Enhanced Neocortical Neural Sprouting, Synaptogenesis, and Behavioral Recovery With d-Amphetamine Therapy After Neocortical Infarction in Rats

R. Paul Stroemer, PhD; Thomas A. Kent, MD; Claire E. Hulsebosch, PhD

Background and Purpose—d-Amphetamine administration increases behavioral recovery after various cortical lesions including cortical ablations, contusions, and focal ischemia in animals and after stroke in humans. The purpose of the present study was to test the enhanced behavioral recovery and increased expression of proteins involved in neurite growth and synaptogenesis in d-amphetamine–treated rats compared with vehicle-treated controls after a focal neocortical infarct.

Methods—Unilateral neocortical ischemia was induced in male spontaneously hypertensive Wistar rats (n=8 per time point per group) by permanently occluding the distal middle cerebral artery and ipsilateral common carotid artery in 2 groups of rats: d-amphetamine treated (2 mg/kg IP injections) and vehicle treated (saline IP injections). To determine the spatial and temporal distribution of neurite growth and/or synaptogenesis, growth-associated protein (GAP-43), a protein expressed on axonal growth cones, and synaptophysin, a calcium-binding protein found on synaptic vesicles, were examined by immunohistochemical techniques, and both density and distribution of reaction product were measured. Since the resulting infarction included a portion of the forelimb neocortex, behavioral assessments of forelimb function using the foot-fault test of Hernandez and Schallert were performed on the same rats used for immunohistochemical studies during the period of drug action and 24 hours later. A Morris water maze and other indices of behavioral assays were also measured similarly. Recovery times were 3, 7, 14, 30, and 60 days postoperatively.

Results—Both GAP-43 and synaptophysin proteins demonstrated statistically significant increases in density and distribution of immunoreaction product as determined by optical density measurements in the neocortex of the infarcted group treated with d-amphetamines compared with vehicle-treated infarcted controls. The GAP-43 was elevated to statistically significant levels in forelimb, hindlimb, and parietal neocortical regions ipsilateral to the infarction only at days 3, 7, and 14. By contrast, the synaptophysin demonstrated no statistically significant changes in expression at 3 or 7 days but demonstrated statistically significant increases at 14, 30, and 60 days in the forelimb, hindlimb, and parietal neocortical regions ipsilateral to the infarction as well as increased distribution in the contralateral parietal neocortex. Behavioral assessment of forelimb function indicated that improved recovery of forelimb placement on the side contralateral to the infarction was statistically significant in the d-amphetamine–treated group compared with the vehicle-treated group (P<0.025). Spatial memory, as measured with the Morris water maze, worsened in the vehicle-treated group compared with the d-amphetamine–treated group at 60 days (P<0.025).

Conclusions—These data support the occurrence of neurite growth followed by synaptogenesis in the neocortex in a pattern that corresponds both spatially and temporally with behavioral recovery that is accelerated by d-amphetamine treatment. While the specific mechanisms responsible for d-amphetamine–promoted expression of proteins involved in neurite growth and synaptogenesis and of enhanced behavioral recovery are not known, it is suggested that protein upregulation occurs as a result of functional activation of pathways able to remodel in response to active behavioral performance. (Stroke. 1998;29:2381-2395.)

Key Words: amphetamines ▪ cerebral ischemia ▪ immunohistochemistry ▪ neuronal plasticity ▪ proteins ▪ synaptophysin ▪ rats

After focal neocortical ischemia, massive primary and secondary neuronal death will result in regions of denervation that provide a stimulus for undamaged neurons to sprout and establish new synaptic connections. We previously reported an upregulation of proteins involved in sprouting and synaptogenesis in ipsilateral and contralateral forelimb...
and parietal neocortices after cerebral infarction that correlated temporally with forelimb behavioral recovery.\textsuperscript{1–3} Although central nervous system (CNS) plasticity after trauma is a subject of controversy, the understanding of this and other mechanisms that provide a substrate for the recovery of function after neural trauma is important to effect improved patient outcome. For example, long-term treatment of patients with cerebral ischemia traditionally consists of symptom management and physical therapy.\textsuperscript{4–8}

A promising approach in recovery from behavioral dysfunction after brain injury is the administration of pharmacological agents that increase release at noradrenergic terminals. One such promising compound, d-amphetamine sulfate (D-AMP), is postulated to involve interactions with noradrenergic transmitter systems in the brain, specifically the catecholamines: norepinephrine, dopamine, and serotonin. D-AMP administration 24 hours after injury in rats, 10 days after injury in cats, and as late as 2 to 4 weeks in stroke patients results in improved function after cortical ablation, cortical contusion, and stroke.\textsuperscript{9–11} Although the mechanisms of D-AMP action remain unclear and some studies show no effect,\textsuperscript{14} behavioral results with D-AMP after cerebral trauma or stroke are promising. The purpose of this study was to measure dysfunction and recovery with the use of behavioral models that test spatial memory and sensorimotor function in D-AMP–treated compared with vehicle-treated rats with unilateral neocortical infarction and to correlate these data with the expression of genes involved in neurite growth and synaptogenesis. The hypothesis to be tested is that D-AMP treatment improves behavioral outcome after neocortical infarction and that one mechanism of D-AMP action involves the upregulation of proteins involved in neuronal remodeling, which is achieved by activation of alternate neural pathways by behavioral use.

Neurite growth, a component of anatomic plasticity, can be identified by the elevated expression of a growth-associated protein identified with a molecular weight of 43 kDa (GAP-43).\textsuperscript{17} GAP-43 is a membrane-bound protein found in the axonal growth cones of sprouting CNS neurites.\textsuperscript{17–25} Another protein useful in the identification of axonal sprouting and synaptogenesis is synaptophysin, a presynaptic vesicle protein (molecular weight 38 000) that is found in all nerve terminals. Levels of synaptophysin within the terminal are believed to remain constant, along with several other vesicle proteins, because of the recycling of vesicle material in the nerve terminal.\textsuperscript{26–28} and they have been used by a variety of laboratories to quantify numbers of terminals during neuroanatomical remodeling and neural development.\textsuperscript{29–34}

It is the objective of this study to use quantitative immunohistochemical techniques to determine increased expression by density and distribution measurements of GAP-43, as an indicator of neurite sprouting, and of synaptophysin, as an indicator of synaptogenesis. The expression of these proteins is measured in the neocortex after distal middle cerebral artery occlusion (dMCAo) with the use of the unilateral tandem occlusion model, in which focal cerebral cortical ischemia is produced by permanent dMCAo in spontaneously hypertensive Wistar rats (SHR).\textsuperscript{35–36} The use of the SHR strain provides spatially consistent and large neocortical infarct volumes because of the lack of anatomic variation in the middle cerebral artery (MCA) and the limited collateral circulation from anterior and posterior cerebral arteries. This results in highly reproducible, well-circumscribed focal ischemic injuries with very little to no penumbral region and no ischemic damage to subcortical structures.\textsuperscript{35–37} Thus, interanimal variability is reduced. Reproducibility of this focal ischemia model provides an important baseline for assessing changes in gene expression and behavioral recovery after treatment.

Since an important outcome measure is recovery of function, it is meaningful to correlate the expression of GAP-43 and synaptophysin with functional outcome in D-AMP–treated compared with vehicle-treated rats. The region of the neocortical ischemia produced in this model includes a portion of the forelimb neocortex (Figure 1). Consequently, forelimb dysfunction on the side contralateral to the ischemia is predicted, and we have previously reported this outcome.\textsuperscript{3} If the temporal expression of the proteins involved in neuroanatomical remodeling is increased in appropriate neocortical regions, i.e., the forelimb, and this increase corresponds with the improvement of forelimb function in D-AMP–treated rats compared with vehicle-treated rats, then these data would provide support that D-AMP enhances neuroanatomical remodeling and provides a mechanism for recovery of function.
in this model. In addition, since the neocortical denervation is extensive, alterations of spatial behavior brought about by secondary and tertiary neuronal death might occur with a time course of several days to weeks. Consequently, another aim of this study is to test spatial memory performance of the rats by monitoring the Morris water maze behavior over time and to compare the temporal behavioral data with the temporal pattern of protein expression in D-AMP–treated rats compared with vehicle-treated controls.

**Materials and Methods**

**Surgical Methods**

Nonfasted male SHR (weight, 260 to 300 g) were anesthetized with halothane (4% induction/1% maintenance), placed on a heating pad, and given an antibiotic (streptomycin, 0.10 mL, 150 mg/mL IP). SHR were used to ensure constant infarction volume and placement because of poor collateral circulation and consistent vascular anatomy. With the use of surgical methods previously described briefly, the right common carotid sheath was ligated with 4-0 suture, and the distal portion of the MCA was permanently ligated with a 10-0 suture knot tied immediately proximal to the frontal branch. Sham controls were prepared for surgery similarly except that the 10-0 suture was not tied into a knot but was left in place. The animals recovered within 2 hours after surgery was completed. If an animal survived 12 hours after surgery, there was no further testing and mortality. Antibiotics (vancomycin, 0.10 mL, 150 mg/mL per day IP) were given for 2 days after surgery. The animals were then housed 2 to a cage. Functional damage could not be detected by gross motor and sensory observations. Animals were allowed to recover for time points of 3, 7, and 14 days and 1 and 2 months with n = 8 in each group (D-AMP and vehicle injections) for each time point. The same animals in which behavioral tests were performed were killed at these time points for immunocytochemical reaction products, as described below. The original design of the experiment was to compare D-AMP– and vehicle-injected dMCAo animals against several control groups: (1) vehicle-injected sham-operated group; (2) vehicle-injected unoperated group; (3) D-AMP–injected sham-operated group; and (4) D-AMP–injected unoperated group. These control groups were designed to rule out confounding behavioral or immunocytochemical changes in response to D-AMP treatment. These control groups showed no statistical differences from naive animals in any test and are not discussed further. All procedures were approved by the University of Texas Medical Branch Animal Care and Use Committee.

**D-Amphetamine Regimen**

Animals were given a single intraperitoneal injection of 2 mg/kg D-AMP dissolved in distilled water (10 mg/10 mL) or vehicle on days 3, 6, and 13 and every third day until day 30. To determine a reasonable test dose of D-AMP for the SHR strain before the experimental design, 5 rats were tested behaviorally at 5 mg/kg, 5 at 3 mg/kg, and 5 at 2 mg/kg D-AMP in a single intraperitoneal injection. Chronic injections of D-AMP have been reported to result in excitotoxic neuronal death. However, the experimental dose used (2 mg/kg) is <1/100 of the dose reported in which excitotoxic neuronal death occurs. In addition, we found no cell loss in catecholaminergic neuronal populations, which are reportedly more susceptible to excitotoxic effects of D-AMP injections, in these animals. Animals were tested on the foot-fault test 1 hour after injections test while the animals were intoxicated, a “training” period according to the above injection regimen. All behavioral tests were performed again, 24 hours after injection, for both vehicle- and D-AMP–injected groups until the animals were killed for immunocytochemistry. The foot-fault behavioral data analyses include data for both the training and 24 hours after intoxication. For the Morris water maze test, the same groups in the foot-fault test were subjected to only 2 days of testing: the behavioral intoxication training period, and behavioral tests 24 hours after the injection (D-AMP or vehicle) on the day of euthanasia. Only data collected on the day of euthanasia were analyzed. This design eliminates any effect of multiple repeated maze testing over time on the Morris water maze and effects that the amphetamine intoxication may have had on swim speed and platform acquisition time. One group each of D-AMP– and vehicle-injected animals was maintained on the injection and training regimen for 30 days, then taken off both the injection and training regimens for 30 days and tested for permanence of recovery (60-day group). The 3-day survival animals had an injection on day 2 after dMCAo. All activity was recorded on videotape for scoring at a later time. Because the treated groups were killed for immunocytochemical analyses on the days described below, the behavioral data between groups were analyzed with the behavior recorded on the day of euthanasia. Thus, the immunocytochemistry and behavioral data were collected from the same group of animals at each time point.

**Immunocytochemistry Methods**

At various time points after surgery (3, 7, 14, 30, and 60 days), 2 groups of animals (n = 8 in D-AMP–treated dMCAo groups and n = 8 for vehicle-treated dMCAo groups) were anesthetized with sodium pentobarbital and perfused transcardially with 4% paraformaldehyde fixative in phosphate buffer containing 0.1% picric acid. The brains were removed, photographed, taken through graded sucrose solutions in fixative up to 30%, blocked and embedded in OCT freezng compound (Miles, Inc), frozen, and stored at −70°C. Cryosections (60 μm thick) were run as sets with sections from infarcted brains and sham control brains run simultaneously, as previously described. Sections were immunostained with a monoclonal antibody to either GAP-43 (Boehringer Mannheim) or synaptophysin (Boehringer Mannheim) at a dilution of 1:500. Every fifth section was mounted on gelatin-coated slides for silver staining. To ensure that immunostaining was specific, control reactions were immunostained in the same solutions with no primary antibody, with glial fibrillary acidic protein primary antibody (1:500, Boehringer Mannheim), or with a leukocyte common antigen primary antibody (1:500, Chemicon). The tissue processed in the absence of primary antibody had little immunostaining. The glial fibrillary acidic protein antibody and the leukocyte common antigen antibody caused immunostaining that was specific for astrocytes or leukocytes, respectively, with relatively homogeneous staining with the exception of a narrow band of immunoreaction surrounding the infarction, which was subtracted from the region analyzed for GAP-43 and synaptophysin. Optical density measurements of immunoreacted tissue were made from an enlarged image (×500) with a Quantex QX-7 Image Analyzer System (Quantex Corp). With methods described earlier, a standard square area was generated (0.2 × 0.2 mm of tissue), and mean radiance (light transmission) was measured on a scale of 0 to 256 relative units. This measurement was repeated 5 times for each area, medial and lateral to the region of damage. Limits of ischemic damage were delineated by microscopic examination of adjacent sections with ammoniacal silver staining. Density measurements were taken in a line perpendicular to a tangent of the surface of the cortex. For comparison, similar measurements were taken from analogous locations of the same anatomic regions from sections with the same atlas coordinates in sham control sections, processed for immunoreactivity at the same time as the experimental sections. Background measures of immunoreactivity for each section (5 measures per section) were determined by measuring in regions of white matter (corpus callosum, anterior commissure, or internal capsule) found on the section. These background measurements were subtracted from the cortical measurements and normalized to establish percent optical densities. Normalized optical densities of the regions in the ischemic brains were then compared with normalized optical densities from corresponding regions in sham control sections. All tissue sections were coded, and data were collected by laboratory personnel shielded from surgical status, time from injury, and treatment status.

**Areas of Increased Immunoreactivity**

To examine increases in the distribution of enhanced immunoreactivity, a threshold level was determined, and all readings above this level were considered increased immunoreactivity.
density were assigned a pseudocolor. The area measurement was then calculated by computer calibration of an input scale relative to pixel size with the “areal” function of the Quanext software. This method was used to calculate the area and distribution of GAP-43 at 3 days after infarction and of synaptophysin at 60 days after infarction in the cortical regions medial and lateral to the infarcted region and in the contralateral cortex.

Quantification of Infarct Area and Volume
To establish the volume damage, photographs of whole brains were taken through a surgical microscope. Prints (enlarged ×8) were then placed under a grid, and the area of the damage was measured (pallid tissue due to poor perfusion of picric acid). The measurements were repeated 3 times for each brain. Infarct volumes were then calculated with the use of cortical depth from similar regions in sham control sections.

Behavioral Methods
For locomotor assessment, the rats were placed on elevated hexagonal grids of 2 sizes to test placement dysfunction of the forepaw with the Hernandez-Schallert foot-fault test. The grids had openings of either 3 cm (small) or 6 cm (large). Both grid sizes were chosen to allow comparison with other studies that use either small or large grids. Rats place their paws on the wire while moving along the grid. The rats were video recorded from below the grid for ease in recording the stepping pattern. With each weight-bearing step, the paw may fall or slip between the wires. This is recorded as a foot fault. The number of faults for the forepaw contralateral to the infarction is recorded along with the number of successful steps and displayed as a percentage of contralateral forelimb foot faults per forelimb steps. Faults were calculated for both sizes of grids.

Results
In the determination of the D-AMP dose response, it is of interest to note that preliminary injections of D-AMP in the amount of 5 mg/kg resulted in a death rate of 80% of the SHR. Lower doses of 3 mg/kg did not result in morbidity but produced extreme stereotypy, that is, the rats performed repetitive head movements and produced little locomotive behavior. We empirically determined that doses of 2 mg/kg IP produced an acceptable level of intoxication in this strain without evidence of D-AMP–induced neuronal toxicity, as determined by pathological assessments of paraffin sections stained with hematoxylin and eosin, thus confirming optimal single doses previously reported.

Histology
At the time of euthanasia, the region supplied by the occluded MCA, principally the parietal and occipital cortex, appeared infarcted, as determined by gross inspection, because of a lack of color from the picric acid in the perfusate and an inconsistent texture compared with the surrounding cortex. The mean±SD value of area of infarction in 12 brains of D-AMP–treated rats, chosen at random, was 59.2±3.2 mm², which can be compared with the untreated mean area of 58.4±2.8 mm² and was not statistically different. The mean volume of the infarction in D-AMP–treated rats was calculated to be 88.8±9.6 mm³, which was not statistically different compared with untreated value of 86.4±8.0 mm³. The region of ischemia was confined to the neocortex (Figure 2). By comparison, the contralateral neocortex regions appeared undamaged at 3 days both by gross inspection and in histological sections. The histology of the hippocampus and other subcortical structures appeared normal in hematoxylin and eosin–stained sections, in thionin-stained sections, and in ammoniacal silver–stained sections. Area cell counts of hematoxylin and eosin–stained sections of the hippocampal CA1 region ipsilateral and contralateral to the infarcted side demonstrated no difference in pyramidal cell number between sham controls and D-AMP– or vehicle-treated infarcted animals or between sides in the infarcted animals. Both of the lateral ventricles and the third ventricle appeared enlarged in both the D-AMP– and vehicle-treated dMCAo groups relative to noninfarcted animals, as reported earlier. Sham-operated animals did not have any damage when examined grossly or histologically as determined by the ammoniacal silver stains or by hematoxylin and eosin stains. For the immunoreaction product, intensity of reaction product varied with each reaction and from animal to animal but was consistent within each animal and within each section. In all comparisons, there was no statistical difference between preoperative and sham control values.
GAP-43 Immunoreactivity

The GAP-43 immunoreactivity in brain slices from vehicle-treated animals was consistent with results from previous studies of GAP-43 at 1 week after occlusion with no injection schedule.2,3 The immunostaining was heavier in areas of the cortex surrounding the infarction compared with control levels of gray matter and with the cortex in the contralateral hemisphere (Figure 3). Immunostaining was also heavy in the hypothalamus and in the cingulate gyrus in some animals (Figure 3). The D-AMP–treated animals demonstrated a greater amount of GAP-43 immunoreactivity in the neocortex both medial and lateral to the infarction compared with contralateral cortex or compared with the same regions in vehicle-treated animals at 3 and 7 days after infarction (P<0.025; Figure 4). The vehicle-treated animals had significantly higher levels of GAP-43 immunoreactivity in the neocortex surrounding the infarction at 3, 7, and 14 days after occlusion (P<0.025). At the 30- and 60-day time points, there was no statistical difference within groups or between groups. With the sham control values and background levels within each animal normalized to 1 to allow between-animal group comparisons, there was a significantly higher increase in GAP-43 immunoreactivity in the D-AMP–treated animals compared with vehicle-treated animals, with a combined normalized optical density of 3.8±0.46 medial and 3.8±0.35 lateral to the infarct region compared with 2.5±0.68 and 2.6±0.37, respectively, at 3 days after infarction and 2.8±0.50 medial and 2.9±0.31 lateral to the infarct region in the D-AMP–treated animals compared with 1.7±0.1 and 1.7±0.35, respectively, at 7 days after infarction (Figure 4). These values are statistically significant between the D-AMP– and vehicle-treated groups (P<0.025). The neocortical regions with elevated density in both vehicle- and D-AMP–treated groups correspond to the forelimb, the hindlimb, parietal regions 1 and 2, and the temporal neocortical regions ipsilaterally. GAP-43 immunostaining was also elevated at these time points in the cingulate and entorhinal cortices of both hemispheres and in the thalamus in D-AMP– and vehicle-treated animals. Elevated staining was also present in the hippocampus, dentate gyrus, and septal/hypothalamic regions in sections of D-AMP–treated and vehicle-treated animals and sham controls at all time points after occlusion.

The mean±SD values of the area measurements for the vehicle-treated group in the peri-infarct regions of increased GAP-43 density at the 3-day time point in sections at bregma −0.30 and interaural 8.70 were 1.01±0.45 mm² medial to the infarct in the hindlimb neocortical region and 2.6±0.57 mm² lateral to the infarct in the parietal 2 neocortical region. The area measurements of GAP-43 density in sections at bregma 0.70 and interaural 9.70 were 0.82±0.062 mm² in the forelimb neocortical region and 1.28±0.35 mm² lateral to the infarction in the parietal 1 neocortical region. For comparison, the area measurements of increased GAP-43 density in the D-AMP–treated group at 3 days were 1.34±0.45 and 2.85±0.52 mm² medial and lateral to the infarction at bregma −0.30 and interaural 8.70, which was not significantly different with immunoreaction product. The tissue is not counterstained, and therefore any density differences are due only to the immunoreaction product. Bar=2 mm.

Figure 2. Serial coronal sections of the rat brain. The regions of the cortex that sustained ischemic damage are shown as the darkened regions. Note that no subcortical regions were involved in the ischemic damage. Sham controls showed no ischemic damage. The location of the section relative to bregma is shown to the right for each section. Adapted from Paxinos and Watson113 (1986).

Figure 3. Light micrographs of Gap-43 immunoreaction product in 60-μm sections from rat brains taken at interaural 9.70 and bregma 0.70 at 3 days after dMCAo from the vehicle-treated group (A) compared with the D-AMP–treated group (B). Note the elevated staining in the lateral septal nucleus (S) and the medial forebrain region (arrow) and the peri-infarct region. Little immunoreaction product is present in white matter structures such as the corpus colossum and the anterior commissure; however, the gray matter stains diffusely with immunoreaction product. Bar=2 mm.
different than the vehicle group. Conversely, at bregma 0.70 and interaural 9.70, the area measurements were 2.54±0.25 and 1.73±0.22 mm² for medial and lateral, respectively, and these 2 areas were increased and significantly different (P<0.05) compared with area measurements of similar regions in the vehicle-treated group.

Synaptophysin Immunoreactivity

The synaptophysin immunoreactivity in brain slices was also consistent with results from previous studies showing increased synaptophysin immunoreactivity in the cortex surrounding the area of infarction and in the contralateral parietal cortex at 1 month.1–3 (Figure 5). The immunostaining was diffuse in both hemispheres, with little staining in the corpus callosum and other areas of white matter in both groups of animals. There was no significant difference in staining in D-AMP– or vehicle-treated rats at 3 and 7 days after infarction compared with sham controls. By contrast, there were significant differences in density compared with sham controls in the peri-infarct region and the contralateral cortex, which corresponds to the parietal 1 cortex, at 14 days, 1 month, and 2 months in both D-AMP–treated and vehicle-treated animals (P<0.025). Although the intensity of reaction product varied from animal to animal, all animals in both the vehicle- and D-AMP–treated DmCAo groups demonstrated increased synaptophysin reaction product in the contralateral parietal 1 cortex as measured by image analyses. The ipsilateral neocortical regions with elevated density in both vehicle- and D-AMP–treated groups correspond to the forelimb, the hindlimb, parietal regions 1 and 2, and the temporal neocortical regions ipsilaterally. Synaptophysin immunoreactivity was also elevated at these time points in the cingulate and entorhinal cortices of both hemispheres and in the thalamus and septal/hypothalamic regions in both D-AMP– and vehicle-treated animals compared with sham controls (P<0.025). There was no statistical difference in the level of synaptophysin immunoreactivity in the cingulate and entorhinal cortices of both hemispheres and in the thalamus and septal/hypothalamic regions in both D-AMP– and vehicle-treated animals compared with sham controls.
synaptophysin immunoreactivity when we compared similar regions between the D-AMP– and vehicle-treated rats at any time point (Figure 6).

The mean±SD values of the area measurements of increased synaptophysin density in the vehicle-treated dMCAo group at the 60-day time point in sections at bregma −0.30 and interaural 8.70 were 1.03±0.21 mm² medial to the infarct in the hindlimb neocortical region, 1.64±0.42 mm² lateral to the infarct in the parietal 2 neocortical region, and 1.58±0.39 mm² in the contralateral parietal 1 and 2 neocortical regions. The area measurements of synaptophysin density in this same group of animals in sections at bregma 0.70 and interaural 9.70 were 0.96±0.27 mm² in the forelimb neocortical region, 1.71±0.25 mm² lateral to the infarction in the parietal and insular neocortical regions, and 1.39±0.11 mm² in the contralateral parietal 1 neocortical regions. For comparison, the area measurements of increased synaptophysin density in the D-AMP–treated dMCAo group at 60 days were 0.83±0.26, 1.19±0.29, and 1.88±0.08 mm² medial, lateral, and contralateral to the infarction at bregma −0.30 and interaural 8.70, while at bregma 0.70 and interaural 9.70 the area measurements were 0.92±0.16, 1.61±0.77, and 2.16±0.34 mm² for medial, lateral, and contralateral to the infarction, respectively. In both sections, the contralateral parietal 1 neocortex of the D-AMP–treated group demonstrated an increased distribution that was significantly different (P<0.05) compared with area measurements of similar regions in the vehicle-treated group.

Behavioral Tests

**Hernandez-Schallert Foot-Fault Test**

D-AMP–treated dMCAo rats had a significantly better performance on the smaller grid than the vehicle-treated dMCAo animals at 2 and 3 days after surgery. This continued throughout the recovery period (P<0.025). Rats were tested for performance 1 hour after injection, which was a training period, and 24 hours later the test was repeated and recorded (Figure 7). The data displayed in Figures 7 and 8 are not data from the same groups over time but represent the results from different groups’ performances either during a training period (at 2, 6, and 13 days and 1 month) or on the day of euthanasia (at 3, 7, and 14 days and 1 and 2 months) for immunocytochemical analysis. The D-AMP–treated dMCAo group had no significant difference in the performance from sham-operated animals at 6 days after infarction. This recovery persisted throughout the time course of the experiment. Vehicle-injected dMCAo groups performed significantly worse on the small grid at all test times compared with sham controls. On large-grid performance, a similar pattern of significant results was obtained.

**Morris Water Maze**

Performance on the Morris water maze, displayed as acquisition time from start to platform, is shown graphed in Figure 8. At 3 days after infarction, the D-AMP–treated group did significantly worse than the vehicle-injected dMCAo group (P<0.025), suggesting that the D-AMP treatment 24 hours earlier during the training session on day 2 affected platform acquisition in the testing sessions. Spatial memory appeared
not to be a factor in these trials because the D-AMP–treated rats frequently bumped into the platform; however, the rats did not ascend onto the platform. The acquisition times of both the D-AMP– and the vehicle-treated groups were not statistically different from each other or from sham-operated animals at 7 and 14 days. However, the acquisition time of the vehicle-treated group did increase significantly with increasing time after surgery, being statistically significant at 1 and 2 months compared with the D-AMP–treated animals but statistically significant from sham controls only at 2 months ($P<0.025$). Swim speeds were measured for all groups, since swim speed could significantly affect behavioral assessments. No statistically significant difference was found between control, vehicle-treated dMCAo, or D-AMP–treated dMCAo groups.

There were statistically significant correlations of contralateral forelimb behavioral performance on the Hernandez-Schallert foot-fault test and GAP-43 immunoreactivity in the forelimb neocortical region in D-AMP–treated groups that were significant for both grid sizes (correlation coefficient = 0.9211, $P=0.0167$ for small grid; correlation coefficient = 0.9177, $P=0.0167$ for large grid). There was a statistically significant negative correlation of improved foot-fault behavior and elevated synaptophysin immunoreactivity in the D-AMP–treated groups over time for the small-grid performance (correlation coefficient = -0.9747, $P=0.0167$) but not for large-grid performance (correlation coefficient = -0.8944, $P=0.0833$). Thus, the numbers of foot faults were at minimal values at the same time points that the synaptophysin optical densities were at maximal values.
Discussion

To summarize the present study, we report that multiple intraperitoneal D-AMP treatments, accompanied by behavioral training during the period of drug effect, enhance behavioral recovery of sensorimotor function of the contralateral forelimb after neocortical infarction. Posttreatment performance on the Morris water maze test was statistically significant between D-AMP–treated and vehicle-treated animals because of the temporal degradation of performance of the vehicle-treated group. There was no difference in infarct volume between D-AMP–treated and vehicle-treated animals. In addition, we report a statistically significant increase in density and distribution of GAP-43 immunoreactivity in the peri-infarct region, including the forelimb neocortical region, and a statistically significant increase in distribution of synaptophysin immunoreactivity in the contralateral parietal cortex of D-AMP–treated rats compared with vehicle-treated rats with neocortical infarction. These data support the occurrence of neurite growth followed by synaptogenesis in the neocortex in a pattern that corresponds both spatially and temporally with contralateral forelimb behavioral recovery that is accelerated by D-AMP treatment. The high correlations between both GAP-43 and synaptophysin measures compared with foot-fault scores support a causal relationship. While the specific mechanism responsible for D-AMP–promoted expression of proteins involved in neurite growth and synaptogenesis and of enhanced behavioral recovery is not known, it is suggested that upregulation of proteins associated with neuronal remodeling occurs because of functional activation of pathways able to remodel in response to active behavioral performance. These data, when considered in the context of other D-AMP studies, suggest the usefulness of multiple-dose regimens of D-AMP treatment during the rehabilitation of patients with cortical ischemia or trauma.

Most postischemic patients show some signs of recovery over time that include both motor and cognitive behavior. Studies in rats show that behavioral recovery may have both short- and long-term components, resulting from both the resolution of acute symptoms such as edema and diaschisis and longer-lasting changes from plasticity in the brain. Although the mechanisms of recovery are controversial, data exist that support long-term recovery in humans as a function of neuronal remodeling in the brain, particularly in the peri-infarct region and in regions homologous to the infarcted region in the contralateral cortex. Behavioral research in our laboratories and others shows recovery in rats after cortical ischemia and indicates that there may be a link between cortical neuronal remodeling and recovery. In this study we demonstrate improved behavioral recovery after neocortical ischemia with D-AMP treatment that is associated with the enhanced expression of molecules involved in neuronal remodeling.

Although the existence of neuronal remodeling, including sprouting and synaptogenesis, in the CNS after injury is controversial, several lines of evidence using behavioral and lesion paradigms in both humans and animal models are consistent with the existence of neuronal remodeling in the neocortex. For example, cortical lesions in rats result in increased dendritic branching or changes of electrophysiological functional maps, which are often accompanied by behavioral recovery. We and others have published results that support the expression of genes involved in neuronal remodeling after ischemia. Consequently, data supporting the existence of cortical plasticity in response to ischemia with accompanying behavioral modifications are accumulating.

A variety of techniques allow the examination of proteins correlated with neuronal remodeling. The 2 proteins used in the present study, GAP-43 and synaptophysin, are associated with neurite growth and synapse formation, respectively. GAP-43 is a membrane-bound protein found in the growth cones of sprouting CNS axons. It is thought that GAP-43 acts with Gi proteins to regulate metabolic responses to signals. By blocking GAP-43 expression with antisense oligonucleotide probes, neurite outgrowth can be eliminated in cultured neurons; conversely, the transfection of fibroblasts to express GAP-43 will result in neuritelike process formation. Antibodies to GAP-43 have been used in the CNS to examine neuronal sprouting and regeneration. Synaptophysin, a presynaptic vesicle protein (molecular weight 38 000), is found in all nerve terminals. Levels of synaptophysin within the terminal are believed to remain constant along with several other vesicle proteins, owing to the recycling of vesicle material in the nerve terminal. Methods developed by Masliah et al allow the estimation of increases or decreases in synaptic numbers with the use of synaptophysin immunostaining and are now used by others in the fields of neuronal remodeling and neural development.

Of interest is the distribution of these proteins in the peri-infarct and contralateral neocortex. Since the increased immunoreactivity of GAP-43 and synaptophysin is interpreted to indicate axonal growth and synaptogenesis, respectively, these data are consistent with enhanced neurite sprouting in these regions, which include the ipsilateral forelimb neocortex and the parietal 1 neocortex both ipsilaterally and contralaterally. It is of interest to compare our results with those obtained after either unilateral occlusion of the proximal MCA or a thrombotic infarction of the vibrissal barrel-field cortex that resides in the parietal neocortices to emphasize the importance of the contralateral cortex. In the unilateral proximal occlusion MCA model, behavioral recovery was demonstrated in tests for functional improvements of the forelimb, hindlimb, beam balance, and spontaneous limb use. Treatment with basic fibroblast growth factor significantly improved behavioral recovery compared with vehicle-treated groups. GAP-43 immunoreactivity was demonstrated in both the ipsilateral peri-infarct region and the contralateral cortex in both groups but demonstrated a selective increase in the contralateral sensorimotor cortex. In the barrel-field cortex of animals with unilaterally thrombotic infarctions of the vibrissal barrel-field cortex, the ability to respond to vibrissal sensory information with a motor task was impaired but recovered over time by 60 days. Bilaterally infarcted animals demonstrated no recovery. These data support the involvement of the intact contralateral cortex.
in the recovery of function. Furthermore, 2-deoxyglucose studies by the same group indicated that 30 days after the unilateral cortical infarction, activation of the vibrissae contralateral to the infarct resulted in a spread of activation anterior and lateral to the infarcted zone and included areas within the ipsilateral somatosensory cortex.50 Our studies confirm the involvement of the neocortex anteriorly, medial and lateral to the infarct and contralateral to the infarct, in that proteins known to be involved in neuroanatomical remodeling are upregulated in these regions34,35 and in response to D-AMP treatment (present study). Thus, the present studies confirm our earlier studies that behavioral recovery in unilaterally infarcted animals is a consequence of functional plasticity with resultant neural circuit reorganization and extend these findings to propose that neuroanatomical reorganization is one mechanism by which the neural circuit reorganization is achieved.48

Furthermore, D-AMP treatment improves behavioral recovery not only in the sensorimotor task of the forelimb behavior but also in the spatial memory tasks. The behavioral results of the Morris water maze indicate an improved long-term benefit in terms of sustained functional recovery of the D-AMP–treated animals compared with the vehicle-treated animals since the vehicle animals exhibited a worsening of behavior. To our knowledge, we are the first to report a delayed worsening of behavior in the Morris water maze test after cortical neurotrauma. By contrast, poor performance after the acute 2-day dose of D-AMP may be related to its monoaminergic agonist role14 directly in the spatial memory task or indirectly as related to a reduction in behavioral “stress” in general and postsurgical stress and/or pain specifically. Therefore, treatment with D-AMP may result in animal behavior in which water swimming is no longer stressful, and thus the motivation to acquire and ascend the platform is absent. Monoaminergic agonists or transport inhibitors are well known to produce feelings of well-being in patients. On the other hand, the worsening of the vehicle-treated groups is not surprising since rats suffering from traumatic brain injury are known to exhibit long-lasting memory deficits45 and rats sustaining a neocortical lesion or ablation demonstrate loss of spatial function,46 presumably as a result of delayed retrograde and orthograde neuronal death as well as the altered circuitry created in response to the loss of significant neuronal populations in the injured cortex. The cortical deafferentation is then followed by sequential loss of projection neurons and/or circuitry in the thalamocortical pathway, followed by loss of appropriate circuitry in the hippocampal-thalamic pathways. We propose that the increased neural sprouting and synaptogenesis induced by D-AMP treatment may have contributed to the improved temporal course of behavior over time due to trophic support from increased neural input onto neurons that would have died otherwise, thus rescuing these vulnerable neuronal populations.

While evidence is mounting that D-AMP administration for 24 hours after injury indicates improved function after cortical ablation, contusion, or ischemia,9,10,12,13 the mechanisms of action remain unclear. One possible mechanism includes the ability of D-AMP to stimulate presynaptic release of the catecholaminergic terminals, which may act by disinhibition of inhibitory circuits, resulting in overall increased facilitation. Thus, cortical ablation or contusion experiments in which administration of norepinephrine improves function may occur through facilitatory, inhibitory, or both mechanisms because the effect of norepinephrine is dependent on the type of receptor activated (inhibition is mediated by β-norepinephrine receptors, while excitation is mediated by α-norepinephrine receptors).46 In support of a facilitatory mechanism, intracerebroventricular injections of norepinephrine but not dopamine are reported to improve recovery.12,37 Furthermore, with this reasoning, lesions of the locus coeruleus, which give rise to massive norepinephrine projections throughout the neuraxis, could result in facilitation of locomotor behavior after cortical injury; however, some experiments indicate facilitation of locomotor recovery,48 and 1 report indicates impairment of motor recovery after locus coeruleus lesions as a result of norepinephrine depletion by systemic administration of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine.89 However, the involvement of norepinephrine in recovery is well documented by many laboratories in which improved recovery with norepinephrine agonists is demonstrated,12 while impairment of recovery with norepinephrine α1-receptor antagonists (haloperidol, prazosin) as well as dopamine antagonists (haloperidol) and serotonin antagonists (p-chlorophenylalanine) is demonstrated after cortical injury.90–92

Another mechanism of D-AMP recovery is that norepinephrine and other catecholamines are involved with postinjury widespread metabolic depression,93 and thus D-AMP treatment, which is known to block posttraumatic depression of cortical metabolism,94 may act through a general mechanism contributing to increased metabolism.58,60,87,95 Another mechanism involves the activation of alternate pathways that, although normally depressed, could substitute to restore lost behavioral function. Thus, administration of D-AMP would result in activation of alternative pathways by, for example, increasing the size of cortical receptive fields responding to specific peripheral stimuli.94 An attractive mechanism involves D-AMP in cellular mechanisms that are responsible for learning and memory since D-AMP is effective if the animals are trained in the sensorimotor test during the D-AMP action.86 The relationship between D-AMP and learning is based on the involvement of catecholamines in the induction of long-term potentiation, a putative cellular mechanism of learning and memory. D-AMP is reported to facilitate the development of long-term potentiation in a dose-dependent manner7 and enhances memory retrieval.98

We propose an additional, nonexclusive, and novel mechanism for the action of D-AMP that involves the upregulation of genes involved in neuronal remodeling. For example, it is known that D-AMP can cause the induction of the immediate early gene c-fos.99–104 This induction is postulated to enhance the expression of proteins, including neurotrophins, that may be involved with dendritic and axonal sprouting in those pathways actively sprouting in response to the denervation. While
sprouting is held by many to be a process that involves weeks, carefully done studies at the ultrastructural level demonstrate that synaptogenesis occurs in CNS regions and is complete in 3 to 6 days in the spinal cord and by 10 days in the red nucleus and is 30% complete in the septal nucleus by 10 days. Although the time course of CNS synaptogenesis may vary after denervation, it is well understood that denervated postsynaptic sites survive the loss of presynaptic elements and induce synaptogenesis, and the new synapses may be either homotypic (from the original innervation source) or heterotypic (from another innervation source). In addition, hierarchies of preference exist in which homotypic synaptogenesis will outcompete heterotypic synaptogenesis, The degree of homotypic versus heterotypic synaptogenesis will have functional consequences on recovery. We propose that D-AMP treatment in concert with performance of behavioral tasks can selectively upregulate neurite growth in those neural circuits able to subserve the behavioral function tested, despite the loss of the original pathways. Thus, physical therapy during the effective window of D-AMP treatment may improve recovery in patients with cortical ischemia and trauma.

Acknowledgments
This study was supported by grants NS 07185, NS 11255, and RR 03979 and by Bristol Myers–Squibb. The authors would like to thank Drs William D. Willis, Myron D. Ginsberg, and the reviewers for their careful reading and constructive criticism of this manuscript.

References


42. Whishaw IQ, Mittleman G. Visits to starts, routes and places by rats (Rattus norvegicus) in swimming pool navigation tasks. J Comp Psychol. 1986;100:422–431.


Despite the widespread pessimistic attitude toward treatment of brain damage in 1970, *Stroke* was founded with the goal of developing treatments from the combined effort of basic scientists and clinicians focused on this intractable problem. In the past decade this attitude has reversed to one of optimism and an expanding research effort devoted to reducing stroke severity. However, this approach of lessening the primary injury is unlikely to help the majority of stroke patients. There is considerable evidence that the therapeutic window for limiting the extent of primary damage is between 3 and 6 hours. Since the signs and symptoms of stroke do not cause pain or external bleeding, most patients consider their symptoms minor and do not seek medical attention within 6 hours after the stroke. For the treatment of the majority of stroke patients first seen after the window for early intervention has closed, it is important to investigate potential therapies that can be initiated late after stroke. To my knowledge, there is only 1 established intervention that enhances both the rate of recovery and level of ultimate outcome after stroke and other types of brain injury: the single or short-term administration of any of the family of drugs enhancing norepinephrine/PT, promotes recovery from hemiplegia in brain-injured hemiplegic rats when treatment is delayed for 10 days. These data have been recently reported by severely affected stroke patients reported significantly improved outcome from hemiplegia when treatment was initiated late after stroke.
initiated as late as 30 days after the infarct, and the ultimate level of recovery remained significantly higher for the treated group compared with placebo/PT controls 1 year after stroke or 8 months after cessation of treatment. In the accompanying article by Stroemer et al, this experimental treatment was begun 3 days after an infarct in a rodent stroke model, and short-term treatment successfully promoted recovery of sensorimotor and cognitive deficits.

A unique aspect of the theoretical approach by Stroemer et al is that the neurophysiological measurements were focused on normal intact tissue adjacent or contralateral to the primary injury, which was unaffected by the treatment regimen. Because this treatment is effective when started days to weeks after injury and also produces a very rapid improvement, previous hypotheses of the mechanism of the norepinephrine/PT effect centered on remote metabolic dysfunction in areas connected to the primary injury. Supporting this hypothesis are studies reporting that delayed D-AMP administration produces an enduring normalization of postinjury hypometabolism that is worsened by α1-noradrenergic antagonists. The study by Stroemer et al evaluated a different hypothesis of processes evoked by this treatment in remote intact tissue. Using immunohistochemistry measures of the growth-associated proteins GAP-43 and synaptophysin, they report that norepinephrine/PT treatment enhances both proteins at different times after injury. By measuring temporal changes of both behavioral recovery and growth-associated proteins, the authors found that D-AMP not only enhances the amount of GAP-43 but that the amount of change was highly correlated with sensorimotor recovery. These observations strengthen the authors’ proposal for a causal role for neural remodeling as a mechanism whereby this treatment enhances functional recovery.

The authors’ suggestion that multiple mechanisms may underlie this treatment effect is quite important. At the behavioral level of analysis, with measures of grid walking for assessment of sensorimotor symptoms and the Morris water maze for assessment of cognitive deficits, recovery of a deficit may appear as a single phenomenon. However, investigations of the mechanisms of this treatment reveal multiple correlated physiological processes. As the authors suggest, the rapid enhancement of recovery from hemiplegia after this intervention may result from alleviation of metabolic depression, but neuronal remodeling may contribute to the later stages of treatment-enhanced recovery.

A few issues must be investigated to clarify and confirm the important observations and neuronal remodeling hypothesis of the norepinephrine/PT treatment proposed by Stroemer et al. First, their conclusion that neural sprouting and synaptogenesis are enhanced by the treatment is only an inference from measures of the amount of growth-associated proteins. It is a likely inference, but direct measures of neuronal growth must be made to confirm their interpretation. This is important because the authors note that GAP-43 and synaptophysin are associated with the release of transmitters, including both noradrenaline and dopamine. Enhanced release of these neurotransmitters could be more important for functional recovery than neuronal remodeling. Second, as noted by the authors, numerous studies have demonstrated that the family of drugs increasing central norepinephrine levels, when combined with PT, promotes recovery. It is not known which catecholamine increases the levels of these growth-associated proteins. Third, even if enduring recovery is attributable to neuronal remodeling, such new wiring must be regulated by α1-noradrenergic receptors. It is important to recall that the relation between norepinephrine and functional recovery has 2 aspects: drugs increasing norepinephrine combined with PT enhance recovery, whereas drugs reducing norepinephrine slow recovery when given early after injury and transiently reinstate deficits in recovered animals. The laboratory and clinical studies of harmful effects on recovery of drugs reducing central norepinephrine levels have been recently reviewed. A short movie of the reinstatement of hemiplegia by an α1-noradrenergic antagonist months after recovery and a second movie illustrating rapid recovery after D-AMP given 24 hours after brain injury can be downloaded from my Web site: http://www.unm.edu/~feeney/index.html. At least for hemiplegia, the state we call recovery remains vulnerable to disruption long after apparent remission of symptoms.

Finally, some comment should be made regarding the increased death of animals given high doses of D-AMP in the pilot studies by Stroemer et al. This is apparently a strain-specific response to high doses. In another study evaluating this treatment in which Sprague-Dawley rats and homologous blood clots were used to produce a severe stroke with high mortality, a significant reduction of mortality was observed in the treatment group compared with saline controls. In addition, when higher doses of D-AMP in a phototherombotic rat stroke model were used, enhanced recovery and evidence for use of alternative circuits were reported, but no increase in mortality was noted. More importantly, no undue side effects have been reported in human studies in which >50 apathic and/or hemiplegic stroke patients were treated with the norepinephrine/PT therapy (D. Walker-Batson, PhD, personal communication, 1998).

Regardless of these issues, the study by Stroemer et al not only adds to the growing literature on the efficacy of this late experimental therapy for stroke but provides a novel hypothesis regarding the mechanisms of the effect.

Note Added in Proof
A recent clinical study provides additional support for the norepinephrine/PT strategy for promoting recovery in stroke patients. The investigation describes enhanced functional motor recovery and less depression after stroke through short-term treatment with methylphenidate in conjunction with PT. This new study is an extension of laboratory studies of methylphenidate and supports the utility of the rat hemiplegia model for predicting drug effects on symptoms in stroke patients.

Dennis M. Feeney, PhD, Guest Editor
Departments of Psychology and Neurosciences
University of New Mexico
Albuquerque, NM
References


Enhanced Neocortical Neural Sprouting, Synaptogenesis, and Behavioral Recovery With d-Amphetamine Therapy After Neocortical Infarction in Rats

R. Paul Stroemer, Thomas A. Kent and Claire E. Hulsebosch

*Stroke*. 1998;29:2381-2395
doi: 10.1161/01.STR.29.11.2381

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://stroke.ahajournals.org/content/29/11/2381

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:

http://stroke.ahajournals.org/subscriptions/