Photochemically induced thalamus infarction impairs cognition in a mouse model

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ABSTRACT
Background Small subcortical infarcts account for up to 25% of ischaemic strokes. Thalamus is one of the subcortical structures that commonly manifest with lacunar infarcts on MRI of the brain. Studies have shown that thalamus infarction is associated with cognitive decline. However, due to the lack of proper animal models, little is known about the mechanism. We aimed to establish a focal thalamus infarction model, characterise the infarct lesion and assess functional effects.

Methods Male C57BL/6J mice were anaesthetised, and Rose Bengal dye was injected through the tail vein. The right thalamus was illuminated with green laser light by stereotactic implantation of optic fibre. Characteristics of the infarct and lesion evolution were evaluated by histological analysis and 7T MRI at various times. The cognitive and neurological functions were assessed by behavioural tests. Retrograde tracing was performed to analyse neural connections.

Results An ischaemic lesion with small vessel occlusion was observed in the thalamus. It became a small circumscribed infarct with reactive astrocytes accumulated in the infarct periphery on day 21. The mice with thalamic infarction demonstrated impaired learning and memory without significant neurological deficits. Retrogradely labelled neurons in the retrosplenial granular cortex were reduced.

Conclusion This study established a mouse model of thalamic lacunar infarction that exhibits cognitive impairment. Neural connection dysfunctions may play a potential role in post-stroke cognitive impairment. This model helps to clarify the pathophysiology of post-stroke cognitive impairment and to develop potential therapies.

INTRODUCTION
Small subcortical infarct, commonly known as lacunar infarct, mostly results from ischaemia in the territory of the small deep perforating arteries and accounts for up to a quarter of ischaemic strokes.1 2 This disorder is closely related to vascular cognitive impairment, which has a devastating impact on life quality.3 Thalamus is one of the subcortical structures that commonly manifests with lacunar infarct on MRI of the brain. Despite the small size, the thalamus consists of multiple groups of nucleus and it has wide interactions with other brain structures, specifically, the anterior thalamic nuclei (ATN), which play a crucial role in learning and memory.4–6

While studies have shown that thalamus infarction is associated with vascular cognitive impairment,7 little is known about the underlying mechanism. One reason for this is that there is not yet a proper animal model that simulates thalamus infarction, hampering the elucidation of mechanisms as well as the development of more effective therapeutic interventions. Up till now, only two infarct models with subcortical deep structures have been well established with photothrombosis in rats, one infarction within the caudoputamen,8 and the other one in the internal capsule.9

Photothrombosis has been well established in animal models of ischaemic stroke. By intravenous injection of photosensitive dyes (Rose Bengal), photo-oxidation will be induced after irradiating the target area. The photo-oxidation causes damage to endothelium, activation of platelet and ultimately leads to occlusion of small vessels.10 11 This technique has the advantage of being able to precisely target the specific brain regions and control the lesion size, particularly subcortical deep brain areas that are more difficult to achieve through the traditional model of middle cerebral artery occlusion (MCAO). Moreover, by...
creating a very focal infarction with minimal surgical invasion, the animals have very low mortality.

In this study, we sought to establish and characterise a mouse model of small infarction in the thalamus by using photochemically induced thrombosis and to understand the mechanism of thalamic infarction induced cognitive impairment.

MATERIALS AND METHODS

Animal

All procedures were conducted in accordance with the Ethics Committee of Experimental Animal Welfare, Beijing Tiantan Hospital. Animal studies were reported following Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines. Experiments were conducted on male C57BL/6J mice (10±2 weeks old from Beijing SPF Biotechnology Co., Beijing, China). Mice were maintained in standard housing conditions on a 12-hour light/dark cycle and were allowed free access to food and water. Animals were randomly assigned to sham or photothrombotic stroke groups.

Photothrombotic thalamus infarction model

Mice were anaesthetised by intraperitoneal injection of 2,2,2-tribromoethanol (300 mg/kg). Each mouse was placed in a stereotaxic apparatus (RWD Life Science, China). Photothrombotic stroke was induced on the right thalamus. The stereotaxic coordinates were 0.8 mm anteroposterior, 0.7 mm mediolateral and −3.5 mm dorsoventral, calculated from the bregma. An implantable optic fibre cannula was made to deliver the light to the target area. The optical fibre has a core diameter of 105 µm, and it was placed into a 27-gauge straight stainless steel needle with epoxy resin filling the space. Attached with a ceramic ferrule with stainless steel sleeve, the optical fibre is connected to a laser source of 532 nm. The Rose Bengal dye (20 mg/kg body weight) was infused via the tail vein. After that, light exposure with an intensity of 3.5 mW was given for 90 s. Sham-operated mice underwent the same procedure, except for an infusion of 0.9% saline instead of Rose Bengal dye. Mice with severe cerebral haemorrhage during the surgery and postoperative infection were excluded from the study.

MRI and analysis

MRI was performed on a 7T animal MRI system (BioClinScan, Bruker, Germany). T2-weighted MRI was acquired with a turbo spin echo sequence (echo time=41 ms, repetition time=2990 ms, number of averages=1320×168 matrix; 18 contiguous, axial, 0.5 mm thick slices). To evaluate the lesion evolution over time, T2-weighted MRI scans were acquired for mice at day 1, day 3, day 7 and day 21 after stroke onset.

Histology

The brain was removed and fixed with 4% paraformaldehyde (PFA) for 6 hours. It was embedded in paraffin, cut coronally in 4-micrometre-thick sections. The H&E staining was carried out at 4 hours, and 1 day, 7 days and 21 days post-ischaemic stroke to evaluate the lesion change over time. Glial fibrillary acidic protein (Servicebio, China) and CD68 staining (Servicebio, China) were performed on day 21 to observe astrocytes and microglia/macrophages, respectively. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL, Servicebio, China) and 4 ',6-diamidino-2-phenylindole (DAPI, Servicebio, China) staining were conducted on day 1 to determine cell death.

Analysis of infarct volume

For infarct volume assessment, images from T2-weighted images and H&E stained sections were used. Quantitative analysis of infarct volume was conducted using ImageJ software (National Institutes of Health, USA). The mean infarct area was multiplied by the longitudinal diameter. The infarct area was calculated as follows: area damaged × (area of contralateral hemisphere/area of ipsilateral hemisphere).

Transmission electron microscopy

Electron microscopy was conducted on the thalamus at 4 hours and 7 days after surgery to observe the ultrastructural features (n=2/group). The tissue was dissected and fixed in a mixture of 2% PFA and 2.5% glutaraldehyde for 2 hours. After dehydrating and embedding in the Epon resin, the thin section was stained with uranyl acetate and lead citrate, observed with the transmission electron microscope (H7650, Hitachi, Tokyo, Japan).

Neurological function assessment

Neurological deficits score

The assessment of neurological deficits was performed at 1 day, 3 days, 7 days and 14 days after surgery based on a 9-neuro score test,12 which was developed to measure the motor, sensory and reflex responses after stroke. Briefly, it includes: (1) spontaneous activity, (2) left drifting during displacement, (3) parachute reflex and (4) resistance to left forepaw stretching. The higher score indicates more severe handicap.

Rotarod test

The rotarod test was conducted to assess motor balance and coordination after ischaemia. The test was carried out before and on day 3, day 7 and day 14 post ischaemia. The mouse was placed on a rotating rod, and the time that the mouse remained on the cylinder was recorded. The speed was accelerated from 4 RPM to 40 RPM over 5 min. The test was repeated three consecutive times.

Open field test

Open field test was used to evaluate locomotor activity and anxiety13 at 3 weeks post surgery. Each mouse was placed in an arena (40×40×40 cm) for 10 min and the behaviour was recorded by a camera. The floor was virtually divided into three areas, which included central, neutral and peripheral zone. Total moving distance, velocity, entry frequency in the centre area, and duration in the centre...
The novel object recognition (NOR) test was conducted to assess recognition at 3 weeks post surgery, which included three phases with 24 hours interval: acclimation, familiarisation and testing phase. The acclimation phase was equal to the open field test. In the familiarisation phase, two identical objects (object A and B) were placed in the arena. Mice were allowed to freely explore for 10 min. Sniffing or touching less than 2 cm around the object was defined as an active exploration. During the recognition phase, the mice were exposed to a novel object (with a different colour, shape and size) paired with the familiar object for 5 min. The objects and arena were thoroughly cleaned using 75% ethanol between each phase to avoid olfactory cues. Object preference was calculated as time to explore novel object × 100/total exploration time for both objects. Mice were recorded by EthVision XT software during the whole test period.

Morris water maze
Spatial learning and memory function were assessed using the Morris water maze test 2 weeks after the surgery, which included a 4-day memory acquisition trial and a 1-day spatial probe test. The test was performed in a round white pool (120 cm in diameter and 40 cm in depth). A platform was placed in the target quadrant and hidden 1 cm below the surface of the water. During the 4 consecutive days of training, each mouse was allowed to swim for 60 s. The time spent on finding out and reaching the platform (escape latency), swimming speed and total distance moved was recorded. If the mouse failed to find the platform within 60 s, the observer would help the animal reach the platform and remain on it for 10 s. On
day 5, mice were tested on a spatial probe trial with the platform removed. Each mouse was released in a quadrant opposite the target quadrant and searched freely for 60s. The time spent in the target quadrant and the number of mice that crossed the platform position were recorded. EthoVision XT V14 tracking software (Noldus Information Technology BV) was used for data collection.

**Retrograde tracing**

To reveal possible mechanisms of thalamus infarction on cognition, we retrogradely label areas that send output fibres to thalamus and quantify those dense projections. The retrograde tracer Retrobeads (Lumafluor) was microinfused immediately after photothrombotic surgery in the thalamus (0.1 µL volume; the stereotaxic coordinates were the same as the photothrombosis). The retrobeads were microinjected using glass pipettes that connected to a Hamilton microsyringe. The glass pipette was left in situ for 5 min before retraction. The mouse brains were harvested 3 weeks after microinfusion to visualise projection neurons. Brains were postfixed with 4% PFA overnight at 4°C, then placed in 30% sucrose for 48 hours, and embedded in OCT Compound (Sakura). Brain tissues were frozen and cut coronally at 50-micrometre thickness using a cryostat (Leica CM1950). Sections were mounted on adhesion microscope slides, subsequently stained and coverslipped with DAPI-antifade fluorescent mounting medium (Cell Signalling). Images were acquired using a fluorescence microscope. Number of retrogradely labelled neurons was counted manually using ImageJ software (National Institutes of Health, USA).

![Figure 2](image1)

**Figure 2** Representative T2-weighted images of infarct lesion at day 1, day 3, day 7 and day 21 post ischaemia. The lesion volume progressively decreased over time. n=3/timepoint.

![Figure 3](image2)

**Figure 3** Histology characteristics at different time points after ischaemia. (A) The normal tissue of the contralateral side. (B) Injured neurons with shrunken and pyknotic nuclei at 4 hours. (C) Prominent microvacuoles form within the neuropil at 24 hours. (D) Infiltration of inflammatory cells surrounding the occlusive vessel (arrow) and the ischaemic area at day 7. Enlarged perivascular space is also noted (arrow). Scale bar, 20 µm. (E) CD68 staining reveals macrophages/microglia notably accumulated at the infarct and peri-infarct zones at day 21. Scale bar, 50 µm. (F)–(H) TUNEL staining showed that extensive TUNEL+ cells were observed in the thalamic stroke group at day 1 after photothrombosis, indicating cell apoptosis. Scale bar, 30 µm. n=3/timepoint. TUNEL, dUTP nick end labelling.
cells were counted across each region of interest and a minimum of two sections per region were counted for each mice.

**Statistical analysis**

Data were expressed as mean±SE, analysed with unpaired Student’s t-test and two-way analysis of variance (ANOVA). GraphPad Prism V.9 software (GraphPad) was used for statistical analysis. A p value of <0.05 was considered statistically significant.

**RESULTS**

**Lesion morphology and evolution**

All mice survived the surgery and none suffered a postoperative infection. Two mice that had severe haemorrhage during the surgery were excluded from the study. In the acute phase, a round-to-ovoid ischaemic lesion was observed in the thalamus 24 hours after photothrombosis; it turned into a small circumscribed infarct on day 21; the central zone was surrounded by reactive astrocytosis, which formed a belt-like glial scar (figure 1). The infarct lesion was noted on 7T-MRI 24 hours after photothrombosis but was not detected in the sham group. Infarct volume analysis showed that the lesion size was progressively reduced within 3 weeks after ischaemic stroke induction (figure 2).

**Ultrastructural features**

Four hours after surgery, the tissue of the contralateral side appeared normal (figure 3A), but that of the ipsilateral side was abnormal with injured neurons and small occlusive vessels in the thalamus (figure 3B–D). H&E staining demonstrated nuclear shrinkage and pyknosis, neurons exhibited numerous vacuolation (figure 3B) and the ischaemic destruction became more evident at 24 hours (figure 3C). Infiltration of inflammatory cells was evident in the infarct region at 7 days (figure 3D). Remarkable macrophages/microglia accumulated in the infarct and peri-infarct regions were detected at 21 days (figure 3E). TUNEL staining showed that extensive TUNEL + cells were observed in the thalamic stroke group at 1 day after photothrombosis, indicating cell apoptosis (figure 3F–H).

Transmission electron microscopy revealed no obvious vessel stenosis in the sham mice 4 hours after surgery (figure 4A). However, severe stenosis of vessels in the thalamic stroke mice was identified (figure 4B). Prominent swelling of astrocytic end-feet and vessel endothelial cell was detected, suggesting the destruction of the microvessel-neural unit (figure 4C). The dark necrotic neuron with chromatin condensation occurred 4 hours post ischaemia (figure 4D). The mitochondria presented with abnormal morphology, cristae broke, suggesting mitochondrial dissolution (figure 4E). By day 7, a cystic cavity began to form and it was surrounded by multiple phagocytes (figure 4F).

**Neurological deficits**

No significant neurological deficit was observed after photothrombosis. The rotarod test showed that the time that the photothrombotic thalamic stroke group stayed on the rod was mildly shorter than the sham group,
especially on day 7, but the two groups reached nearly the same at 2 weeks (figure 5A).

Anxiety-like behaviour and recognition memory
In the open field test, no difference in locomotor was found between groups (online supplemental figure S1A). In the familiarisation phase of the NOR test, there was no difference in preference for the familiar object. However, the thalamic stroke mice spent less time exploring the novel object compared with the sham group (online supplemental figure S1B).

Spatial learning and memory function
There was no difference in the swimming speed between both groups, indicating no impairment in motor ability in the probe test on day 5 (figure 5B). In the memory acquisition trial, the escape latency showed a downward trend in both groups; however, the thalamic stroke mice showed a longer time to find the platform compared with the sham group, and the trends reached statistically significant on day 4 (figure 5C). For the spatial probe trial, the time stayed in the target quadrant and the number crossed the platform area in the thalamic stroke group was significantly reduced (figure 5D,E). As depicted in figure 5F, the sham group mice spent more time in the platform quadrant, while the thalamic infarct mice swam randomly.

Projections to thalamus
We identified retrogradely labelled neurons in anterior cingulate cortex, retrosplenial cortex and mammillary body (figure 6), which is consistent with previous data. Specifically, quantitative analysis showed that the number of retrogradely labelled neurons in the retrosplenial granular cortex were altered and decreased in thalamic infarct mice compared with sham mice, indicating that this region had damaged projections to the thalamus (figure 6 and online supplemental figure S2).

DISCUSSION
In the present study, we established an experimental mouse model of small infarction in the thalamus manifested with cognitive impairment. We further clarified the possible mechanism of post-stroke cognitive impairment and found that the direct innervations from retrosplenial granular cortex to thalamus were decreased.
Currently, commonly used animal models of ischaemic stroke are limited to cortical large-area infarction. Well-established models of subcortical small infarction are rare. This thalamic infarction model has minimal invasiveness, low mortality and good reproducibility. Interestingly, it does not have motor deficits that may confound cognitive evaluation. In contrast, MCAO is a widely used technique for the induction of large cortical ischaemic stroke in rodents. However, this method may cause hippocampal lesions, and animals that underwent MCAO had severe sensorimotor deficits, which may lead to the inaccurate evaluation of cognitive functions.

Up to now, only a few studies have well-generated models of photothrombotic infarction in subcortical regions, including caudoputamen and internal capsule in rats.\textsuperscript{8} 9 Aside from considerable proficiency, several key points need to be emphasised. To successfully produce a small infarction in the target location, it is essential to effectively deliver the laser light to the target area with a minimal lesion to the unrelated brain issue. To accomplish this, we placed a thin optical fibre into a matched straight stainless steel needle, thus efficiently preventing not only the bending of the optic fibre but also the scattering of light along the needle track. Furthermore, the degree of damage caused by the needle tract was much smaller than the photothrombosis, and there were no significant neurological deficits in the sham group that received the optic fibre insertion and light exposure. Other factors such as the intensity of laser light, time of light irradiation and fine-tuned targeting are also essential to establish the model.

The method used in the study can be modified and extended to other subcortical infarct models. By adjusting the laser intensity and duration of illumination, it is feasible to produce various sizes of infarct lesions, depending on the needs of the experiment.\textsuperscript{8} 9 Combined with a stereotaxic instrument, the implantable optic fibre cannulae can precisely target any specific subcortical region such as the basal ganglia and brain white matter.\textsuperscript{9} This highlights the flexibility and convenience of the method, which opens a door to understanding the pathophysiology of subcortical ischaemic stroke and facilitates the development of new therapeutic and rehabilitative approaches.

Several lines of evidence suggest that the thalamus is involved in cognitive function apart from sensory processing.\textsuperscript{18–20} Thalamus has traditionally been considered as relaying sensory information passively but is now becoming widely recognised as regulating the information actively, which can impact the cortical activity strongly.\textsuperscript{18} Cognitive impairment has been observed in patients following thalamic lesions.\textsuperscript{27, 28} Similarly, our prior study has shown that patients with thalamic ischaemic stroke increased risk of worse cognitive performance.\textsuperscript{29} The thalamus can be classically divided into five groups of nuclei based on the anatomical location: anterior, posterior, medial, lateral as well as ventral nuclei.\textsuperscript{4} The anterior thalamus is positioned vitally and it has wide interactions with the hippocampus, cortex and subcortex. Being a central component of the Papez’ circuit,\textsuperscript{30} the ATN is generally assumed to play critical roles in learning and memory.\textsuperscript{4, 6, 30–33} Based on the evidence, we selected the ATN as the target location for implantation. Interestingly, our study found that the mice following a thalamic stroke had a bad performance on Morris water maze test, which is in line with the previous study which showed that a lesion in the ATN caused spatial learning and memory deficits.\textsuperscript{32}

\textbf{Figure 6  Retrograde tracing.} The left panel shows the schematic of the RSG, ACC and MB. The right panel demonstrates retrobeads-labelled cells in the RSG, ACC and MB (arrows). Scale bar, 200µm at low magnification, 20µm at high magnification. ACC, anterior cingulate cortex; MB, mamillary body; RSG, retrosplenial granular cortex.
Furthermore, we found that retrogradely labelled neurons in retrosplenial granular cortex were decreased in thalamic infarct mice, suggesting the afferent dense inputs from that region to the thalamus were injured, which may have a potential influence on post-stroke cognitive decline. Indeed, ATN and retrosplenial cortex have reciprocal connections. Evidence has shown that retrosplenial granular cortex is involved in spatial working memory, lesion of this structure can impair spatial learning and navigation. Pathology in ATN is associated with anterograde amnesia, and the possible factor is the disruption of functional connectivity between ATN and retrosplenial cortex. Taken together, the cognitive change induced by thalamus ischaemic stroke could be taken into account to ATN neurocircuitry dissociation and dysfunction. Future studies on functional networks, neurotransmitter expression levels and neuroplasticity with this model are warranted to further clarify the mechanism and develop therapeutic strategies.

There are several limitations in this study. As cognitive functions are very complicated, we only performed Morris water maze and NOR tests, which may not fully characterize the infiltration of immune cells. Future studies with comprehensive cognitive function evaluation, especially in the chronic stage, combined with the flow cytometry is recommended to better understand the pathophysiological mechanism and the potential impact.

In conclusion, the current study established a model of lacunar infarction in the thalamus that manifested cognitive impairment. This model will shed light on the mechanism and provide new insights into treatment approaches for lacunar infarction and associated vascular cognitive impairment.

Contributors JS and CZ formulated the study concept and design. CZ collected the data and wrote the manuscript. SJ revised the manuscript. YW, JS and SL supervised and organised the project. Responsible for the overall content as the guarantor: JS

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Figure S1. Behavioral assessment. A, Open field test. B, Novel object recognition. Sham, n = 9; Thalamic stroke n = 10; Data are shown as the mean ± SEM. ns indicates not significant; *p < 0.05.
**Figure S2.** Retrograde tracing. **A,** Injection site of ipsilateral thalamus. **B,** Quantitative analysis of retrobeads-labeled cells in the retrosplenial granular cortex (RSG), anterior cingulate cortex (ACC), and mamillary body (MB). The number of retrobeads+ cells in the RSG within the group of thalamic stroke were reduced compared with sham (n = 3 per group). Data are shown as the mean ± SEM. ns indicates not significant; *p < 0.05. Scale bar, 200 μm. AD, anterodorsal thalamic nucleus; AM, anteromedial thalamic nucleus; AV, anteroventral thalamic nucleus.