Nitric-Oxide Synthase Mediates the Ability of Darbepoetin Alfa to Attenuate Pre-Existing Spatial Working Memory Deficits in Rats Subjected to Transient Global Ischemia

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ABSTRACT

Erythropoietin has been reported to improve the behavioral performance of healthy mice in tests thought to depend on synaptic plasticity in the CA1 region of the hippocampus. We show here for the first time that a single injection of the erythropoietin analog darbepoetin alfa reverses pre-existing cognitive deficits in adult rats that had been subjected to transient global ischemia produced by four-vessel occlusion (4-VO). Quantification of neuronal density demonstrated that 12 min of 4-VO selectively killed more than 90% of CA1 neurons in the dorsal hippocampus. Rats that had sustained a bilateral loss of hippocampal CA1 neurons in this range (4-VO rats) displayed more errors and longer escape latencies in the Barnes maze compared with sham-operated controls. A single injection of darbepoetin alfa (5000 U/kg i.p.) 4 h before behavioral testing decreased deficits in escape latency for 4-VO rats but not sham-operated controls. This improvement in spatial working memory performance was correlated with increased levels of nitric-oxide metabolites in the ventral hippocampus. Systemic administration of the nitric-oxide synthase inhibitor N(G)-nitro-L-nitro-arginine methyl ester reversed the increase in nitric-oxide metabolites and improvements in spatial working memory produced by darbepoetin alfa (5000 U/kg, i.p.) at a dose (10 mg/kg, i.p.) that did not impair the spatial working memory performance of intact rats. Taken together, these findings suggest that darbepoetin alfa reverses pre-existing spatial working memory deficits resulting from transient global ischemia by increasing the activity of nitric-oxide synthase, an enzyme implicated in synaptic plasticity.

Stroke is one of the leading causes of death and a major cause of adult disability in North America (Lloyd-Jones et al., 2009). Approximately half of patients who survive a stroke will still suffer from cognitive impairments 2 years later (Serrano et al., 2007). At present, an effective treatment for cognitive deficits resulting from stroke has yet to emerge. Although memantine and cholinesterase inhibitors have been approved for the treatment of cognitive impairments associated with Alzheimer’s disease, their ability to ameliorate cognitive impairments resulting from vascular dementias is questionable (Kivirajar and Schneider, 2007). Cognitive deficits resulting from stroke therefore represent a major unmet medical need.

Erythropoietin (EPO) and the chemical analog darbepoetin alfa (D alfa), long approved for the treatment of anemia secondary to kidney failure, were first reported to enhance cognition in anemic patients, raising the possibility of EPO being useful in neurological disorders that affect cognition (O'Shaughnessy, 2002). This led to clinical trials that confirmed EPO improved the cognitive performance of patients with schizophrenia and multiple sclerosis (Ehrenreich et al., 2007a,b). In healthy adults, EPO has been shown to enhance hippocampal activity associated with improvements in a visuo-spatial working memory task (Miskowiak et al., 2007). This effect on hippocampal activity appeared to be independent of any changes in tissue oxygenation because hematocrit levels were unaltered. These findings suggest that EPO and its chemical analogs may also improve cognition in patients who have survived a stroke.

Preclinical studies have convincingly demonstrated that administration of EPO or D alfa at the time of cerebral ischemia or shortly after this event reduces the loss of neurotological function and cognitive deficits by decreasing infarct...
the ventral hippocampus and the reversal of pre-existing alfa-induced increases in NO metabolite concentrations in a single systemic administration of the NOS inhibitor levels in the hippocampus. Next, we examined the ability of effects of systemic administration of D alfa on NO metabolite D alfa. To address this hypothesis, we first determined the information was responsible for the cognitive-enhancing effects of present study was therefore to determine whether NO for-

production of NO in the hippocampus. A second aim of the mation was responsible for the cognitive-enhancing effects of normal blood flow for 12 min. During occlusion a heating pad was used to maintain the body temperature at 37.8 ± 1°C. Core body temperature was measured with an anal thermometer. Sham-operated rats underwent the same surgical procedures except for the 12 min of occlusion with the aneurysm clips.

Assessing Spatial Working Memory Performance in 4-VO and Sham-Operated Rats. Evaluation of spatial working memory using the Barnes maze was performed as reported previously (Bar-

essence, 1979) with modifications to permit behavioral assessment of working rather than reference memory. The major modification was that each day the location of the escape box was chosen at random to evaluate spatial working memory. The Barnes maze apparatus consists of a wooden circular platform 1 m in diameter placed on a tabletop 1.2 m above the floor. Around the perimeter of the platform are 18 holes 9 cm in diameter. The platform has a single escape box positioned under one of the 18 holes. The platform freely rotates on the tabletop while the escape box remains in the same position relative to the room. At the beginning of a trial, a rat was placed at the center of the table under a tin can. The tin can was attached to a rope and raised by a pulley system to release the rat. An aversive bright light (1000 lux) suspended above the table motivated the rodents to find the escape box.

Each trial began with the rat placed under the tin can for 1 min. The can was then raised by an observer out of sight of the rat. The rat was then free to explore the maze and find the hidden escape box. The latency to locate the escape box and the number of nose pokes into holes that did not contain the escape box was counted by an observer unaware of the treatment conditions. Sessions were recorded by a video camera placed above the maze. After each trial the platform and escape box were sprayed with 70% ethanol, and a clean plastic container was placed in the escape box. The platform was then spun to randomly disperse any remaining odor cues. The escape box below the table was fixed and remained in the same position relative to extra maze cues.

On the first day of experimentation, the rats were habituated to the maze by a 4-min exploration period. If a rat did not enter the escape box within 4 min, the experimenter gently placed the rat into the escape box. The rats remained in the escape box for 1 min and were then returned to their home cage. The next 5 test days comprised two trials per day 1 h apart. Each day the location of the escape box was chosen at random. Each of the 5 days of training consisted of a single acquisition trial and a single test trial. The acquisition trial occurred first in which rats learned the location of the escape box. The test trial occurred 1 h after the acquisition trial. The dependent measures were the time required to locate the escape box (latency) and the total number of nose pokes in holes that did not contain the escape box (errors). The trial ended when the rat poked its nose into the hole containing the escape box. In the event that the rat did not find the escape box within the first 4 min the rat was placed in the escape box for 30 s before being returned to its home cage. Rats that remained in the same spot of the table for more than

Materials and Methods

Animal Care. All experiments involving the use of animals were approved by the University Committee on Laboratory Animals and done in accordance with guidelines for the Canadian Council on Animal Care. The animal holding rooms were on a 12-h dark/light cycle, and water and food were provided ad libitum. Male Sprague- Dawley rats 250 to 330 g obtained from Charles River Canada (Montreal, QC, Canada) were used for experimentation.

Four-Vessel Occlusion. To induce transient forebrain ischemia the two-step version of the four-vessel occlusion method was used as described by Davoli et al. (2002). Briefly, rats were anesthetized by using 2% isoflurane (Baxter Corporation, Mississauga, ON, Canada) then placed in the supine position. A ventral midline incision was made on the neck, and both common carotid arteries were located and had loose sutures placed around them. The rats were then placed in a stereotaxic apparatus, and a midline incision was made on the dorsal portion of the rat’s neck. The alar foramina were located, and an electrocautery pen was inserted into both foramina to cauterize the underlying vertebral arteries. The next day the ventral midline incision was reopened and the carotids were raised by using the sutures placed around the carotids the previous day. An aneurysm clamp was placed on each common carotid to occlude blood flow for 12 min. During occlusion a heating pad was used to maintain the body temperature at 37.8 ± 1°C. Core body temperature was measured with an anal thermometer. Sham-operated rats underwent the same surgical procedures except for the 12 min of occlusion with the aneurysm clips.

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1 min in the test trial were considered not to be exploring the maze. A rat that exhibited this behavior for 2 or more days was excluded from the analysis (two sham-operated rats and three 4-VO rats).

Assessment of Spatial Working Memory Performance After Drug Treatment. For all animals, the effects of drug administration on spatial working memory performance in the Barnes maze was assessed 24 h after the last training session (day 6). The effects of D alfa on Barnes maze performance was first assessed in sham-operated rats. Two groups, composed of 13 to 15 rats, were injected intraperitoneally with either D alfa (Amgen, Thousand Oaks, CA) (5000 U/kg; vehicle, phosphate-buffered saline [PBS] containing 0.1% bovine serum albumin [BSA]) or vehicle (1 ml/kg i.p.; PBS) 4 h before the acquisition trial. As in previous testing days, latency and errors were recorded by an observer blind to the treatment conditions. Unlike the 5 training days, on the drug test day (day 6) all animals received two consecutive test trials spaced 1 h apart. The average scores of the two test trials were used for statistical analysis [(test trial 1 + test trial 2)/2]. In a second experiment, we compared the effects of a single injection of saline or l-NAME on the spatial working memory performance of sham rats. Two groups, composed of seven to eight animals, received an intraperitoneal injection of either l-NAME (Sigma-Aldrich, St. Louis, MO; 10 mg/kg in 0.9% saline) or saline (1 ml/kg) 4 h before the acquisition trial. Spatial working memory performance was assessed as described for the previous experiment. In a third experiment, we determined whether D alfa improved the spatial working memory performance of 4-VO rats and whether l-NAME reversed the effects of D alfa. Based on performance during the last 2 training days (number of test trial errors), rats were ranked and allocated to one of three groups to ensure that the average spatial working memory performance for each was similar. On the drug test day, three groups, composed of 12 to 13 4-VO rats each, received either D alfa (5000 U/kg i.p.) or vehicle (1 ml/kg i.p.; BSA) or D alfa (5000 U/kg i.p.) and l-NAME (10 mg/kg i.p.; 0.9% saline) 4 h before the acquisition trial. Behavioral performance was assessed as described above. A diagram showing the experimental protocol is presented in Fig. 1A.

Assessment of CA1 Pyramidal Neuron Loss: Neuronal Nuclei Immunohistochemistry and Cresyl Violet Staining. Rats were euthanized with sodium pentobarbital (240 mg/kg i.p.) and transcardially perfused with 0.9% saline and 4% paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed, postfixed in 4% paraformaldehyde in PBS for 48 h, and then placed in 30% sucrose in PBS for 48 h. Brains were sectioned on a freezing microtome to yield 30-μm-thick free-floating coronal sections. To visualize viable neurons, the sections were stained immunohistochemically for neuronal nuclei (NeuN) protein by using a mouse anti-NeuN primary antibody (MAB377; Millipore Corporation, Etobicoke, ON, Canada) at a 1:2000 dilution. The sections were blocked and permeabilized by rinsing sections in PBS containing 0.02% Triton-X (Sigma-Aldrich) and 5% horse serum (Vector Laboratories, Burlington, ON, Canada) before overnight incubation with the primary antibody. An antimouse biotin-conjugated secondary antibody (Vector Laboratories) was used at a dilution of 1:500, and the tissue was incubated in an avidin–biotin complex (Vector Laboratories) at a dilution of 1:1000. Incubations with secondary antibody and the avidin–biotin complex were both 1 h in duration. Diaminobenzidine (Sigma-Aldrich) (0.5% in PBS) was used as the chromagen. After mounting stained sections on glass slides, the CA1 subsection of the hippocampus at 3.6 mm anterior to Bregma was captured at 50×. Images were analyzed with ImageJ software (http://rsweb.nih.gov/jj/download.html). The dense packing of pyramidal neurons in the dorsal hippocampus precluded cell counts in sections 30-μm thick. Neuronal loss was therefore estimated by measuring the area occupied by NeuN-positive cells. To perform this task, images were converted to a gray scale and the image binary tool was selected so that only positively labeled cells were black on a white foreground. The CA1 section was then outlined and the area of labeled cells was calculated with the measurement function. The areas on both sides of the brain were summed for analysis. Only animals that displayed more than a 90% loss of the CA1-positive neurons in both hippocampi compared with sham-operated controls were considered to be adequately lesioned and used for further analysis.

For cresyl violet staining, coronal sections were mounted on glass slides and dehydrated through a graded series of ethanol dilutions of increasing concentration. The slides were then placed in xylenes and rehydrated by placement through a graded series of ethanol dilutions of decreasing concentration. To achieve the desired staining intensity, the slides were placed in 1% cresyl violet (Sigma-Aldrich) solution for 10 min and the tissue was destained in 1% acetic acid. The slides were dehydrated again and cleared in xylenes before overslipping.

Nitrate/Nitrite Assay. Seven hours after an intraperitoneal injection of D alfa (5000 U/kg), vehicle (1 ml/kg), or D alfa (5000 U/kg) + l-NAME (10 mg/kg) rats were euthanized by a lethal injection of sodium pentobarbital (240 mg/kg i.p.), and brains were re-
moved rapidly from the cranial vault and stored at −80°C until measurement of nitrate/nitrite levels. For determination of hippocampal nitrate and nitrite levels, the ventral hippocampi were rapidly dissected from the brains and weighed. The dissected hippocampi were homogenized in 1× PBS and spun at 10,000 g for 20 min at 4°C. The supernatant was placed on 30 kD filters (Millipore Corporation) and spun at 14,000 g for 30 min at 4°C. The filtrate was collected to measure nitrite levels. The final products of NO in vivo are nitrite (NO₂⁻) and nitrate (NO₃⁻). The relative proportions of NO₂⁻ and NO₃⁻ are variable and cannot be predicted with certainty. Thus the best measure of total NO production is the sum of both NO₂⁻ and NO₃⁻. This was achieved by using a commercial kit from Cayman Chemical (Ann Arbor, MI) that first converts all nitrate to nitrite by using nitrate reductase. The second step used a Griess reaction to convert all nitrite into a deep purple azo compound that was detected at an absorbance of 562 nm with a microplate reader. Known concentrations of nitrite were run in tandem to create a standard curve for the quantification of nitrite levels in each homogenate sample derived from the hippocampus. Levels of nitrite were standardized according to tissue wet weight to yield mM nitrite/g tissue.

**Statistical Analysis.** A two-way analysis of variance (ANOVA) with repeated measures was used to test for statistically significant differences between the Barnes maze performance of sham- and 4-VO-operated animals during the 5 training days (latency and errors) (Fig. 2). The effects of drug treatments (D alfa and L-NAME) relative to vehicle controls (BSA and saline) on the spatial working memory performance (latency and errors) of sham animals were analyzed by using unpaired t tests (Fig. 3). The effect of different drug treatments on spatial working memory performance (Fig. 4, A and B) and nitrite levels (Fig. 5) in 4-VO operated animals were analyzed by using one-way ANOVAs. Post-hoc comparisons were performed by using Bonferroni post-tests. To determine whether D alfa increased the speed of locomotion for 4-VO rats, we compared average times to find the escape box on acquisition trials for the 5

**Fig. 2.** Two weeks after sham surgery or 12 min of exposure to 4-VO, rats were tested on the Barnes maze for 5 consecutive days to evaluate short-term spatial working memory performance. A, rats subjected to 4-VO (n = 41) displayed a greater escape latency than the sham-operated rats (n = 52) across the 5 days. B, rats subjected to 4-VO also committed more errors in the test trials compared with sham-operated controls across the 5 days. The difference in test trial and acquisition trial latency and errors were calculated on the 5 test days to yield intertrial difference scores. C and D, rats subjected to 4-VO had lower intertrial latency differences (C) and lower intertrial error differences (D) compared with sham-operated controls across the 5 testing days. Data are expressed as mean ± S.E.M. Two-way ANOVAs were used to analyze the data. **p** ≤ 0.01; Bonferroni post-test, single-day comparison.

**Fig. 3.** A and B, 1 day after the end of training (day 6), two groups of sham-operated rats were injected with D alfa (5000 U/kg i.p.; n = 15) or vehicle (1 ml/kg i.p.; BSA, n = 13). C and D, two additional groups of sham-operated rats were injected with L-NAME (10 mg/kg; n = 8) or vehicle (1 ml/kg i.p.; 0.9% saline, n = 7). All animals were injected 4 h before the acquisition trial. The animals were then tested again 1 and 2 h later (test trials). The latency and number of errors during the two test trials were averaged and used to gauge spatial working memory performance. There was no significant difference found between the D alfa- and vehicle-treated rats or L-NAME and vehicle with respect to latency (A and C) or errors (B and D). Data are expressed as mean ± S.E.M.
training days and drug-treated day. These comparisons between average acquisition latencies while not on D alfa (training, days 1–5) and with D alfa on board (drug test day, day 6) were analyzed by using a paired t test (Fig. 4C). To assess whether D alfa improved the spatial working memory performance of 4-VO rats on the drug test day, we compared the average number of errors committed on the acquisition and test trial [(test trial 1 + test trial 2)/2] by using paired t tests (Fig. 4D). The 0.05 level of significance was adopted for all comparisons. Statistical testing was performed with GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA).

Results

Rats Subjected to 12 min of Transient Forebrain Ischemia Displayed a More than 90% Loss of Dorsal CA1 Hippocampal Neurons. Male Sprague-Dawley rats 250 to 330 g were subjected to 12 min of forebrain ischemia produced by 4-VO, and the brains were harvested 3 weeks later for histological analysis. Thirty-micrometer-thick coronal sections were labeled immunohistochemically with an antibody against the neuron-specific marker NeuN to visualize viable neurons in the hippocampus (Fig. 1, B and C). Rats subjected to transient forebrain ischemia (Fig. 1C) displayed a marked reduction of NeuN-positive cells in the CA1 region of the dorsal hippocampus compared with sham-operated controls (Fig. 1B). Sections were also stained with the nonspecific cellular marker cresyl violet (Fig. 1, D and E). Cresyl violet staining confirmed that 4-VO resulted in a loss of CA1 neurons (Fig. 1E) compared with sham-operated rats (Fig. 1D). The increase in small and intensely labeled cells is indicative of gliosis in the damaged area. The mean ± S.E.M. area of NeuN-positive labeling in the left and right hippocampal region of the dorsal hippocampus compared with sham-operated controls (Fig. 1B) was 3.415 ± 0.035 mm² for the 4-VO–operated rat and 10.69 ± 0.89 mm² for the sham-operated control. This difference corresponded to a 66% loss of CA1 neuronal density (Fig. 1F).

Rats Subjected to 12 min of 4-VO Displayed Deficits in Short-Term Spatial Working Memory as Assessed by the Barnes Maze. Two weeks after global cerebral ischemia or sham surgery, rats were tested on the Barnes maze to evaluate spatial working memory performance. Testing occurred over 5 days (training), each day comprising two trials 1 h apart with the escape box at a new location each day. The latency to locate the escape box and number of errors committed before entering the escape box were recorded during both the acquisition and test trial for each of the 5 training days. Test trial and intertrial differences (acquisition trial – test trial) for the two measures were compared between both groups. A two-way ANOVA was used to determine the effects of treatment (4-VO and sham) on mean escapes latencies and errors over time (days 1–5). For mean escapes latencies, the effects of both treatment [F(4, 91) = 6.49; p < 0.01] and time [F(4, 91) = 3.52; p < 0.01] were significant (Fig. 2A). Bonferroni post-tests indicated that 4-VO animals required longer than sham animals to locate the escapes box on day 2 (t = 3.415; p < 0.01) and day 3 (t = 3.282; p < 0.01) (Fig. 2A). The
treatment x time interaction was not significant \[F(4,91) = 0.42; p = 0.637\], suggesting that both sham and 4-VO groups improved over time at a similar rate (Fig. 2A). In the case of mean errors, only the main effect of treatment was significant \[F(1,91) = 1.88; p < 0.01\], indicating that over the course of the 5 training days, 4-VO rats committed more errors than the sham animals (Fig. 2B). Bonferroni post-tests did not, however, reveal any differences between the sham and 4-VO groups for each of the 5 training days. Further analysis comparing the intertrial differences across the 5 days determined that sham-operated rats displayed a greater improvement from the acquisition to test trial compared with 4-VO rats with respect to both latency \[F(1,91) = 4.1; p < 0.05\] and errors \[F(1,91) = 7.7; p < 0.01\] (Fig. 2, C and D). The average performance of animals subjected to 4-VO for both latency \((5.5 \pm 6.1 \text{ s})\) (Fig. 2C) and errors \((-0.3 \pm 0.4\) \(\text{Fig. 2D})\ was at levels no different from chance for the 5 days of training. By contrast, sham animals displayed average performance levels that were better than chance for both latency \((21.7 \pm 2.5 \text{ s})\) (Fig. 2C) and errors \((1.9 \pm 0.4\) \(\text{Fig. 2D})\). Consequently, although we did detect significant effects of 4-VO on mean errors over the course of the 5 training days, mean escape latencies were a more sensitive measure of impaired spatial working memory. These results suggest that rats subjected to transient forebrain ischemia by the method of 4-VO had impaired spatial working memory 2 weeks after injury relative to sham-operated controls.

**Systemic Injection of Either D Alfa at 5000 U/kg or L-NAME at 10 mg/kg Did Not Alter Spatial Working Memory Performance of Sham-Operated Rats.** After the 5 days of training on the Barnes maze, the effects of drug treatment on the spatial working memory performance of sham rats was examined on day 6 (drug test day). All animals received an acquisition trial followed 1 h later by two test trials that were spaced 1 h apart. Performance measures (latency and errors) were averaged over the two test trials. To determine the effect of D alfa on spatial working memory performance, the rats were injected with either D alfa (5000 U/kg i.p.) or vehicle (1 ml/kg i.p.; BSA) 4 h before the acquisition trial. An unpaired \(t\) test determined that there was no significant difference between the two groups with respect to latency \((t_{26} = 1.4; p = 0.163\) and errors \((t_{26} = 0.45; p = 0.653)\) (Fig. 3, A and B). In a separate experiment, rats were injected with the NOS inhibitor L-NAME (10 mg/kg i.p.) or vehicle (1 ml/kg i.p.; saline) 4 h before the acquisition trial. An unpaired \(t\) test determined that there was no significant difference between vehicle- and L-NAME-treated rats with respect to test trial latency \((t_{12} = 1.24; p = 0.240)\) or errors \((t_{12} = 0.23; p = 0.822)\) (Fig. 3, C and D). Hence, relative to their respective vehicle controls, neither treatment with D alfa (5000 U/kg i.p.) nor treatment with L-NAME (10 mg/kg i.p.) altered spatial working memory performance.

**Systemic Injection of D Alfa Increased Spatial Working Memory Performance of Rats Subjected to 4-VO that Is Reversed by Coadministration with L-NAME.** Rats that had been subjected to 4-VO 2 weeks previously were trained for 5 days with a short-term spatial working memory task using the Barnes maze. On the 6th day (drug test day), rats were injected with D alfa (5000 U/kg i.p.), vehicle (1 ml/kg i.p.), or D alfa + L-NAME (5000 U/kg i.p. + 10 mg/kg i.p.) 4 h before the acquisition trial. They were then subjected to two test trials, and the latency and errors were averaged over both trials as described above. A one-way ANOVA comparing test trial latencies was significant \[F(2,37) = 3.32; p < 0.05\]. Bonferroni post-tests determined that there was a difference in test-trial latency between the vehicle and D alfa groups \((t_{23} = 2.30; p < 0.05\) and between the D alfa and D alfa + L-NAME groups \((t_{23} = 2.87; p < 0.05\) (Fig. 4A). There was no significant difference with respect to test trial errors between the groups \[F(2,37) = 1.063; p = 0.357\] (Fig. 4B). The latency in the acquisition trial was compared with the average acquisition trial latency over the 5 training days for the rats given D alfa to address whether D alfa was decreasing latency by increasing locomotor activity. A paired \(t\) test determined that there was no significant difference between the average training trial acquisition latency and the acquisition latency after D alfa administration \((t_{11} = 1.034; p = 0.329)\) (Fig. 4C). When comparing performance in acquisition trial versus test trials for each group, paired \(t\) tests determined that only the rats treated with D alfa made significantly fewer errors during test trials \((t_{12} = 0.22; p = 0.825\) for vehicle; \(t_{11} = 2.4, p < 0.05\) for D alfa; and \(t_{11} = 0.24, p = 0.81\) for D alfa + L-NAME) (Fig. 4D). These results suggest that systemic injection of D alfa increased spatial working memory performance that was reversed by L-NAME.

**Systemic Injection of D Alfa Increased Nitric Oxide Products in the Ventral Hippocampus of Rats Subjected to 4-VO that Is Reversed by L-NAME.** After testing on the Barnes maze a subset of 4-VO rats treated with vehicle, D alfa, or D alfa + L-NAME were sacrificed, and the ventral hippocampi were collected to measure tissue concentrations of the NO metabolites nitrite and nitrate. Nitrate was converted to nitrite by nitrate reductase and then measured by using a Griess reaction that converts nitrite into a compound that can be read with a spectrophotometer. A one-way ANOVA determined that there was a statistically significant difference between nitrite concentrations for the three groups \[F(2,14) = 6.32; p < 0.001\] (Fig. 5). Post-hoc tests reveal a significant difference between the vehicle and D alfa groups \((t_{23} = 2.75; p < 0.01\) and between the D alfa and D alfa + L-NAME groups \((t_{23} = 2.26; p < 0.05\). These findings indicate that D alfa increased NO metabolite concentrations that were normalized by L-NAME.

**Discussion**

The first aim of the present study was to determine whether rats subjected to forebrain ischemia have impaired spatial working memory that can be detected with the Barnes maze. Two weeks after the ischemic insult or sham surgery, rats were tested over 5 consecutive days. Rats subjected to forebrain ischemia in which more than 90% of CA1 neurons were lost displayed longer latencies and committed more errors compared with sham-operated rats. These results indicate that spatial working memory, as assessed with the Barnes maze, was impaired by transient forebrain ischemia. Furthermore, rats with CA1 neuronal loss had neutral intertrial differences, indicating their performance was at chance, whereas sham-operated rats displayed positive intertrial differences, indicating better than chance performance. These results are consistent with a large body of evidence indicating that dorsal CA1 neurons are implicated in spatial working memory processing (Nelson et al., 1997; Hartman et
al., 2005; von Euler et al., 2006). Using the Barnes maze, transient global ischemia has previously been shown to impair the ability of rats to learn a new escape box location in a reversal test once an initial location has been learned (Milani et al., 1998). The present results extend these observations by showing that the Barnes maze may also be used to examine the disruptive effects of transient global ischemia on a behavioral task of short-term spatial working memory.

The task most often used to assess spatial working memory in rats subjected to forebrain ischemia is the Morris water maze (Block, 1999). The greatest advantage of the Barnes maze over the Morris water maze is that it is less stressful. The water maze causes a larger increase in plasma glucocorticoids than the Barnes maze, an indication of increased stress (Harrison et al., 2009). Elevated stress may confound interpretation of results, because performance in memory tasks is impaired by increased plasma glucocorticoid levels (Mclay et al., 1998).

A major finding of the present study was that D alfa improved spatial working memory of rats with pre-existing deficits. Rats with a loss of dorsal hippocampal CA1 neurons that received a single dose of D alfa (5000 U/kg i.p.) 4 h before testing required less time to find the escape box (decreased latency) compared with vehicle-treated controls (Fig. 4A). This decrease was not associated with an increase in the rate of locomotion because escape latencies for acquisition trials during training trials was the same as acquisition trial after administration of D alfa (Fig. 4C). Consequently, the reduction in escape latency produced by D alfa did not appear to result from an increase in locomotor activity. A comparison of test trial errors, however, did not reveal a significant difference between 4-VO rats that had received vehicle (1 ml/kg i.p.), D alfa (5000 U/kg i.p.), or L-NAME (10 mg/kg i.p.) plus L-NAME (10 mg/kg i.p.) (Fig. 4B). In the present study, it was difficult to detect a significant effect using errors as the dependent measure when comparisons were made for frequency over just a single day. For example, although there was a main effect of treatment (4-VO and sham) with respect to mean errors over the course of 5 days of training, Bonferroni post-tests performed on the number of errors committed on individual days did not detect any statistically significant differences (Fig. 2B). This indicates that errors were a less sensitive measure of spatial working memory performance deficits than latency. To address this problem, we measured intertrial differences during training days and the test drug day. During the first 5 training days 4-VO rats displayed neutral intertrial differences indicating these animals were not learning from subsequent training, D alfa (5000 U/kg i.p.) did decrease the number of test trial errors relative acquisition trial errors on the drug test day unlike other groups (Fig. 4D). Taken together, these findings indicate that D alfa can reverse pre-existing spatial working memory deficits in 4-VO rats.

D alfa administration did not affect the performance of the sham-operated rats. This may have been caused by a ceiling effect. For example, spatial working memory performance of sham-operated rats may have been at an optimal level or improvements produced by D alfa undetectable by methods used in the current study. In keeping with this hypothesis, we have reported that D alfa-induced improved object recognition memory in animals with ventral hippocampal lesions but not in sham-operated controls (Hori et al., 2007). Cerebral ischemia may also improve the ability of the brain to respond to D alfa. EPO receptors are up-regulated after cerebral ischemia (Spandou et al., 2004). Therefore, D alfa may exert a greater effect in rats that have sustained brain damage after forebrain ischemia. This could account for the memory-enhancing effects seen in rats that had sustained brain injury but not in sham controls.

D alfa produced improvements in spatial working memory performance within hours of a single systemic injection. This indicates that the mechanism of action must involve rapid signaling events rather than a rise in hematocrit or neurogenesis that require days to weeks to occur. We hypothesized that memory enhancement occurred via activation of the NOS pathway. It has been demonstrated that local application of EPO increases extracellular concentrations of NO in the hippocampus (Yamamoto et al., 2004), and a large body of literature has heavily implicated NO is memory formation (Fin et al., 1995; Huang and Lee, 1995; Meyer et al., 1998; Pitsikas, 2009). This first result was confirmed and extended in the present study by measurement of NO metabolites in the ventral hippocampus of 4-VO-operated rats. Rats subjected to transient global ischemia displayed increased NO metabolites in the hippocampus after systemic administration of D alfa compared with vehicle treated 4-VO controls. This D alfa-mediated increase was reversed by systemic administration of the inhibitor of NOS, L-NAME (Fig. 5). This reversal of D alfa-mediated NO metabolite levels was accompanied by a decrease in the ability of D alfa to improve pre-existing spatial working memory deficits. A dose of L-NAME (10 mg/kg i.p.) was chosen that did not affect the Barnes maze performance in sham-operated rats. Furthermore, NO metabolite levels in the hippocampi of 4-VO rats treated with both D alfa (5000 U/kg i.p.) and L-NAME (10 mg/kg i.p.) were not less than vehicle-treated animals. It is therefore unlikely that L-NAME reduced the ability of D alfa to enhance spatial working memory performance by interfering with basal NOS signaling.

The majority of CA1 pyramidal neurons of the dorsal hippocampus in rat models of transient forebrain ischemia are irreversibly injured after several days of reperfusion, whereas CA3 pyramidal neurons that project to the CA1 area remain morphologically intact during this period (Pulsinelli and Brierley, 1979; Smith et al., 1984). In the present study, we measured nitrate and nitrite levels in the ventral hippocampus and observed that D alfa increased tissue concentration of these NO metabolites in this region. These results suggest that D alfa may have improved spatial working memory performance by enhancing nitric-oxide levels in the ventral hippocampus. There is considerable evidence that the CA3 subregion of hippocampus, which is spared after transient forebrain ischemia, plays an important role in the encoding of new spatial working information within short-term memory with a duration of seconds to minutes (Kesner, 2007). Nitric oxide is thought to promote memory consolidation by stimulating production of the second messenger cGMP (Kleppisch and Feil, 2009). Phosphodiesterase 5 inhibitors such as sildenafil that raise cGMP levels improve object recognition memory in association with increased cGMP levels in varicosities in the CA3 region of the hippocampus (Rutten et al., 2005). Taken together, these findings suggest that the ability of D alfa to improve spatial working memory performance after transient forebrain ischemia may be linked
to enhanced nitric oxide-mediated signaling in CA3 neurons, perhaps located in the ventral hippocampus.

In summary, the results of the present study demonstrate that transient global ischemia resulting in more than 90% loss of CA1 neurons in the hippocampus produced behavioral performance deficits in the Barnes maze. During 5 days of training, unlike sham operated rats, 4-VO rats failed to learn the location of a hidden escape box. A single dose of Δf (5000 U/kg i.p.) improved spatial working memory deficits in Barnes maze performance of 4-VO, but not sham-operated, rats within hours of administration. Improved Barnes maze performance was associated with increased tissue concentrations of NO metabolites, suggesting that Δf may have enhanced spatial working memory performance by activating this signaling pathway implicated in synaptic plasticity. In agreement with this proposal, administration of the NOS inhibitor, L-NAME, at a dose that did not impair spatial working memory performance in sham-operated rats, reversed Δf-mediated improvements in Barnes maze performance. Taken together, these findings suggest that erythropoietins such as Δf may improve cognitive performance in humans by activating the NOS signaling pathway.

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References


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