Progressive Cognitive Deficits in a Mouse Model of Recurrent Photothrombotic Stroke

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Background and Purpose—In spite of its high disease burden, there is no specific treatment for multi-infarct dementia. The preclinical evaluation of candidate drugs is limited because an appropriate animal model is lacking. Therefore, we aimed to evaluate whether a mouse model of recurrent photothrombotic stroke is suitable for the preclinical investigation of multi-infarct dementia.

Methods—Recurrent photothrombotic cortical infarcts were induced in 25 adult C57BL/6 mice. Twenty-five sham-operated animals served as controls. The object recognition test and the Morris water maze test were performed >6 weeks to assess cognitive deficits. Afterward, histological analyses were performed to characterize histopathologic changes associated with recurrent photothrombotic infarcts.

Results—After the first infarct, the object recognition test showed a trend toward an impaired formation of recognition memories (P=0.08), and the Morris Water Maze test revealed significantly impaired spatial learning and memory functions (P<0.05). After recurrent infarcts, the object recognition test showed significant recognition memory deficits (P<0.001) and the Morris water maze test demonstrated persisting spatial learning and memory deficits (P<0.05). Histological analyses revealed remote astrogliosis in the hippocampus.

Conclusions—Our results show progressive cognitive deficits in a mouse model of recurrent photothrombotic stroke. The presented model resembles the clinical features of human multi-infarct dementia and enables the investigation of its pathophysiological mechanisms and the evaluation of treatment strategies. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.115.008905.)

Key Words: animal model • cognition • poststroke dementia • stroke

After a first-ever stroke, ≈10% of patients develop poststroke dementia, which is defined as dementia occurring after stroke in patients without dementia before stroke.1 After recurrent stroke, the prevalence of poststroke dementia rises from 10% after a first-ever stroke to >30%, thus having coined the term multi-infarct dementia.2 With a 1-year recurrent stroke rate of >10%, multi-infarct dementia has an unequivocally high contribution to the long-term disability of stroke patients.3 But in spite of its high disease burden, there is no specific treatment for multi-infarct dementia. One reason for the lack of specific treatments is that the underlying pathophysiological mechanisms of multi-infarct dementia are unclear. The secondary gliosis in remote brain regions, which share synaptic connections with the lesion site, might play an important role in the pathophysiology of multi-infarct dementia.4 5

However, an appropriate animal model of multi-infarct dementia, which enables the investigation of its pathophysiological mechanisms and the preclinical evaluation of potential treatments, is lacking, to date. In the current work, we present a mouse model of recurrent photothrombotic stroke that leads to increasing cognitive impairment and resembles the clinical features of human multi-infarct dementia.

Methods

Animals
Our experiments were performed using adult male C57BL/6 mice. All experiments were conducted in accordance with animal welfare regulations, and experimental protocols were approved by the local ethics committee. All experiments were performed in a randomized and blinded design.

Ischemia Model
Focal cerebral ischemia was induced in the right parietal cortex of 25 adult male C57BL/6 mice by photothrombosis of cortical microvessels (detailed Methods are available in the online-only Data Supplement).6 At an interval of 10 days, a second photothrombotic
Assessment of Cognitive Deficits

The object recognition test and the Morris water maze test were used to assess cognitive deficits. The object recognition test, which is based on the preference of rodents to explore novel objects rather than familiar objects, was performed 3 days after the first infarct and 3 days after the recurrent infarct (Figure IA in the online-only Data Supplement) as previously described. In brief, animals (n=15 per group) were exposed to 2 identical objects during a familiarization trial. After a delay of an hour, one of the familiar objects was replaced by a novel object. As a measure of object recognition, a previously used score was calculated as follows: (novel object interaction time−familiar object interaction time). Detailed Methods are available in the online-only Data Supplement.

In another group of animals, the Morris water maze test was performed during the first week after the first infarct, during the first week after the recurrent infarct, and with a delay of 4 weeks after the recurrent infarct (Figure IB in the online-only Data Supplement). In brief, animals (n=10 per group) learned to use spatial cues to find a hidden escape platform located at a fixed position below the water surface. During the acquisition trials, the latency to reach the platform and the swimming speed were recorded using Ethovision XT tracking software (Noldus Information Technology). In the probe trial, which was performed without an escape platform, the time the animals navigated through the former platform quadrant and the amount of platform crossings were recorded.

Object Recognition Test

After the first infarct, the object recognition test revealed a trend toward recognition memory deficits among lesioned mice (P=0.08, Student t test with Bonferroni correction; Figure 1A). After recurrent infarcts, the animals’ ability to distinguish between novel object and familiar object was significantly impaired (P=0.0003, Student t test with Bonferroni correction; Figure 1B). The total exploration time did not differ between both groups.

Water Maze Test

After the first infarct, lesioned animals had reduced spatial learning abilities, as shown by a significantly increased latency to reach the platform in the acquisition trials of the Morris water maze test (P<0.05, repeated measures ANOVA; n=10 per group; Figure 2A). The swimming speed did not differ between groups, demonstrating that the observed learning deficits were not attributable to impaired motor functions (Figure 2A). In the probe trial, the amount of platform crossings and the time spent in the target quadrant did not differ between both groups (Figure II A in the online-only Data Supplement). After a recurrent infarct, lesioned animals exhibited stable learning deficits. The latency to reach the hidden platform was significantly increased among lesioned animals (P<0.05; n=10 per group; Figure 2B), and the swimming speed did not differ between groups (Figure 2B). There were no differences in the probe trial (Figure II B in the online-only Data Supplement). Four weeks after a recurrent infarct, lesioned animals had an increasingly shallow learning curve, indicating significant learning deficits (P<0.05; n=9 or 10 per group; Figure 2C). The swimming speed remained unchanged (Figure 2C). The probe trial showed a trend toward a reduced amount of platform crossings among lesioned animals (P=0.07, Student t test with Bonferroni correction; Figure II C in the online-only Data Supplement).

Infarct Volumes

Histological analyses were performed 4 days after recurrent stroke. The mean volumes of reorganized first infarct and comparisons, the P value was adjusted according to Bonferroni’s method.

Physiological Parameters

Physiological parameters such as body weight and body temperature did not differ between groups.

Histological Analyses

A subgroup of animals (n=8 per group) was used for histological analyses. For infarct volume assessment, coronal sections (10 μm) that was collected at 200-μm intervals were stained with toluidine blue (Sigma, St Louis, MO), and the edema-corrected mean infarct area was multiplied by the longitudinal diameter. To verify the hypothesis that secondary gliosis in remote brain regions plays an important role in the pathophysiology of multi-infarct dementia, we performed immunohistochemical analyses using anti-GFAP (1:500; Dako, Hamburg, Germany) and anti-F4/80 (1:500; Serotec, Biozol, Germany) double staining. Mitochondrial damage was assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (Roche, Basel, Switzerland) and Neuronal Nuclei (NeuN, 1:200; Millipore, Schwabach/Ts, Germany) double staining (detailed Methods are available in the online-only Data Supplement).

Statistical Analysis

Statistical analyses were performed using the Statistical Package of Social Sciences (version 18.0; SPSS Inc, Chicago, IL). The results are presented as means±SEM. Behavioral tests were analyzed by 2-way repeated measures ANOVA. One-way ANOVA followed by Fisher’s protected least significant difference post hoc test was used for comparisons with ≥2 groups. Student t test was used for comparisons between 2 groups. P<0.05 was considered as significant.
recurrent infarct were 6.86±0.77 mm³ and 12.56 ±3.06 mm³, respectively (n=8 per group). Moreover, the toluidine blue stained coronal sections demonstrated that the infarct area did not involve the hippocampus.

Immunohistochemical Analyses
The number of GFAP-expressing astrocytes and of F4/80-expressing activated microglial cells and macrophages was assessed in 4 regions of interest (peri-infarct cortex, subcortical white matter, perirhinal cortex, and hippocampus) 4 days after the recurrent infarct.

When compared with sham level, the number of astrocytes was elevated in the peri-infarct cortex, subcortical white matter, perirhinal cortex, and hippocampus of both hemispheres (P<0.001, ANOVA followed by Fisher’s protected least significant difference test; Figure 3). The subcortical astrogliosis ipsilateral to the recurrent infarct (200.36±27.06 per mm²; Figure IVA in the online-only Data Supplement) did not differ from the subcortical astrogliosis ipsilateral to the first infarct (159.63±17.23 per mm²; P=0.22; Figure IIIA in the online-only Data Supplement). The hippocampal astrogliosis ipsilateral to the recurrent infarct (205.04±20.14 per mm²; Figure 3) did not differ from the hippocampal astrogliosis ipsilateral to the first infarct (240.62±23.68 per mm²; P=0.27; Figure 3), and astrogliosis in the perirhinal cortex ipsilateral to the recurrent infarct (59.17±5.61 per mm²; Figure 3) did not differ from astrogliosis in the perirhinal cortex ipsilateral to the first infarct (55.43±3.82 per mm²; P=0.72; Figure 3), either. The cortical astrogliosis, by contrast, was more intense ipsilateral to the recurrent infarct (173.67±8.4 per mm²; Figure 3) than ipsilateral to the first infarct (75.84±5.87 per mm²; P<0.001; Figure 3), indicating a decreasing astrogliosis in the peri-infarct cortex when compared with persisting astrogliosis in the hippocampus, perirhinal cortex, and subcortical white matter.

Compared with sham level, an increased microglial activation was observed in the peri-infarct cortex ipsilateral to the recurrent infarct (176.02±12.52 per mm²; P<0.001; Figure 3). The microglial activation ipsilateral to the first infarct was not increased (9.83±3.16 per mm²; Figure 3). The microglial activation in the peri-infarct cortex was more intense ipsilateral to the recurrent infarct than ipsilateral to the first infarct (P<0.001; Figure 3), indicating a transient microglial activation in the peri-infarct cortex. Compared with sham level, microglial activation in the perirhinal cortex was increased in both hemispheres (P<0.05; Figure 3). There was no difference between microglial activation in the perirhinal cortex ipsilateral to the recurrent infarct (8.43±3.22 per mm²; Figure 3) and microglial activation in the perirhinal cortex ipsilateral to the first infarct (10.17±2.91 per mm²;
The hippocampal and subcortical microglial activation were not increased either ipsilateral to the first infarct (20.13±10.33 per mm² and 7.02±3.43 per mm², respectively) or ipsilateral to the recurrent infarct (20.60±7.62 per mm² and 17.32±6.40 per mm², respectively). Terminal deoxynucleotidyl transferase dUTP nick-end labeling and NeuN double staining revealed neuronal apoptosis in the peri-infarct region (Figure IV in the online-only Data Supplement). We did not observe apoptotic neurons in the hippocampus or in the perirhinal cortex.

**Discussion**

After a first photothrombotic cortical stroke, the object recognition test revealed a trend toward an impaired formation of recognition memories among lesioned mice. Spatial learning and memory functions were significantly impaired after the first stroke, as illustrated by an increased latency to reach the platform in the Morris water maze test. After recurrent stroke, cognitive deficits were more pronounced. The object recognition test showed significant recognition memory deficits, and the Morris water maze test showed significantly impaired spatial learning and memory functions. Four weeks after recurrent stroke, lesioned animals had an increasingly shallow learning curve. The swimming speed remained unaffected during the whole experiment, we did not observe circling or thigmotactic swimming, and a cued maze trial did not show differences between both groups, either. These findings demonstrate that...
deficits in the animals’ water maze performance were not attributable to confounding motor disturbances, but a result of truly impaired memory functions. Our histological analyses showed remote astrogliosis in the hippocampus and the perirhinal cortex of both hemispheres.

The occurrence of cognitive impairment after experimental stroke has been described in several previously published articles. However, deficits in water maze tasks have often been attributed to confounding sensorimotor disturbances after middle cerebral artery occlusion, the most common model of stroke. In the present work, we confirm that photothermotic cortical infarcts, which cause minor sensorimotor deficits, are well suited for the investigation of cognitive deficits. Moreover, we show that recurrent photothermotic infarcts lead to progressive memory deficits comparable with human multi-infarct dementia, which is characterized by a rise in incidence after each recurrent stroke. Beyond, animals in our model exhibit deficits in memory, visuo-spatial abilities, and executive function, thus resembling the clinical features of multi-infarct dementia. Another advantage of our model is the use of adult mice without comorbidities, which excludes confounding risk factors for vascular dementia and non-stroke-related cognitive impairment. A limitation of the presented model is that large sample sizes are needed to detect behavioral improvement. As an example, a sample size of 29 animals would be needed to detect a 40% improvement in the water maze test performed 4 weeks after recurrent stroke.

Our histological analyses revealed remote astrogliosis in the hippocampus, which encodes spatial memories, and in the perirhinal cortex, which is of importance for the formation of recognition memories. The finding of remote astrogliosis in nonischemic brain regions that share synaptic connections with the lesion site was previously reported and explained by orthograde and retrograde degeneration of neocortical projections. Considering that memory formation requires an intact interplay between primary and associative cortical areas, hippocampal-cortical networks, and cortico-cortical networks, the degeneration of neocortical projections and subsequent remote histopathologic changes in the hippocampus and the perirhinal cortex may, in part, explain the observed memory disturbances in our model.

Conclusions
The presented model of recurrent photothermotic stroke in mice led to the progressive memory disturbances, thus resembling the clinical features of human multi-infarct dementia. Remote histopathologic changes in the hippocampus and the perirhinal cortex were identified as a potential pathophysiological mechanism of multi-infarct dementia. Altogether, the presented model represents a research tool to further investigate the pathophysiology of multi-infarct dementia and to establish specific therapies.

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Disclosures
None.

References
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Supplemental Methods

Photothrombotic stroke
Focal cerebral ischemia was induced in adult male C57BL/6 mice by photothrombosis of cortical microvessels in inhalation anesthesia with 1.5% isoflurane in 30% O₂/70% N₂O. A thermostat-controlled heating pad served to maintain a constant body temperature (37°C±0.5°C). The skull was exposed via a dorsal midline incision, and a cold light source (KL1500, Zeiss, Jena, Germany) with a diameter of 4 mm was positioned 3 mm posterior to the bregma and 3 mm right from the midline. Following an intraperitoneal injection of 0.15 ml Bengal rose (10 mg/ml) the skull was illuminated for 20 min. Sham-operated animals received the same treatment, including Bengal rose injection, without illumination of the skull. Subsequently, the skin was sutured and mice were allowed to recover from anesthesia. At an interval of 10 days, a second photothrombotic infarct was induced in the contralateral cortex.

Object recognition test
The object recognition test, which is based on the preference of rodents to explore novel objects rather than familiar objects, was employed to compare the animals’ recognition memory. The object recognition test was performed 3 days after the first stroke and 3 days after recurrent stroke. To avoid stress and a potentially interfering neophobic response, animals were allowed to explore the arena during two 10-min habituation trials two days before testing. Each test session consisted of a 10-min familiarization trial and a 5-min test trial. At the beginning of each trial, animals (n=15 per group) were placed near the front wall of the arena with their heads oriented away from the objects. During the familiarization trial animals were exposed to two identical objects. After a delay of an hour, one of the familiar objects was replaced by a novel object. As a measure of object recognition a previously described difference score was calculated as follows: (novel object interaction – familiar object interaction). Interaction with an object was defined as follows: Touching the object or directing the head at a distance of less than 2 cm towards the object. The objects did not have a natural meaning to mice. Between trials the objects and the arena were cleaned with 70 % ethanol to minimize olfactory cues.

Water maze test
In another group of animals, the water maze test was performed to assess spatial learning and memory functions. We used a modification of the protocol described by Morris et al.. The maze consisted of a circular pool with a diameter of 1.50 m. The water temperature was maintained at 21 °C. The day before the first acquisition session, animals were allowed to explore the pool during three habituation trials (supplemental figure 1). Each acquisition session comprised three trials with a maximum duration of 90 s and an intertrial interval of 60 s. During acquisition, the animals (n=11 per group) learned to use spatial cues to navigate to a hidden escape platform located at a fixed position below the water surface. The animals were released into the maze from varying positions. The trials were recorded by a video camera connected to a computer equipped with Ethovision XT tracking software (Noldus information technology). The latency to reach the platform and the swimming speed were determined. The acquisition sessions were followed by a probe trial (60 s) without an escape platform. In the probe trial, the time the animals navigated through the former platform quadrant and the amount of platform crossings were recorded. The water maze test was performed after the first stroke, after recurrent stroke, and with a delay of four weeks after recurrent stroke to investigate persistent memory deficits. A cued maze trial was performed to test for confounding sensorimotor impairment and to exclude a confounding lack of incentive among lesioned animals. The cued maze trial was performed with a visible platform.
Tissue preparation
Animals used for histological analyses (n=8 per group) were deeply anesthetized with xylazine/ketamine and perfused through the left ventricle with 0.9% NaCl, followed by 4% buffered paraformaldehyde (pH 7.4) for 10 minutes. Subsequently, brains were removed, postfixed in 4% buffered paraformaldehyde (3 h at 4°C), immersed in 20% sucrose solution overnight, frozen on dry ice and stored at -80°C. Coronal sections (10 µm) were cut with a cryostat (Leica AM 3050, Nussloch, Germany), mounted on glass slides (SuperFrost, Langenbrinck, Germany) and stored at -20°C.

Infarct volume assessment
For infarct volume assessment, coronal sections (10 µm) collected at 200 µm intervals were stained with toluidine blue (Sigma, St Louis, MO). On each section, the infarct area and the area of both hemispheres were measured using a computer-assisted analysis technique (ImageJ). To estimate the infarct volume the mean infarct area was multiplied by the longitudinal diameter. For infarct volume assessment in the acute phase (four days after stroke), an edema corrected infarct area was calculated as follows: Infarct area x (area of contralateral hemisphere/area of ipsilateral hemisphere).

Immunohistochemical analyses
Immunohistochemical analyses were performed to verify the hypothesis that the secondary gliosis in remote brain regions play an important role in the pathophysiology of multi-infarct dementia. Immunohistochemical stainings were performed with mounted coronal sections (10 µm) postfixed with 4% paraformaldehyde for 15 minutes. Slides were rinsed in 3% H2O2/Methanol for 10 minutes to block endogenous peroxidases and thereafter incubated in Blocking Reagent (Roche Diagnostics, Mannheim, Germany) for 15 minutes to prevent unspecific protein binding. The primary antibodies used were anti-GFAP (1:500, Dako, Hamburg, Germany) and anti-F4/80 (1:500, Serotec, Biozol Diagnostica, Eching, Germany) as primary antibodies. A mounting medium containing 4',6-diamidino-2-phenylindole (Vector, Burlingame, CA) was used for nuclear counterstaining. The immunofluorescence signal was visualized with a fluorescent microscope (Nikon Eclipse 80i, Nikon GmbH, Duesseldorf, Germany). For digitizing, we used the Stereo Investigator Software (MicroBrightField Inc., Williston, VT, USA) and ImageJ software (public domain). For further investigation of photothrombosis-induced, secondary histopathologic changes, we performed terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL, Roche, Basel, Ch) and Neuronal Nuclei (NeuN, 1:200, Millipore, Schwalbach/Ts, D) double staining.
**Figure I: Experimental design. A: Object recognition test.** The object recognition test was performed three days after the first stroke and three days after recurrent stroke (n=15 per group). Two days before testing animals were allowed to explore the apparatus during habituation sessions. **B: The Morris water maze test.** In another group of animals, the Morris water maze test was performed in the week after the first stroke, after recurrent stroke and with a delay of four weeks after recurrent stroke (n=9 or 10 per group). The day before testing animals were allowed to explore the apparatus during habituation sessions.
Figure II: Results of the probe trials. A: Results of the probe trial after a first infarct. There were no significant differences in the amount of platform crossings and in the time spent in the target quadrant (n=10 per group). B: Results of the probe trial after recurrent infarcts. There were no significant differences in the amount of platform crossings and in the time spent in the target quadrant (n=10 per group). C: Results of the probe trial four weeks after a recurrent infarcts. There was a trend towards a reduced amount of platform crossings among lesioned animals (p=0.07, student t-test with Bonferroni correction, n=9 or 10 per group).
Figure III: Immunohistochemical analysis of astrogliosis and microglial activation in the subcortical white matter. A: Subcortical astrogliosis. The subcortical astrogliosis ipsilateral to the first infarct and ipsilateral to the recurrent infarct was significantly increased compared to sham level (p<0.001). There was no difference between the hemispheres ipsilateral to the first infarct and ipsilateral to the recurrent infarct, indicating persisting subcortical astrogliosis. B: Subcortical microglial activation. The subcortical microglial activation was not increased compared to sham level. Results are presented as mean±SEM, n=7 to 8 per group. *** indicates p<0.001 vs. sham.

Supplemental figure IV: Neuronal apoptosis in the periinfarct area. A: Neuronal nuclei (NeuN) staining showing cortical neurons in the periinfarct area. B: Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) illustrating apoptotic cells. C: NeuN and TUNEL double staining reveals neuronal apoptosis in the periinfarct area.
References


