text{Left} + \text{Right} + \text{simultaneous})^{20-22}$ . *Grid-walking test* was performed as described<sup>4,5</sup>. Mice walked on an elevated wire grid for 5min while being video-recorded. Total normal steps and foot-faults were counted for each limb, and ratio between foot-faults and total-steps calculated.

### Sensory mapping

OIS imaging (5 animals/group) was performed 7 days before and 7,14,21 and 28 days after focal ischemia, as described<sup>18</sup>. Briefly, electrical stimulation (50Hz, 0.001s pulse width, 0.14-0.22mA) was delivered to the forepaw and hindpaw (FP, HP) through pairs of subdermal needle electrodes, while 617nm 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 > 50% of maximum response. Image analysis was performed using either plugins or custom routines written for ImageJ<sup>23</sup>.

### Histology

Full histological methods appear in the **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- $\mu\text{m}$  frozen sections were prepared as described<sup>4,24</sup>. Every third section was collected to quantify infarct volumes using Nissl stain (4 animals,  $\geq 6$  sections each per group). Brightfield immunohistochemistry (4 animals,  $\geq 6$  sections each per group) was performed using biotinylated secondary antibodies, biotin-avidin-peroxidase complex and diaminobenzidine as the developing agent<sup>25</sup>. Primary antibodies were: rabbit anti-GFAP, rat anti-PECAM-1, bio-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 $\mu\text{m}$ , divided into 6 regions for analysis. 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

### Western blot

Brains were placed in a mouse matrix in which a 2mm coronal section was cut, -1mm to +1mm from bregma. This section included the whole ischemic area and cognate regions from the contralateral hemisphere (4-6 animals/group; **Figure 1A,B**). Hemispheres were separately frozen in dry ice. Protein extracts were prepared using standard methods<sup>26</sup>. Primary antibodies used for Western blots were anti-VEGF, anti-GDNF, anti-BDNF, anti-Trk-B, anti-phospho-Trk-B. Quantitative densitometric analyses were performed using the Gel Analyzer Plugin (ImageJ).

### Statistical Analysis

Experiments were performed in accordance with ARRIVE and NINDS guidelines<sup>27,28</sup>. Group sizes were determined from a combination of power analysis (see **Data Supplement**) and our previous work in similar models<sup>4,5,18</sup>. Animals were randomized to treatment vs. sham groups, and experimenters and raters were blinded to group identity. Comparisons

were made with one- or two-way-ANOVA with post-hoc Tukey test for pairwise comparisons. A  $p < 0.05$  was considered statistically significant. Data are expressed as mean  $\pm$  standard error of the mean (SEM).

## Results

### Identical infarct size and neuronal density in MEM- and vehicle-treated animals

Consistent with previous work<sup>4,18</sup> an infarct centered 1.5mm lateral to Bregma affected primary motor cortex as well as FP and HP sensory cortex<sup>29</sup>, with HP sensory cortex being more severely affected. There was no difference in infarct size between MEM- and vehicle-treated animals (**Figure 1B**), consistent with other work in which MEM was given more than 2 hours after ischemia<sup>10,12</sup>. NeuN staining revealed no difference in neuronal density between treated and untreated animals (**Figure 1C,D**). Though histological measures alone do not rule out all types of neuroprotection<sup>30</sup>, we inferred that MEM was not exerting significant neuroprotective effects using our treatment regimen.

### Improved forepaw behavioral recovery in MEM-treated animals

Despite identical infarct volumes, there was significant improvement in behavioral measures of recovery in MEM-treated compared to vehicle-treated animals. Though both MEM- and vehicle-treated animals showed a significant increase in forelimb use asymmetry after stroke, MEM-treated animals showed a progressive recovery of the impaired limb on cylinder test, which was significant at 28 days post-stroke (**Figure 2A**). For grid-walking test, there was a significant increase in FP and HP foot faults after stroke and a subsequent slow recovery of function. MEM-treated animals showed a greater reduction in FP (but not HP) foot-faults, which became significant 28 days after stroke (**Figure 2B,C**). Taken together, these data show that MEM treatment was associated with improved recovery of FP, but not HP, function after stroke.

### Improved recovery of forepaw sensory maps with MEM treatment

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

Though the behavioral and functional activation data were collected in separate animals, we examined whether there was a correlation between the two measures. There was a significant negative correlation between OIS response area and forepaw use asymmetry (Pearson correlation coefficient ( $r$ )=-0.9413;  $r^2=0.8860$ ;  $p=0.0169$  (two-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 MEM-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:  $1.13 \pm 0.12 \text{ mm}^3$ ; stroke+MEM:  $1.22 \pm 0.10 \text{ mm}^3$ ), encircled by an border of compact glial scar and a surrounding zone of reactive astrogliosis<sup>31</sup> that exhibited a gradient of elevated GFAP expression (highest near the lesion; indistinguishable from control hemisphere  $>1770 \mu\text{m}$  from the lesion border; **Figure 4B,C**). Vascular density, measured by PECAM1 staining of the endothelium, was maximally decreased in the immediate vicinity of the infarct lesion and became indistinguishable from control hemisphere at distances  $>2 \text{ mm}$  from the lesion border (**Figure 5B,C**). GFAP and PECAM1 immunoreactivity were significantly altered in MEM-treated animals. Total GFAP-expressing-cell area was significantly reduced in each region of interest, becoming indistinguishable from control hemisphere at  $1050 \mu\text{m}$  from the lesion border (**Figure 4D**). PECAM1 expression was significantly *increased* in MEM-treated vs. vehicle treated animals within the first three zones of tissue (0-1050 $\mu\text{m}$ ) adjacent to the lesion border (**Figure 5D**).

### Increased BDNF pathway signaling in peri-infarct cortex of MEM-treated animals

BDNF expression is increased during stroke recovery<sup>5,32,33</sup>, 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 p-TrkB expression in MEM-treated compared to vehicle-treated animals (**Figure 6**).

## Discussion

We have shown that chronic treatment with a clinically tolerated medication, dosed to deliver concentrations comparable to human use, improves stroke outcome. The improvements occurred despite the fact that MEM 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 two-fold. Firstly, it suggests a treatment for stroke recovery that is clinically feasible. Secondly, it suggests that stroke recovery can be improved without the stringent time-dependency of neuroprotective strategies.

### Recovery-promoting vs. neuroprotective effects of orally-dosed MEM

MEM is neuroprotective when given before<sup>12</sup>, and sometimes within the first two hours after stroke<sup>8-10,12,14</sup>. Though we cannot completely rule out all aspects of neuroprotection (e.g. changes in synaptic physiology or dendritic structure/function)<sup>30</sup>, we are fairly confident that neuroprotection did not play a significant role in our results, as we observed no significant difference between MEM- and vehicle-treated animals in either cell number,

behavior, or OIS maps during the first seven days after stroke. This is important as it allows us to specifically assess MEM effects on post-stroke recovery.

### Improved sensorimotor recovery in MEM-treated animals

Cylinder and grid walking tests document stroke recovery<sup>4,5</sup> and OIS imaging has been used to demonstrate sensory map plasticity after stroke<sup>18,34</sup>. We observed improvements in both behavior and OIS following MEM compared to vehicle treatment, with a significant correlation between the two measures (albeit collected from separate groups of animals). Interestingly, both tests showed improvement in FP, but not HP, behavior and sensory maps. This is likely due to greater destruction of HP cortex by our stroke technique (**Figures 1,3**). Alternatively, MEM is an activity-dependent blocker of NMDA receptors<sup>35</sup>: differential use of the FP compared to HP (e.g. for exploratory activity) might account for a greater effect on FP sensory maps and behavioral function than HP. There are also intrinsic differences in FP and HP excitability and plasticity which might explain a difference between the two cortices<sup>36</sup>.

### Increased vascular density

OIS maps are generated primarily by increases in blood volume or oxygenation specific to the activated region of cortex<sup>37</sup>, and functional stroke recovery is correlated with recovery of vascular density in peri-infarct regions<sup>38</sup>. We found that PECAM1 staining, which outlines vascular endothelium, was increased adjacent to the lesion border in MEM-treated compared to vehicle-treated animals. This area corresponds to regions activated on OIS mapping (**Figures 1,3**). Vascular integrity is a prerequisite for survival of peri-infarct tissue and subsequent functional recovery<sup>24,38</sup>. Given the use-dependent nature of both post-stroke angiogenesis<sup>39</sup> and the neurovascular coupling relationship, it is likely that OIS map plasticity, increased vascular density, and behavioral recovery were mutually dependent processes in MEM-treated animals. Regarding mechanism, BDNF signaling is involved in angiogenesis<sup>40,41</sup>, and BDNF polymorphisms, associated with poor stroke outcome in humans, show reduced angiogenesis in animal models<sup>42</sup>. Our peri-infarct BDNF increases may be relevant in this regard.

### Decreased GFAP expression

GFAP expression is a hallmark of reactive astrogliosis<sup>31</sup>. Our observation of a decrease in GFAP immunoreactivity in peri-infarct cortex after MEM-treatment is consistent with other reports associating improved stroke recovery with reduced astrocytic reactivity<sup>43,44</sup>. Moreover, in a similar animal model, BDNF treatment was associated with a significant decrease in astrogliosis, which is consistent with our results<sup>45</sup>. 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 outcome<sup>46-48</sup>. In human studies, BDNF polymorphisms affect stroke outcome<sup>49</sup>. We observed an increase in BDNF and p-TrkB expression in MEM- compared to 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.

MEM may supplement an endogenous tendency toward BDNF increase after stroke: treatment at levels comparable to ours has been shown to increase BDNF mRNA expression across the brain<sup>15,50</sup>. The mechanism of MEM-induced BDNF increase is not clear: BDNF increase has been associated with both activation<sup>51,52</sup> and suppression<sup>15,53</sup> of NMDA receptor activity. Moreover, such effects might be independent of MEM's NMDA antagonism<sup>54</sup>.

## Conclusions and translational relevance

We have shown that chronic MEM treatment improves stroke outcome in an apparently non-neuroprotective manner, concomitant with sensory map recovery, decreased reactive astrocytosis, increased vascular density, and increased BDNF/TrkB expression. MEM has been used for many years in treatment of Alzheimer's and other neurological diseases, and has proven well tolerated in an elderly, medically complex population. Recently, MEM has been used to successfully treat aphasia<sup>55</sup>, showing its promise in post-stroke populations. Further evaluation of MEM in the clinical setting may be warranted.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Experimental design: HLV,ANC,MVS,STC,KCB; OIS, behavior, Western blot: HLV; surgeries, histology, behavior: ANC; immunohistochemistry: HLV,YA; imaging tools: AC; data analysis: HLV,ANC,YA,MVS,STC,KCB; wrote manuscript: HLV,ANC,MVS, STC,KCB.

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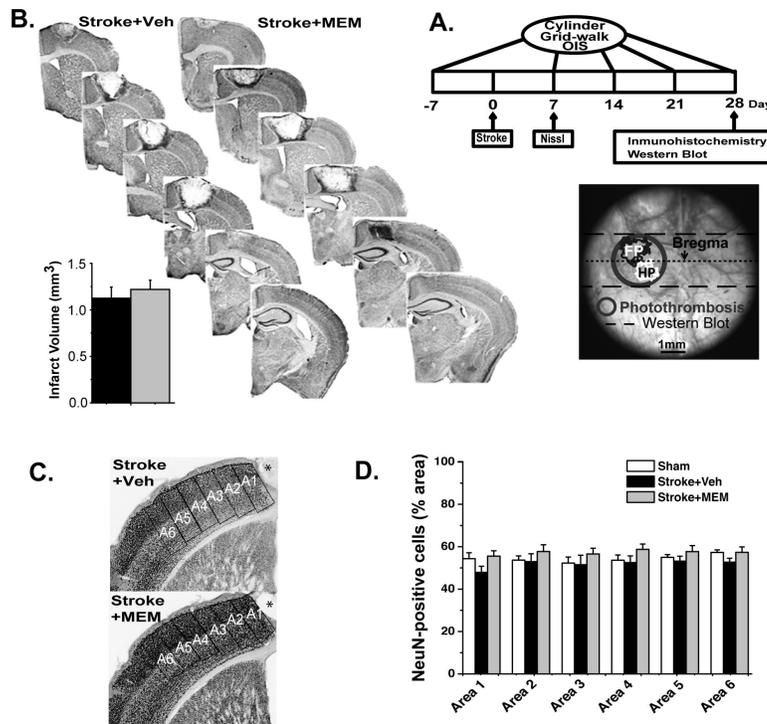
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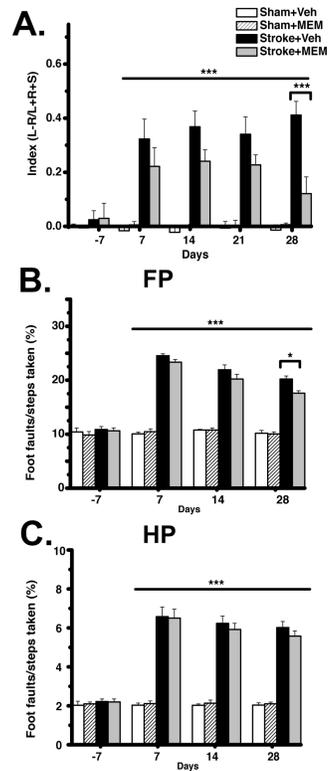
**Figure 1.**

**A.** Timeline. Separate groups of animals were used for each kind of experiment, following identical stroke, recovery, and treatment procedures. Schematic: OIS functional mapping preparation, showing photothrombosis and boundaries of cortex taken for Western blot.

**B.** Post-stroke MEM treatment shows no structural or cellular evidence of neuroprotection. Representative 40μm Nissl-stained sections collected 7 days after infarct for vehicle- and MEM-treated animals. Inset: no significant difference in mean ( $\pm$ SEM) infarct area in mm<sup>3</sup>; 4 animals,  $\geq 6$  sections per group.

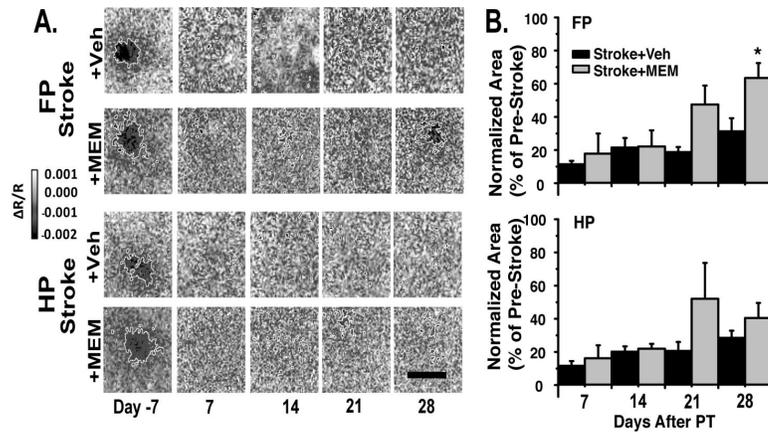
**C.** Representative NeuN-stained sections from vehicle and MEM-treated animals. A1-A6: regions-of-interest (ROI); \*infarct core.

**D.** Percent-area occupied by NeuN-positive cells in 350μm-wide ROIs, increasing distances from infarct core. There was no significant difference in area between groups (4 animals,  $\geq 6$  sections per group).



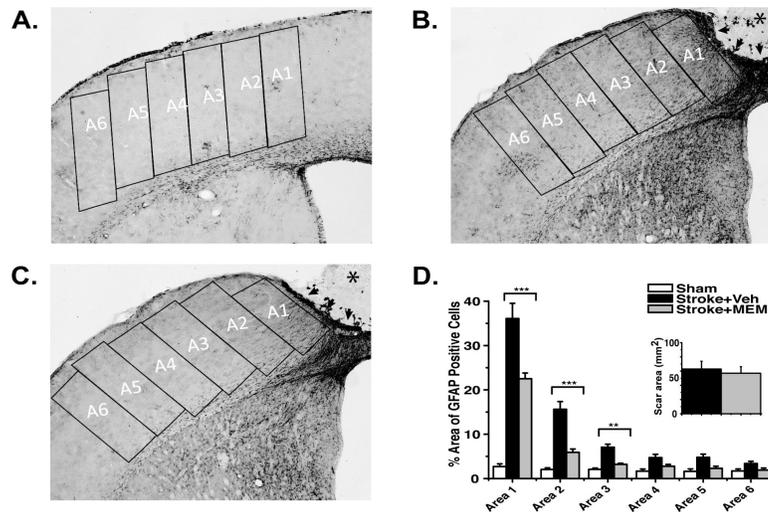
**Figure 2. Improved behavioral recovery from stroke with MEM treatment**

**A.** Cylinder test. Both MEM- and vehicle-treated animals showed significant forepaw use asymmetry after stroke. There was significant improvement in forepaw asymmetry at 28 days of MEM vs. vehicle treatment. **B,C.** Grid-Walking test. Both MEM- and vehicle-treated animals showed a significant increase in foot-faults after stroke. There was a significant reduction in forelimb but not hindlimb foot-faults at 28 days of MEM compared to vehicle treatment (\*\* $p < 0.001$ ; \* $p < 0.05$ , Repeated-measures two-way ANOVA; post-hoc Tukey Test; 8 animals per group).



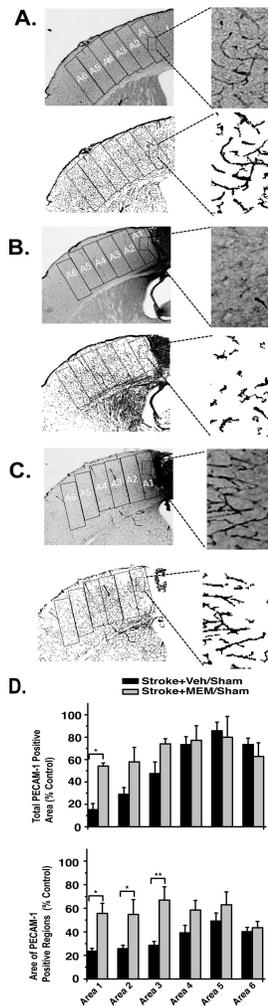
**Figure 3. Significant recovery of forepaw sensory maps with MEM treatment**

**A.** FP and HP sensory maps in representative vehicle 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 of MEM treatment compared to vehicle-treated animals (\* $p < 0.05$ , twoway repeated-measures ANOVA; post-hoc Tukey Test; 5 animals per group).



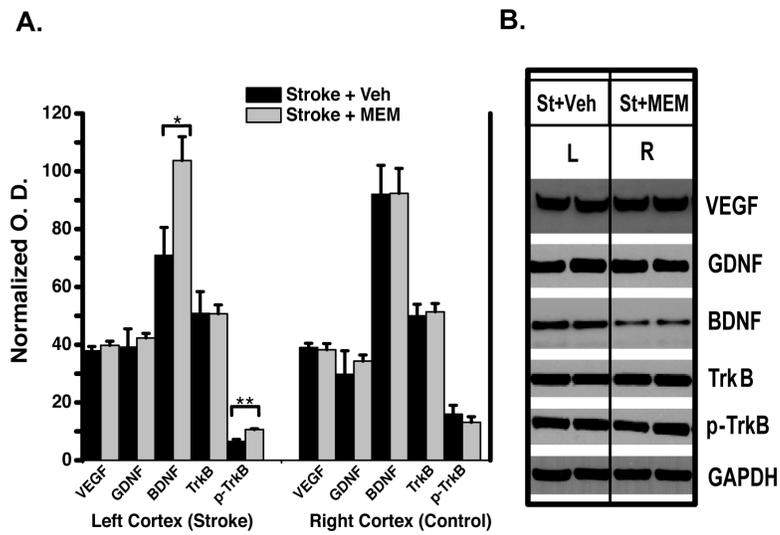
**Figure 4. Decreased reactive astrocytosis in MEM-treated animals**

**A,B,C.** Representative GFAP immunohistochemistry from sham-, vehicle- and MEM-treated groups 28 days after photothrombosis. A1-6: 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 to 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 (One-way ANOVA with post-hoc Tukey Test \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; 4 animals;  $\geq 6$  sections each per group).



**Figure 5. Increased vascular density in MEM-treated animals**

**A,B,C.** Micrographs show representative PECAM-1 immunohistochemistry for sham-, vehicle- and MEM-treated animals, 28 days after photothrombosis. Skeletonized figures in each panel are the same figures after image processing. **D.** PECAM-1 quantification. Percent area occupied by PECAM-1 immunoreactivity in 350 $\mu$ m wide regions of interest beginning at the glial scar margin is a proxy for vascular density; average area of each PECAM-1-positive image region is a proxy for vascular length and diameter. There was a significant difference in both measures between MEM- and vehicle-treated animals (indicated by asterisks). (ANOVA with post-hoc Tukey Test; \* $p < 0.05$ , 4 animals,  $\geq 6$  sections each per group).



**Figure 6. Increased BDNF pathway signaling in MEM-treated animals**

**A.** Quantification (mean optical density, normalized to GAPDH) of Western Blots (representative blots in **B.**). There was a significant increase in BDNF and phospho-TrkB expression, consistent with activation of the BDNF pathway. (\* $p < 0.05$ , Student's t-test; 4-6 animals per group).