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Post-treatment with amphetamine enhances reinnervation of the ipsilateral side cortex in stroke rats

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Abstract

Amphetamine (AM) treatment has been shown to alter behavioral recovery after ischemia caused by embolism, permanent unilateral occlusion of the common carotid and middle cerebral arteries, or unilateral sensorimotor cortex ablation in rats. However, the behavioral results are inconsistent possibly due to difficulty controlling the size of the lesion before treatment. There is also evidence that AM promotes neuroregeneration in the cortex contralateral to the infarction; however the effects of AM in the ipsilateral cortex remain unclear. The purpose of this study was to employ T2-weighted imaging (T2WI) to establish controlled criteria for AM treatment and to examine neuroregenerative effects in both cortices after stroke. Adult rats were anesthetized, and the right middle cerebral artery was ligated for 90 min to generate lesions in the ipsilateral cortex. Animals were separated into two equal treatment groups (AM or saline) according to the size of infarction, measured by T2WI at 2 days after stroke. AM or saline was administered to stroke rats every third day starting on day 3 for four weeks. AM treatment significantly reduced neurological deficits, as measured by body asymmetry and Bederson's score. T2WI and diffusion tensor imaging (DTI) were used to examine the size of infarction and axonal reinnervation, respectively, before and following treatment on days 2, 10 and 25 after stroke. AM treatment reduced the volume of tissue loss on days 10 and 25. A significant increase in fractional anisotropy ratio was found in the ipsilateral cortex after repeated AM administration, suggesting a possible increase in axonal outgrowth in the lesioned side cortex. Western analysis indicated that AM significantly increased the expression of synaptophysin ipsilaterally and neurofilament bilaterally. AM also enhanced matrix metalloproteinase (MMP) enzymatic activity, determined by MMP zymography in the lesioned side cortex. qRT-PCR was used to examine the expression of trophic factors after the 1st and 2nd doses of AM or saline injection. The expression of BDNF, but not BMP7 or CART, was significantly enhanced by AM in the lesioned side cortex. In conclusion, post-stroke treatment with AM facilitates behavioral recovery, which is associated with an increase in fractional anisotropy activity, enhanced fiber growth in tractography, synaptogenesis, upregulation of BDNF, and MMP activity mainly in the lesioned cortex. Our data suggest that the ipsilateral cortex may be the major target of action in stroke brain after AM treatment.

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INTRODUCTION

During stroke, an ischemic brain area receives inadequate blood and oxygen supply, causing apoptosis, infarction, and loss of brain function. Much research is being conducted to identify and optimize pharmacological treatments for stroke, with a focus on limiting the extent of neuronal injury and on enhancing recovery post-stroke. However, most treatments, such as the use of thrombolytic agents, are limited by a narrow therapeutic time window.

Both clinical and pre-clinical studies have indicated that administration of a high dose of amphetamine (AM) is associated with ischemic brain injury. AM or its analogs facilitate ischemic damage in rodent or human brain. We have also reported that administration of methamphetamine, at high doses (40 mg/kg), augments ischemic injury by inhibiting the expression of protective neurotrophic factors and by up-regulating apoptotic markers in the brain (Shen et al., 2008; Wang et al., 2001). These data suggest that high dose AM or its analogs potentiate ischemic injury in brain.

AM, at lower doses, has differential responses after ischemic brain injury. The behavioral responses after post-stroke AM treatment vary depending upon the model of stroke used and size of the lesion. For example, AM (2 mg/kg), given every third day after small lesions (<10 mm³ per rat), produced by focal injection of endothelin-1, facilitated recovery of skilled forelimb use on the paw-reach or foot-fault tests (Adkins and Jones, 2005; Gilmour et al., 2005). On the other hand, AM failed to improve the qualitative aspects of reaching movements in animals with small lesions induced by devascularization of blood vessels in the forelimb area of the motor cortex (Alaverdashvili et al., 2007).

The beneficial effects of AM have also been reported in stroke animals with relatively large lesions. Treatment with AM after stroke, induced by injecting emboli into the internal carotid artery (Rasmussen et al., 2006) or aspiration of sensorimotor cortex (Ramic et al., 2006), improved cognitive performance in rats. A more recent study also demonstrated that AM enhanced long-term improvement in forelimb motor function in stroke rats after permanent unilateral occlusion of the common carotid and MCA (Papadopoulos et al., 2009). However, in all these studies, volume and location of infarction were not monitored before treatment; animals were randomly assigned to receive either AM or vehicle. Although the size of lesion was found to be not significantly different between AM- and vehicle- treated animals at 10 weeks post- MCAo, after excluding the animals with subcortical lesion and/or no lesion in the brain forelimb region (Papadopoulos et al., 2009) or 28 days after stroke (Rasmussen et al., 2006), it is still not clear if chronic AM treatment altered the size of lesion since the infarction volume was not known before treatment. The marked variability of infarction was also demonstrated in another study where the location of infarction extended to striatum and/or cortex (Rasmussen et al., 2006). Moreover, AM is an indirect dopaminergic agonist, AM-mediated behavioral improvement may be influenced by the dopaminergic innervation of striatum. In this study, we used a transient distal MCA ligation technique to generate unilateral lesions mainly in the temporoparietal cortex, which has less dopaminergic innervation in rats. To reduce variation in the size of the lesion, animals were grouped according to the size of infarction using T2-weighted imaging (T2WI) before treatment. T2WI can delineate ischemic lesions as a hyperintense area with high tissue contrast without image distortion artifacts.

Another confounding factor in evaluating behavioral recovery after repeated AM administration in stroke animals is that AM itself alters behavioral responses. Acute administration of AM releases dopamine from presynaptic terminals and augments locomotor behaviors. Repeated administration of AM induces behavioral sensitization (Tien et al., 2007). On the other hand, high doses of AM analogs deplete dopamine and lesions

presynaptic terminals (Chou et al., 2008). To reduce pharmacological ambiguity by AM in stroke animals, neurological symptoms were scored using an elevated body asymmetry test (Borlongan et al., 1998) and Bederson's neurological test (Bederson et al., 1986) after repeated AM administration. For comparison, we also used activity chambers to examine locomotor activity (Luo et al., 2009; Shen and Wang, 2010; Tomac et al., 1999), which can be influenced by AM itself (Tien et al., 2007).

Up to now, there have been no reports of any MRI study to examine structural changes after AM treatment in stroke animals. Diffusion tensor imaging (DTI) has been widely used for noninvasive investigation of white matter structure and plasticity (Basser and Pierpaoli, 1996; Le et al., 2001). Fractional anisotropy (FA), an index derived from the diffusion tensor, can be used to quantify the diffusion of water in axonal fibers, reflecting the microstructure and integrity of white matter. The orientation and density of axonal fibers can be visualized by tractography, based on similarities of the shape and orientation of the diffusion ellipsoids among neighboring voxels (Basser et al., 2000). In this study, FA and tractography were used to examine time-dependent changes in plasticity of white matter structures following AM treatment in stroke animals.

The mechanism of AM-mediated regeneration has not been thoroughly investigated. AM enhanced cortico-efferent projections from the opposite, non-damaged motor cortex to the deafferented basilar pontine nuclei after sensorimotor cortex lesions in rats (Ramic et al., 2006). This response was further potentiated if combined with physical rehabilitation. Similarly, administration of AM combined with focused activity and enriched environment increased axonal sprouting into deafferented subcortical areas from contralateral projection neurons in rats with permanent MCA and unilateral common carotid occlusion (Papadopoulos et al., 2009). These data suggest that AM activates neuronal sprouting from the contralateral cortex. Since anatomical tracers were injected only into the contralateral cortex in these studies, the role of AM on neural regeneration on the lesioned side cortex has not been fully investigated.

We, and others, have shown that AM regulates expression of certain genes in dopaminergic and non-dopaminergic cells. For example, AM increases the expression of brain-derived neurotrophic factor (BDNF) (Meredith et al., 2002) and cocaine- and amphetamine-regulated transcript (CART) in striatum, hippocampus, and cortex (Douglass et al., 1995). Administration of CART peptide increases BDNF expression in brain (Wu et al., 2006). Since CART and BDNF have protective or trophic activity on CNS neurons (Jia et al., 2008; Xu et al., 2006), it is possible that AM-mediated regeneration may involve upregulation of BDNF, CART, or other trophic factors.

In this study, we used MRI to evaluate the changes in brain tissue integrity in a well controlled distal MCAo model of stroke in rats. All animals underwent MRI examinations to measure the size of lesion and to evaluate the changes in brain tissue integrity before and after AM or vehicle treatment. Our data suggest that a potent trophic response was induced by AM in the ipsilateral cortex after distal MCAo.

MATERIALS AND METHODS

Surgery and Drug Administration

Adult male Sprague-Dawley rats, purchased from Charles River Laboratories Inc., were housed in an enriched environment by providing a toy (nylabone) or crinkle paper in their home cages with a 12 hour dark (6pm to 6 am) and 12 hour light (6 am to 6 pm) cycle approved by the NIDA ACUC. Rats were anesthetized with chloral hydrate (0.4 g/kg, i.p.). The right MCA was ligated with a 10-0 suture and common carotids were clamped

bilaterally by nontraumatic arterial clips to generate focal infarction in the cerebral cortex. The ligature and clips were then removed after 90-min ischemia to allow reperfusion. The volume of infarction was measured at 2 days after MCA occlusion (MCAo) by T2-weighted imaging (T2WI, see below). The inclusion criterion was set at a lesion volume of 100 mm³, measured by T2WI, on the 2nd day after MCAo. Three rats with lesion volume (LV) less than 100 mm³ were excluded from this study. Animals were then separated into 2 groups with similar lesion sizes (Fig 1), and were treated with AM (2 mg/kg, i.p., lesioned volume = 195.33 ± 14.09 mm³) or saline (lesioned volume = 191.34 ± 13.89 mm³) every third day for four weeks. Lesion volume was not different between these two groups before treatment ($p=0.869$, t test). No animal died during surgery or during post-stroke drug treatment.

Magnetic Resonance Imaging (MRI)—Rats underwent T2WI and DTI on day 2, 10 and 25 after stroke onset under isoflurane anesthesia (3% for induction and 1.8% for maintenance) in air/O₂ (80:20). MRI experiments were performed on a Bruker Biospin 9.4 T animal MRI scanner (Bruker Medizintechnik, Karlsruhe, Germany) equipped with an actively shielded gradient coil. The inner diameter of the gradient coil was 0.12 m, and the maximum gradient strength was 400 mT/m. A birdcage coil driven in linear mode was used for radiofrequency excitation, and a single-turn circular surface coil (2 cm in diameter) was used for signal reception (Lu et al., 2010). The animals were secured on a custom made holder equipped with a nose cone for administration of anesthetic gases and ear bars to minimize head motion. Rectal temperature was maintained at 37.5±0.5°C with a feedback-controlled, water-circulated heating pad. ROI analyses in all MR images were analyzed by an experienced researcher blinded to the treatment information while tracing the regions of interest. Intraobserver reliability was assessed by calculating 1-way random intraclass correlation coefficients with p value less than 0.001 for LV and FA measurements.

T2WI were acquired using a Rapid Acquisition with Relaxation Enhancement (RARE) sequence with the following parameters: field of view = 3.2×3.2 cm², matrix size = 192×192 (zero-filled to 256×256), repetition time (TR) = 2750 ms, echo time (TE) = 13.3 ms, and 23 slices with 1 mm thickness. DTI were acquired using a single-shot echo-planar imaging (EPI) sequence. Scan parameters were: 19 slices with 1mm thickness, FOV = 3.2×3.2 cm², matrix size = 96×96, TR = 9500 ms and TE = 38 ms. Thirty diffusion-weighted images with diffusion gradients along independent orientations ($b=1000$ s/mm²) and 1 baseline image ($b=0$) were acquired for each slice, and the acquisition was repeated three times to improve signal-to-noise ratio. The total acquisition time was approximately 40 minutes. Geometric distortions in EPI were corrected using the Phase Labeling for Additional Coordinate Encoding (PLACE) scheme (Xiang and Ye, 2007). This was done by acquiring an additional set of EPI data with the phase-encoding blip offset by -1 and 0. This dataset was used to derive pixel-wise displacement maps, which were subsequently applied to correct for images acquired with the same EPI parameters. Diffusion tensors and FA were analyzed, and fiber-tract maps were created using the “dTV II” diffusion tensor imaging software developed by Image Computing and Analysis Laboratory, Dept. Radiology, Univ. Tokyo Hospital, Japan (<http://www.ut-radiology.umin.jp/people/masutani/dTV.htm>; (Masutani et al., 2003). For each voxel, the diffusion tensor elements were diagonalized to obtain three eigenvectors (e_1 , e_2 and e_3) and their corresponding three eigenvalues (λ_1 , λ_2 and λ_3). The eigenvector associated with the largest eigenvalue was referred to as the primary eigenvector indicating the maximum diffusion, whereas the eigenvector with the minimum eigenvalue was associated with the direction with the least diffusion (Basser and Pierpaoli, 1996). The degree of diffusion anisotropy was quantified by the fractional anisotropy (FA) (Masutani et al., 2003), which is defined as:

$$FA = \sqrt{\frac{3}{2}} \frac{\sqrt{(\lambda_1 - \lambda_M)^2 + (\lambda_2 - \lambda_M)^2 + (\lambda_3 - \lambda_M)^2}}{\sqrt{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}}$$

where $\lambda_M = (\lambda_1 + \lambda_2 + \lambda_3)/3$.

The direction of the principal eigenvector of the diffusion tensor, was used to represent the major orientation of the axonal fiber bundle in the image voxel. The tracking algorithm moved along the principal vectors of the diffusion tensors that have high anisotropy values. Tracking lines were propagated until the line encountered a voxel with an FA value less than 0.18 or the tracking length was larger than 160 steps. Tractographies were displayed with colors of a directional code: red for left-right, blue for cranial caudal, and green for dorsal-ventral directions.

Ischemic injury size was determined from the hyper-intensity region in the T2-weighted images. An area containing ischemic tissue and peri-lesioned boundaries was first manually traced and mirrored onto the contralateral side in each slice. Voxels in the lesion area with image intensities higher than the mean + 2 standard deviations of the intensity in the mirrored contralateral area were defined as the infarct zone (Chan et al., 2009). The volume of infarction in each animal was obtained from the product of the slice thickness (1mm) and the sum of lesion areas in all slices. To reduce variability among animals, the change in lesion (relative lesion volume or rLV) on days 10 and 25 post-MCAo was analyzed by comparison to the volume of infarction on day 2 in each animal.

For FA analysis, a region-of-interest (ROI) was selected within the peri-lesioned region in the ipsilateral white matter area (external capsule, coronal slices located approximately from 1.64 to -5.63 mm relative to bregma) based on its relatively clear gray/white matter boundaries (Fig. 2A). The voxel size for DTI was $0.33 \times 0.33 \times 1 \text{ mm}^3$. An additional ROI was placed in the area in the contralateral white matter for comparison.

For tractography, analysis of the tracked fibers was performed using fiber density, which was calculated as the total number of successfully tracked lines divided by the seeding area (i.e. fibers/voxel). The seeding area was selected in the lesion area of the imaged slice with the maximal lesion size shown in the FA maps, which includes both the ischemic tissues and the lesion boundary with elevated FA values. The fiber density measured from the contralateral side with a similar size of seeding area was used as a reference for comparison.

Behavior—Three behavioral tests were used to examine recovery after treatment:

- a. Body asymmetry was analyzed using an elevated body swing test (Borlongan and Hida, 1998). Rats were examined for lateral movements/turning when their bodies were suspended 20 cm above the testing table by lifting their tails. The frequency of initial turning of the head or upper body contralateral to the ischemic side was counted in 20 consecutive trials. The maximum impairment in body asymmetry in stroke animals is 20 contralateral turns/20 trials. In normal rats, the average body asymmetry is 10 contralateral turns/20 trials (i.e. the animals turn in each direction with equal frequency).
- b. Neurological deficits were also evaluated using Bederson's score (Bederson et al. 1986). In a postural reflex test, rats were examined for the degree of abnormal posture when suspended by 20–30 cm above the testing table. They were scored according to the following criteria.

- 0 Rats extend both forelimbs straight. No observable deficit.
 - 1 Rats keep the one forelimb to the breast and extend the other forelimb straight.
 - 2 Rats show decreased resistance to lateral push in addition to behavior in score 1 without circling.
 - 3 Rats twist the upper half of their body in addition to behavior in score 2.
- c. Locomotor activity: Each animal was individually placed in a 42×42×31 cm activity monitor for 24 hours (Omnitech Electronic Inc, Columbus, OH) one day after the 2nd and 4th doses of AM injection. Water and food were provided in the chambers. The monitor contained 16 horizontal and 8 vertical infrared sensors spaced 2.5 cm apart. Locomotor activity was calculated using the number of infrared beams broken by the animals.

Tissue harvesting

The brains were sliced coronally at 2-mm intervals. We have previously demonstrated that the largest lesion, as examined by TTC staining, occurred in the 3rd and 4th slices from the rostral end (Airavaara et al., 2009; Wang et al., 2005). These slices were collected for Western, MMP and Rt-PCR analysis.

Western Blotting

Brain tissues (right and left cortex) were homogenized in RIPA buffer containing 50mM Tris HCl pH 7.4, 1% NP40, 0.25% deoxycholic acid, 150 mM sodium chloride, 1 mM EDTA and 1% protease inhibitor (Sigma). Lysates were centrifuged at 14000 × g for 5 min at 4°C and the supernatant was collected. Total protein concentration in each sample was determined by DC assay (Biorad) using bovine serum albumin (BSA) as a standard curve. Lysates were separated by 4–20% Tris-HCl gel (Bio-Rad); proteins were transferred to polyvinylidene difluoride membranes (Biorad). After pre-blocking in Odyssey blocking buffer (Li-Cor, Lincoln, NE, USA), membranes were incubated at 4°C with the primary antibody to synaptophysin (1:5000, Millipore), mouse anti-neurofilament (1:500, Chemicon), or rabbit anti-actin (1:500) overnight, then incubated for 1 h at room temperature in Donkey-anti-rabbit IR-800nm or Donkey anti-mouse IR-700nm secondary antibodies (1:2500, Rockland). Membranes were scanned using an Odyssey Infrared Imager (Li-Cor). Fluorescence density was measured using image analysis.

Gelatin Zymography for MMP activity

Brain tissues were homogenized in lysis buffer containing 1% TritonX-100 and 1% protease inhibitor cocktail (Sigma). Samples were electrophoresed on Novex 10% Glycogel (Gelatin) Gel (Invitrogen). Gels were renatured overnight at room temperature, incubated in developing buffer at 37°C overnight, and then stained with Coomassie brilliant blue R-250 for 30 min at room temperature.

Gelatinolytic activity representing MMP-2 was observed as a clear band of digested gelatin against a blue background of undegraded gelatin (Sariahmetoglu et al., 2007). Images were acquired using a UVP imaging system and the pixel density of MMP bands quantified using Image J.

Expression of BDNF, BMP7 and CART

Cortex from the lesioned and contralateral hemispheres, as well as from non-stroke rats corresponding to the MCAo region, were dissected and frozen in isopentane on dry ice.

Tissue was stored at -80°C until RNA isolation. Tissue was homogenized (Polytron, Kinematica) in Trizol reagent (Invitrogen) containing 5 $\mu\text{g}/\text{ml}$ linear polycrylamide (GenElute) and RNA extracted according to the manufacturer. RNA was DNase treated and cleaned using RNeasy MinElute Cleanup Kit (Qiagen). One μg of RNA was reverse transcribed using iScript Select cDNA Synthesis Kit (Bio-Rad) in 20 μl reactions. The cDNA samples were diluted 1:5 with nuclease-free water and analyzed by qRt-PCR using Taqman primers and probes as follows.

Ube2i: 5'GCCACCACTGTTTCATCCAAA3' (fwd),
 5'FAM-CGTGTATCCTTCTGGCACAGTGTGC-BH1 3' (probe),
 5'GCCGCCAGTCCTTGTCTTC3' (rev);
BMP7: 5'TCTCTCACTGCCCACCTTGG3' (fwd),
 5'FAM-ACAGACCAAGCACCTCTCCTGAGCCTTC-BH1 3'(probe),
 5'TGAAAGTATGCTGCTTATCAACCAC3' (rev);
CART: 5'CGAGCCCTGGACATCTACTC3' (fwd),
 5'FAM-TGCCGTGGATGATGCGTCCC-BH1 3'(probe),
 5'CCTTGGCAGCTCCTTCTCAT3' (rev) and

BDNF sequences were as previously described (Gomez-Pinilla et al., 2002).

Results of the qRt-PCR were calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Schmittgen and Livak, 2008). Briefly, the threshold cycle (Ct) of CART, BMP7 and BDNF was normalized to the Ct of the reference gene, Ube2i, for each sample, which was used to determine fold-changes in mRNA expression between left and right hemispheres in the various treatment groups.

Statistical Analysis

All data were expressed as means $\pm$ SEM. Data were analyzed using Student's t test (for LV, tractography, Bederson's neurological test, Western, and zymography analysis) or two way ANOVA and post-hoc Newman-Keuls tests (for rLV, FA, body asymmetry, and RTPCR analysis). All analyses were done using SigmaStat software ver 3.0.

Results

MRI

A total of 32 stroke rats were used for the MRI study. At 2 days after MCAo, an increase in intensity of T2-weighted images was found in cortex, ipsilateral to the lesioned side, in all animals. Based on the volume of infarction, animals were evenly distributed into two treatment groups, AM and veh (Fig 1A, 1B). Thus, lesion volume (LV) was not different between these two groups before treatment (AM group: $195.33 \pm 14.09 \text{ mm}^3$; Veh group: $191.34 \pm 13.89 \text{ mm}^3$, $p = 0.869$, t test). The size of lesion was further examined after AM treatment (days 10 and 25 after MCAo). Relative lesion volume (rLV) of each animal on days 10 and 25 was calculated as the sum of the lesion area across all slices, divided by the lesion volume measured at the 2nd day after stroke. Using a two-way ANOVA, we found a time ($p < 0.0001$) and treatment ($p < 0.0001$) dependent reduction in rLV. There is a significant interaction between treatment and follow-up time points ($p < 0.0001$). Post hoc analysis showed that AM significantly reduced rLV on 10 and 25 days after stroke ($p = 0.0003$ and $p = 0.0002$ for 10 and 25 days, respectively, post hoc test, Fig 1C). Typical T2-weighted images from one animal treated with AM or veh are shown in Fig 1D1 and Fig 1D2.

The calculated FA maps in normal rat brain regions are consistent with myelin-stained histological images which can be obtained from the public Brainmaps database (website www.brainmaps.org). Using a two way ANOVA, we found that FA values in the contralateral white matter area (Fig 2A) were not significantly ($p > 0.8$) altered before AM treatment on day 2 (veh: 0.75 ± 0.44 vs. AM: 0.74 ± 0.58) as well as after AM treatment on day 10 (veh: 0.73 ± 0.43 vs. AM: 0.75 ± 0.20) and day 25 (veh: 0.75 ± 0.22 vs. AM: 0.74 ± 0.31). FA on the lesioned side hemisphere was normalized by comparison with the contralateral white matter in the same slice (Fig 2A). FA was significantly enhanced by AM treatment ($p < 0.0001$) or follow-up time ($p < 0.0001$, 2-Way ANOVA). There was a significant interaction between treatment and follow-up time ($p < 0.0001$). AM significantly increased FA on days 10 and 25 days after stroke ($p = 0.0064$ and $p = 0.001$ for 10 and 25 days, respectively, post hoc test, Fig 2B). Typical FA maps from each group are shown in Fig 2C and 2D.

Axonal fibers were measured by tractography on day 25 after MCAo. There was an increase in fiber growth in AM –treated (upper panels, Fig 3), as compared to veh –treated (lower panels, Fig 3), animals. Fiber density in tractography was further analyzed by comparing number of fibers per seeding area. No significant difference in fiber density was found between AM and Veh groups (AM: 0.99 ± 0.001 vs. veh: 0.99 ± 0.005 , $P = 0.67$, two-sample t-test, Fig 3) in the contralateral area. AM treatment significantly enhanced density of fibers in the lesion side cortex in stroke brains (AM: 0.78 ± 0.03 vs. veh: 0.67 ± 0.04 , $P = 0.02$, two-sample t-test, Fig 3).

Behavior

Three tests were used to examine behavioral recovery before and after treatment. Using an elevated body asymmetry test, we found that all stroke animals ($n=16$) developed prominent body asymmetry by biased movement of the upper body to the side contralateral to the lesion in 20 trials on day 2 after MCAo (Fig 4A). Post-treatment with AM ($n=8$) significantly reduced the frequency of body asymmetry in stroke rats starting from day 9 after MCAo ($*p < 0.05$, 2-Way ANOVA+Post-hoc Newman Keuls test, Fig 4A). Bederson's neurological tests were carried out on day 3 (before treatment) and on day 12 (after treatment). Bederson's score was significantly reduced by AM treatment ($p < 0.05$, Fig 4B, t test). 24-hour locomotor activity was examined on days 7 and 13 after treatment (i.e. 1 day after the 2nd and 4th doses of AM). No significant difference in horizontal activity, total distance travelled, and movement time were found between animals treated with AM and saline ($p > 0.05$, t test).

Expression of Neurofilament and Synaptophysin

The immunoreactivity of neurofilament (NF) and synaptophysin was examined by Western blotting in 16 rats on day 27 after MCAo (or 2 days after the 3rd MRI exam). AM treatment significantly increased NF protein, normalized to actin, in the ipsilateral ($p = 0.036$, t-test) and contralateral cortices ($p = 0.021$, t-test; Fig 5A). These data suggest that post-stroke treatment with AM increases the levels of NF protein in cerebral cortex.

Compared to saline treatment, AM increased synaptophysin protein, normalized to actin, in the lesioned-side cortex (Fig 5B). The significant increase in synaptophysin expression was found in the lesioned ($p < 0.001$, t test, Fig 5B), but not the contralateral ($p = 0.554$, t test), cortex after AM treatment. Our data suggest that post-stroke treatment with AM increases synaptophysin protein levels in the ipsilateral cortex.

Gelatinolytic activity

MMP-2 activity was examined using gelatin zymography. Cortical tissue was harvested at 4 weeks after AM or saline treatment. No detectable MMP2 activity was found in control non-stroke animals (Fig 6A). Similar to previous reports, MMP-2 enzymatic activity was enhanced in the ischemic side cortex. Treatment with AM, compared to vehicle, further enhanced MMP-2 activity (Fig 6A). MMP-2 band intensity was quantified with ImageJ analysis. AM significantly increased gelatinolytic activity in the ipsilateral cortex of stroke rats (t-test, $p=0.05$, Fig 6B).

Expression of BDNF, BMP7, and CART

A total of 31 stroke and 10 non-stroke animals were used to examine the expression of BDNF, BMP7, and CART within one day after the 1st and 2nd AM or saline injections. Since there was no lesion in the non-stroke animals, data from the left and right cortices in these animals were combined. There was a significant difference in BDNF mRNA expression in the lesioned side cortex ($F_{2,42}=10.721$, $p<0.001$, two way ANOVA). Post-hoc Newman Keuls analysis indicates that BDNF mRNA was significantly reduced after MCAo ($p<0.001$, non-stroke vs. saline+stroke). Treatment with AM significantly increased BDNF expression in the ischemic cortex ($p=0.030$, saline+stroke vs. AM+stroke, Fig 7A1). No difference was found in the contralateral cortex ($F_{2,42}=0.584$, $p=0.562$, Fig 7A2). Similar to our previous reports (Chang et al., 2003), we found that ischemia enhances BMP7 mRNA expression in the ischemic cortex ($F_{2,43}=21.866$, $p<0.001$, two way ANOVA, Fig 7B1). No difference was found in the stroke animals treated with AM or vehicle ($p=0.767$, Newman-Keuls test, Fig 7C, D). CART mRNA expression in the lesioned side cortex was significantly reduced after MCAo ($p<0.001$, $F_{2,42}=0.7.707$, Fig 7C1). However, AM treatment did not alter CART expression in the lesioned ($p=0.241$) and non-lesioned side cortices ($p=0.316$, Fig 7C2).

Discussion

In this study, we used T2WI and a transient MCA ligation model to generate consistent unilateral ischemic brain lesion in the cortex in rats. Post-stroke treatment with AM reduced body asymmetry and neurological scores while increasing FA, measured by DTI. AM upregulated expression of BDNF mRNA as well as the axonal proteins synaptophysin and neurofilament. Collectively, our data indicates that post-treatment with low doses of AM provides beneficial outcomes after the onset of stroke.

We used MRI to evaluate the structural changes in peri-lesioned areas non-invasively at 2, 10 and 25 days after stroke. We found the lesion volume, measured by T2WI, was not different before AM or vehicle treatment on day 2 but was significantly reduced by AM on days 10 and 25 after stroke. We postulate that the reduction in cerebral infarction was mediated by endogenous reinnervation in the lesioned cortex, which is further supported by DTI.

FA has been used to monitor the maturation of white matter (Provenzale et al., 2007) and the degree of myelination in human brain developmental studies (Lobel et al., 2009), as well as white matter damage in stroke patients or experimental stroke animals (Jiang et al., 2010; Watanabe et al., 2001). There are limited studies using FA to examine reorganization of white matter in brain repair (Jiang et al., 2010). In correspondence with previous immunohistochemical studies, in which sprouting of fibers from surviving neurons and formation of new synapses were found in peri-infarct cortical zones (Dijkhuizen et al., 2001; Kawamata et al., 1997), we demonstrated an increase in FA ratio in the peri-lesioned area in stroke rats. Our results are also in close agreement with recent studies showing that axonal

projections were reorganized and overall orientation of the axonal projection in DTI was parallel to the lesion boundary with a significant increase in FA values in stroke animals after neurorestorative treatments (Ding et al., 2008; Jiang et al., 2010; Li et al., 2009). AM treatment further and time-dependently enhanced FA in the lesioned cortex. Using fiber tractography, the increase in fiber growth was found mainly in the ipsilateral cortex. Taken together, these MRI data suggest that AM enhances neuronal connectivity in peri-lesioned tissue, which may contribute to the recovery of neurological function after ischemic stroke (Granziera et al., 2007).

Post-stroke treatment with AM has been shown to induce recovery of certain skilled forelimb behaviors in animals (Adkins and Jones, 2005; Gilmour et al., 2005). However, AM failed to improve the qualitative aspects of reaching movements after ischemic brain injury (Alaverdashvili et al., 2007). The inconsistency of behavioral recovery may be attributed to the size of infarction, striatal vs. cortical lesion, and others as reported previously. In this study, we controlled the size of lesion by MRI to ensure there was no difference in the infarct volume between the two groups before starting treatment. We also limited the lesion to the ipsilateral cortex by using a distal MCAo model to generate infarction. We found that neurological indices, such as body asymmetry (Borlongan et al., 1998) and Bederson's neurological scores (Bederson et al., 1986), were reduced by AM. However, AM did not alter 24-hour locomotor behavior, including horizontal and vertical activity, in stroke animals. These data suggest that AM induced some, but not all, behavioral improvements. It has been long known that AM can alter locomotor behavior by releasing or depleting DA from nerve terminals (Chou et al., 2008). Repeated administration of AM facilitates locomotor sensitization (Tien et al., 2007). It is likely that the lack of behavioral recovery in locomotor activity may be confounded by AM actions on dopaminergic neurons. In contrast, the change in neurological score, as examined by the elevated body asymmetry and Bederson's neurological tests, has been successfully used to evaluate behavioral recovery after cortical stroke (Airavaara et al., 2010; Luo et al., 2009; Shen and Wang, 2010) and, thus, maybe a more reliable method to study effects of repeated AM administration.

Previous studies have indicated that AM treatment, combined with focused activity and enriched environment or exercise, improves AM-induced behavioral recovery and increases axonal sprouting from contralateral projection neurons as measured by injection of the anterograde tracer BDA into the contralateral sensorimotor cortex in stroke rats (Papadopoulos et al., 2009; Rasmussen et al., 2006), suggesting AM can induce sprouting from the contralateral hemisphere. In the current study, all animals were housed in an enriched environment without providing further rehabilitation or exercise. An increase in neurofilament expression was found in both the ipsilateral and the contralateral cortex after repeated AM treatment. These data suggest that AM-mediated behavioral recovery involves axonal regeneration from cortex. Interestingly, the synaptic marker synaptophysin (Calhoun et al., 1996) and BDNF mRNA were upregulated only in the lesioned side cortex in AM-treated stroke rats. Together with increase in FA and reduction of lesion volume in the ipsilateral cortex, these data suggest that treatment with AM alone induced regeneration mainly in the lesioned side cortex. The potentiation of AM-mediated behavioral responses and axonal sprouting in contralateral cortex after physical therapy or exercise (Brown et al., 2004; Rasmussen et al., 2006) may involve other factors, because exercise by itself increases BDNF expression in the contralateral hemisphere in stroke animals (Kim et al., 2005).

The increase in synaptophysin and FA ratios is accompanied by increased BDNF mRNA expression in the lesioned cortex. This response is selective because BMP7, a trophic factor with neuroregenerative properties in stroke animals (Chang et al., 2003; Chou et al., 2006), and CART, which regulates BDNF expression (Wu et al., 2006), were not altered by AM.

BDNF can regulate dendritic branching in cortex (McAllister et al., 1995; McAllister et al., 1996). Transplantation of bone marrow cells, together with BDNF, enhanced cell differentiation and improved motor recovery in stroke rats (Chen et al., 2000). Taken together, these data suggest that AM selectively upregulates BDNF expression in lesioned cortex and may contribute to axonal differentiation in this region.

Our data also suggest that AM treatment increases MMP-2 activity in the ipsilateral cortex. MMPs, a group of enzymes which control proteolysis of extracellular matrix, have multiple roles after brain ischemia (Cunningham et al., 2005). At an early stage after reperfusion, increases in MMP lead to cleavage of PARP-1 and apoptosis. MMP inhibitors attenuated the early increases of oxidized DNA (Yang et al., 2010). On the other hand, MMP increases nerve sprouting (Saygili et al., 2010) and promotes reinnervation of the lesioned area in the recovery stage after stroke (Lee et al., 2006). In this study, we found MMP-2 activity was enhanced in the lesioned side cortex at 4 weeks after stroke during recovery, which is consistent with a role in promoting reinnervation in the lesioned cortex. The increase in MMP-2 activity and BDNF expression may facilitate neural sprouting in the lesioned cortex.

In conclusion, our data indicate that post-treatment with low-dose AM facilitates functional behavioral recovery, which may be mediated by reinnervation and upregulation of BDNF in the lesioned side cortex of the stroke brain.

Acknowledgments

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List of abbreviation

AM	amphetamine
BDNF	brain-derived neurotrophic factor
BMP7	Bone morphogenetic protein 7
BSA	bovine serum albumin
CART	cocaine- and amphetamine-regulated transcript
DTI	Diffusion tensor imaging
EPI	echo-planar imaging
FA	fractional anisotropy
MCA	middle cerebral artery
MCAo	middle cerebral artery occlusion
MMP	matrix metalloproteinase
MRI	Magnetic resonance imaging
PLACE	Phase Labeling for Additional Coordinate Encoding
RARE	Rapid Acquisition with Relaxation Enhancement
rLV	Relative lesion volume
ROI	region-of-interest
T2WI	T2-weighted imaging
TE	echo time

TR repetition time

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Fig 1.

T2W images in stroke animals. (A) T2W imaging was used to group animals on day 2 after MCAo. Typical T2W images taken on day 2 after MCAo in two rats to receive AM (right panel) and vehicle (left panel) subsequently. Infarction was found only in the lesioned side cortex in both animals. (B) Animals were separated into two groups according to the area of infarction in T2W imaging. There was no difference in volume of infarction on day 2 between these two groups before treatment. (C) AM treatment significantly reduced relative lesion volume (rLV) on days 10 and 25 after stroke. Representative T2WI from animals treated with (D1) AM or (D2) saline taken on day 2, 10 and 25 after MCAo. * $p = 0.0003$ and $p = 0.0002$ for 10 and 25 days, respectively, two way ANOVA + Post-hoc Bonferroni test.

Fig 2.

Post-stroke treatment with AM increases FA. (A) ROI was placed in regions of perilesion (red) and contralateral white matter (yellow) in the coronal fractional anisotropy (FA) map. (B) Time course changes of FA. AM treatment significantly increased FA values on days 10 and 25 after stroke (* $p < 0.001$). (C) FA maps of an AM-treated animal (upper panels), and an animal receiving veh (lower panels).

Fig 3.

AM treatment increases fiber growth in the ipsilateral cortex. Axonal fibers were measured by tractography on day 25 after MCAo. There is an increase in fiber growth in AM (upper panels), as compared to veh (lower panels), -treated animals. The normal-appearing contralateral side showed a similar density of fiber tracking between AM and veh-treated animals.

Fig 4.

Post-treatment with AM reduced (A) body asymmetry and (B) Bederson's neurological score. (A) In 20 trials, all stroke animals developed prominent body asymmetry by swinging the upper body to the side contralateral to the lesion on day 2 after MCAo. Treatment with AM (arrows) significantly reduced the frequency of body asymmetry. (B) Neurological deficits, measured by Bederson's test, were examined on day 3 after MCAo (before AM treatment) and were significantly reduced by AM treatment on day 12. * $p < 0.05$.

Fig 5.

AM treatment increases expression of neurofilament (NF) and synaptophysin in cerebral cortex in stroke animals. Protein expression was examined by Western blotting at 4 weeks after stroke. (A) NF (green) was enhanced by AM, compared to saline treatment. Lower panel represents quantitation of NF protein expression normalized to actin. AM treatment significantly increased NF expression in the ipsilateral (right) and the contralateral (left) cortex. (B) Post-stroke AM treatment increased synaptophysin protein levels in the lesioned-side cortex. AM treatment increased synaptophysin (green) in the lesioned side cortex (upper panel). AM treatment significantly increased synaptophysin protein levels in the lesioned (right) cortex after AM treatment. AM did not alter synaptophysin expression in the contralateral (left) cortex. * $p < 0.05$.

Fig 6. Post-stroke treatment with low-dose amphetamine increased activation of MMP-2 in the ipsilateral cortex. (A) A representative gelatin zymogram demonstrates that AM treatment increased MMP-2 enzymatic activity (arrow, 64kDa) in the lesioned-side cortex of stroke rats. There was no detectable activation of MMP-2 in a non-stroke control sample (far right). (B) MMP-2 gelatinolytic activity from each sample was quantitatively analyzed and was normalized to the mean activity from the saline-treated animals. AM significantly increased activation of MMP-2 in the lesioned cortex. $p=0.05$.

Fig 7.

Post-stroke treatment with AM increased BDNF mRNA expression in the lesioned side cortex. Brain samples were collected within one day after the 1st or 2nd dose of AM or saline. All data were normalized to the non-stroke controls. (A1) In the ipsilateral cortex, BDNF mRNA activity was significantly reduced after MCAo. Treatment with AM significantly increased BDNF expression. (A2) BDNF mRNA levels were not affected by AM in the contralateral side cortex. (B) Ischemia enhanced BMP7 mRNA expression in the lesioned side cortex (B1). AM did not alter BMP7 mRNA expression on either side. (C) CART mRNA expression in the lesioned side cortex was significantly reduced after MCAo (C1). AM treatment did not alter CART expression in the lesioned (C1) and non-lesioned side cortices (C2). *p < 0.05, 2-way ANOVA.