INTERNAL CAPSULE STROKE IN THE COMMON MARMOSET

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Abstract—White matter (WM) impairment and motor deficit after stroke are directly related. However, WM injury mechanisms and their relation to motor disturbances are still poorly understood. In humans, the anterior choroidal artery (AChA) irrigates the internal capsule (IC), and stroke to this region can induce isolated motor impairment. The goal of this study was to analyze whether AChA occlusion can injure the IC in the marmoset monkey. The vascular distribution of the marmoset brain was examined by colored latex perfusion and revealed high resemblance to the human brain anatomy. Next, a new approach to electrocoagulate the AChA was developed and chronic experiments showed infarction compromising the IC on magnetic resonance imaging (MRI) scanning (day 4) and histology (day 11). Behavioral analysis was performed using a neurologic score previously developed and our own scoring method. Marmosets showed a decreased score that was still evident at day 10 after AChA electrocoagulation. We developed a new approach to induce damage to the marmoset IC that may be useful for the detailed study of WM impairment and behavioral changes after stroke in the nonhuman primate. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: anterior choroidal artery, internal capsule, motor impairment, nonhuman primate, white matter stroke.

INTRODUCTION

Stroke is a devastating disease, being the major cause of acquired disabilities around the world (Donnan et al., 2008). To understand the injury mechanisms and develop new strategies aimed to improve the motor conditions of stroke survivors, several animal models have been developed (Canazza et al., 2014). Owing to the heterogeneous nature of stroke and additional features such as age, sex, race and comorbidities that vary among patients, there is no ideal animal model of human stroke (Mergenthaler and Meisel, 2012); however, the developed models have tried to mimic as much as possible the human condition. Because the middle cerebral artery (MCA) is the most commonly affected artery among stroke patients (Rordorf et al., 1998), one of the most common models for stroke research is MCA occlusion (MCAO) in rodents (Tamura et al., 1981; Kohno et al., 1995). Although these models have helped to unveil the effects of cortical ischemia (Astrup et al., 1981; Neumann-Haefelin et al., 2000; Dijkhuizen et al., 2001), they mislead the researchers' attention to gray matter (GM) injury. Owing to the fact that the GM/white matter (WM) ratio found in the rat neocortex (GM:WM = 87:13) is significantly higher than in humans (GM:WM = 61:39, Zhang and Sejnowski, 2000), the rodent MCAO stroke model induces large infarcts affecting mainly the GM. This feature has inspired the development of neuroprotective agents focused on GM protection aiming for neuron rescue; although such therapies succeed in the rodent recovery after stroke, they fail in clinical trials (Xu and Pan, 2013). This discrepancy between rodent models and human trials has drawn the attention to the essential brain structural differences between both species: the WM ratio.

Recent imaging studies done in stroke survivors have highlighted the importance of WM damage, demonstrating that corticospinal tract (CST) integrity can be considered as a reliable predictor of stroke severity and clinical outcome (Thomalla et al., 2004; Puig et al., 2011; Rosso et al., 2013). Additionally, there is evidence that motor dysfunction after MCA stroke is more dependent on WM than GM damage (Rosso et al., 2011). Because both GM and WM differ importantly in the initial

Abbreviations: ACA, anterior cerebral artery; AChA, anterior choroidal artery; AChAO, anterior choroidal artery occlusion; AMG, autometallographic; BA, basilar artery; CST, corticospinal tract; DW, distilled water; FA, flip angle; FOV, field of view; FS, Fretet neurologic score; GM, gray matter; HSD, honestly significant difference; IC, internal capsule; ICA, internal carotid artery; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MNS, marmoset neurologic score; MRI, magnetic resonance imaging; NHP, nonhuman primate; OR, orbital rim; OT, optic tract; PB, phosphate buffer; PCA, posterior cerebral artery; PcomA, posterior communicating artery; SCA, superior cerebellar artery; TE, echo time; TM, temporal muscle; TR, repetition time; WM, white matter.

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responses to ischemia (Hughes et al., 2003), some researchers suggest that WM ischemia may have a longer therapeutic window (Muñoz Maniega et al., 2004; Koga et al., 2005). Therefore, it is imperative to deepen the research on WM ischemia owing to its potential for the development of new therapies for stroke patients.

To investigate the effect of subcortical WM injury, different animal models have been developed; a rodent model (Frost et al., 2006; Lecriux et al., 2008) that induces direct damage on the internal capsule (IC), and a mini-pig model (Tanaka et al., 2008) that attempts to impair the IC by occlusion of the anterior choroidal artery (AChA). Although both approaches induced motor impairment, the damage was subtle and transient in contrast to human strokes that compromise the AChA territory (Derflinger et al., 2013). Stroke of the AChA in the human brain can lead to infarction of the posterior limb of the IC, which induces isolated motor deficits due to disruption of the CST (Rascol et al., 1982; Nelles et al., 2008; Likitjaroen et al., 2012). The development of relevant animal models to study this condition may help to identify critical factors related to WM changes after ischemia and for the development of new approaches focused on the rescue of WM (Sozmen et al., 2012).

Because nonhuman primates (NHPs) are phylogenetically closer to the human, where the WM volume is larger than rodents’ (GM/WM ratio: 79/21. Zhang and Sejnowski, 2000; Bailey et al., 2009; Okano et al., 2012), the development of an alternative model of WM ischemia in such species may provide relevant information about WM responses after stroke and improve further translational research. The common marmoset (Callithrix jacchus) is a NHP similar to Homo sapiens, with a brain five times larger than the rat’s, representing approximately 2.7% of its body weight, which is equivalent to human proportions (Abbott et al., 2003; Okano et al., 2012). Moreover, the neocortical GM/WM ratio is smaller in comparison with rodents (Zhang and Sejnowski, 2000), and marmoset ergonomics are closer to the human’s. We consider that the similarities in WM proportions and ergonomics between marmosets and humans may offer a promising scenario for the study of WM changes after ischemia.

The aim of this study was to establish whether a vessel homologous to the human AChA exists in the marmoset brain and to evaluate the effect of its occlusion on the IC. To our knowledge, there is no established method to induce an infarct in the marmoset IC as a model of WM stroke.

**EXPERIMENTAL PROCEDURES**

**Animals**

Twenty-two laboratory-bred adult common marmosets (C. jacchus) ~4.5 years old at the start of the experiments were used. Two already euthanized marmosets (fixed and long-term freeze-preserved: cadaveric preparations) were used for carotid artery cannulation and colored latex intravascular perfusion (Alvernia et al., 2010). Twelve marmosets were used to evaluate brain vascular anatomy (non-operated side) and test the reproducibility of the AChA occlusion (AChAO; operated-side) by the injection of colored latex perfusion after surgery (acute experiments). The remaining eight marmosets were divided into two groups to perform AChAO (n = 5) and sham operation (n = 3). These animals were observed for 11 days before euthanasia (chronic experiments). All monkeys were kept within a large colony to allow good visual and auditory interaction with other marmosets. All procedures were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Research Committee at the National Institute of Neurosciences in Tokyo, Japan.

**AChA identification**

To identify the vascular anatomy of the marmoset, liquid latex was used as previously described (Alvernia et al., 2010), with some modifications as follows: For cadaveric preparations, the animals were unfrozen at room temperature, and bilateral dissection of the common carotid arteries was performed. Cannulation was achieved using an 18-gauge catheter (18G × 2” catheter; Nipro, Osaka, Japan), and both catheters were perfused with tap water followed by liquid red latex solution (Ward’s Natural Science 37-2571, Columbus Chemical Industries, Columbus WI, USA) using a 10-cc syringe until leakage from the contralateral carotid artery and vertebral arteries was evident. After 20 min, the brain was dissected carefully and vascular exploration was performed. To evaluate the consistency of the vascular patterns, pictures were taken, hand drawings from the right side of the intracranial vessels emerging from the internal carotid artery (ICA) were performed, and the distance between the AChA and ICA bifurcation was measured. The same evaluation was performed for the non-operated side of animals used to test the surgical procedure accuracy (see below). In total, 14 animals were used for the evaluation of the vascular pattern.

**Surgical procedures**

**Surgical preparation.** Marmosets were anesthetized with Isoflurane (1–2% (v/v); Mylan Pharmaceutical Co., Ltd. Morgantown WV, USA) delivered initially via an animal face mask, then through endotracheal intubation (6 Fr catheter, length 6.5 cm). Two g/kg of D-Mannitol (20% (w/v); Yoshindo Inc., Toyama, Japan) were slowly injected from the catheterization of the femoral vein (26G × 3/4” catheter; Nipro, Osaka, Japan) followed by continuous infusion (0.7 ml/h) containing Remifentanil (0.18 µg/h; Ultiva 5 mg; Janssen Pharmaceutical, Tokyo, Japan) and Rocuronium Bromide (24 µg/h; Eslax, 25 mg/2.5 ml; MSD Co., Ltd., Tokyo, Japan) before starting artificial ventilation (A.D.S. 2000; Engler, Hialeah FL, USA) (flow rate: 1.65 ± 0.4 l/min; peak inspiratory pressure: 15 cm of H2O; respiratory rate: 9.5 ± 1 breaths per minute). During surgery, heart rate (189.7 ± 16.4 beats per minute) and arterial oxygen saturation (SaO2: 96.4 ± 2.5%) were monitored with a pulse oximeter (8600V NONIN medical Inc., Plymouth MN, USA). Electrocardiographic traces
were also recorded during all procedures (Grass Astro-Med Inc., Warwick RI, USA). Temperature was recorded and maintained around 36.7 ± 0.25 °C with a heater pad thermocouple (BVT-100A BRC, Tokyo, Japan) or an air-circulation heating system (3M Bair Hugger warming unit 750, Arizant Healthcare, St. Paul, MN, USA). The variables did not change significantly during the procedures. For infection prophylaxis, antibiotics were applied intramuscularly before starting the surgery (cefovecin sodium, Convenia 8 mg/kg, Pfizer Japan Inc., Tokyo, Japan).

**AChAO.** The marmoset head was fixed in a stereotaxic frame (Narishige SR-6C; Japan), and under aseptic conditions the scalp was cut between the ears and retracted anteriorly and posteriorly. The left temporal muscle was then detached from the bone and retracted. A large cranial flap from the orbital rim to the occipital bone was opened and the dura was incised. The stereotaxic frame was tilted ~30° laterally and the anterior lobe was lifted up gradually by placing small cotton balls into the space underneath. Dissection was performed in the convergence of the anterior and temporal lobe, finding the MCA origin and following the ICA until the AChA was found over the optic tract. The vessel was electrocoagulated and transected completely (20 W) using bipolar forceps (Surgitron F.F.P.F. EMC; Ellman, NY, USA). Coagulation was performed by episodes of approximately 1 s in length until the vessel was completely sectioned. Between coagulations, saline solution irrigation was used to cool down the tissues surrounding the vessel. For chronic experiments, artificial dura (Gore-Tex DM-03020, Tokyo, Japan) was used to cover the brain surface and sutured without inducing pressure; then, the temporal muscle was placed over the artificial dura and the skin was sutured (n = 5). For acute experiments, 12 marmosets were euthanized immediately after surgery and received transcardial perfusion with heparinized saline solution followed by 4% (w/v) cold paraformaldehyde and latex perfusion (as described above) to study the vascular structures (non-operated side) and to confirm the accuracy of AChA identification and occlusion (operated side). To avoid brain deformation during perfusion, the bone flap was returned over the brain and fixed using a suture between the temporal muscles before euthanasia.

**Sham operations.** In three animals, the protocol developed for AChAO was used, but without electrocoagulating the AChA.

**Postoperative management.** After surgery, a bolus injection of sugammadex (Bridion IV, 0.16 g/kg, MSD Co., Ltd., Tokyo, Japan) was administered to reverse the muscle relaxant effect. After marmosets started to breathe spontaneously, artificial ventilation and isoflurane were ceased, extubation was performed, and animals were allowed to recover in an incubator (29 °C, O2: 20% (v/v)). All animals were nursed and hand-fed after the procedure until they were able to care for themselves.

**Behavioral assessment**

**Hand preference.** Before starting the chronic experiments, marmosets were evaluated to determine their hand preference as follows: a transport cage was attached to the marmoset home cage with a modified cover consisting of a transparent acrylic panel with a rectangular window. Perpendicular to the acrylic, six PVC tubes were placed horizontally and attached to each other (inner diameter 2 cm, length 5 cm) with one opening over the window. To test the marmosets, a black panel was used to cover the marmoset side; meanwhile a sweet treat was loaded in one of the tubes. When the black panel was removed, the preferred hand to retrieve the treat was recorded. The procedure was repeated 30 times (five times per tube) on three different days using a random pattern. All marmosets used in the chronic phase of the experiments preferred the right hand. Although the same evaluation was attempted after surgery, some animals were reluctant to perform the task; for this reason, after surgery hand preference was evaluated only during volitional attempts that each marmoset made during feeding.

**Freret neurologic score (FS).** Before surgery and 1, 4, 7 and 10 days after surgery, the neurologic status of each animal was assessed using a neurologic score previously described by Freret et al. (2008) (FS). This test consisted in the evaluation of the absence (score = 2), scarce occurrence (score = 1) or presence (score = 0) of the following abnormal movements and postures: forelimbs/hindlimbs slipping or dangling under the perch at rest or movement, hand crossing the chest, head tilting and reaction to a visual stimulus. The highest scores were 24 points for “total score” and 10 points for “hemilateral score” (left or right evaluation of forelimb/hindlimb slipping or dangling under the perch at rest or movement and ipsilateral hand crossing the chest).

**Marmoset neurologic score (MNS).** Because the FS provides a gross impression of marmoset status, we considered that an additional, more detailed evaluation of natural behavior was necessary to provide a comparison point of neurologic condition in each animal. For this reason, we designed a new test (MNS) aimed to determine the presence (score = 0) or absence (score = 1) of several aspects before and at days 1, 4, 7 and 10 after surgery (Table 1). The marmoset was evaluated in the home cage before breakfast; after removing all the cage contents, an acrylic door was placed instead of the regular cage door and a camera was located in front of the cage. The initial 10 min involved video recording the spontaneous natural behavior of the marmoset. Following this, a perch and a loft were introduced into the cage, and little treats were distributed over the cage to encourage the marmoset to move around. An additional 5 min were recorded with these conditions. Finally, the marmoset was retrieved from the cage and allowed to stand in the experimenter’s arm. General evaluation, “stick test” and “limb stimuli
Table 1. Marmoset neurologic score (MNS)

<table>
<thead>
<tr>
<th>General evaluation</th>
<th>During holding in the examiner’s arm</th>
<th>Hemilateral evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stays in back of the cage</td>
<td>Inadequate grasping to the examiner’s arm&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Body tilting</td>
</tr>
<tr>
<td>Stays still for 1 min</td>
<td>Poor body balance&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Head tilting</td>
</tr>
<tr>
<td>Cannot stand in the perch</td>
<td>Inaccurate food targeting</td>
<td>Hand waving</td>
</tr>
<tr>
<td>Dysemetria&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>Repeated touching before grasp cage bars</td>
</tr>
<tr>
<td>Required assisted feeding&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td>Hand crossing the chest</td>
</tr>
<tr>
<td>Circling behavior</td>
<td></td>
<td>Hand slipping from the cage bars</td>
</tr>
<tr>
<td>Left palpebral ptosis</td>
<td></td>
<td>Hand dangling from the cage bars</td>
</tr>
<tr>
<td>No jumping from cage walls&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
<td>No grasping a stick when presented</td>
</tr>
<tr>
<td>No rearing without hand support</td>
<td></td>
<td>Cannot hold a stick more than 3 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Absent retrieve reflex to hand stimuli&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Total score: 40 points (general, 9 points; holding, 3 points; hemilateral, 14 points per side).

1 Dysemetria was evaluated during feeding. Marmosets trying to eat alone that could not coordinate hand–mouth movement, or attempted to aim a piece of food to the mouth but missed, were considered symmetric.

2 When marmosets could not finish a quarter of their food in 1 h, manual feeding was performed.

3 Before surgery, marmosets usually jumped from one wall of the cage to another; when this behavior was absent during observation after surgery, a point was taken off.

4 Lack of force to hold evidenced by easy slipping from the examiners arm, or resistance was minimal when the animal was grabbed by the tail and gently pulled away, in comparison to presurgical status.

5 During holding, when the marmoset was located over the examiner’s arm and the arm was moved away from the examiner’s chest, if the marmoset moved from right to left while approaching the examiner’s chest, a point was taken off.

6 “Retrieve reflex” refers to the fast retrieval of the marmoset extremity when the examiner holds the animal and without intruding in the marmosets’ visual field touch an extremity using a brush.

tests” (see Table 1) were performed by the experimenter. A score was calculated from the features observed in the video and a record made by the experimenter during the marmoset holding. The highest score was 40 for “total” (all points) and 14 for “hemilateral” (points for only one side of the body) evaluation.

Magnetic resonance imaging (MRI)

Prior to and 4 days after surgery, MRI images of the brain were obtained from eight marmosets. Each animal was anesthetized using pentobarbital sodium (Somnopentyl, 25 mg/kg IM., Kokuritsu Seiyaku Corp., Tokyo, Japan), and SaO2 was monitored. Temperature was maintained with a warm gel pad throughout the scanning procedure. Heads were fixed in an MRI-compatible stereotaxic frame and scanned using a 4-channel array coil on a 3 Tesla MRI (Siemens Trio, Erlangen, Germany). A three-plane localizer image was obtained to ensure correct positioning of the target images (repetition time (TR) = 100 ms, echo time (TE) = 5 ms, flip angle (FA) = 40°, field of view (FOV) = 120 mm, slice thickness = 3 mm). A three-dimensional T1-weighted image was then taken using a magnetization prepared rapid gradient echo sequence (TR = 2300 ms, TE = 2.8 ms, inversion time (TI) = 1000 ms, FA = 12°, FOV = 67 mm, image matrix = 192, in-plane voxel size = ~0.3 mm). T2-weighted images (TR = 4000 ms, TE = 520 ms, FOV = 43 mm, image matrix 128, in-plane voxel size = ~0.3 mm) were also taken.

Histology

Eleven days after surgery, marmosets were deeply anesthetized with pentobarbital sodium (Somnopentyl, 35 mg/kg IV, Kokuritsu Seiyaku Corp., Tokyo, Japan) and transcardially perfused with 300 cc of heparinized saline solution followed by 300 cc of cold 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were dissected, post-fixed overnight and then immersed in 30% (w/v) sucrose in PB. Forty-micrometer-thick slices were cut across the infarcted zones confirmed by the MRI images using a freezing stage sledge microtome (REM-710, Yamato Kohki Industrial, Saitama, Japan). To assess the infarcted area, Nissl staining was performed on one in every four sections. Sections were incubated with 0.1 M PB containing 0.5% (v/v) Triton-X100 for 30 min at room temperature, washed with 0.05 M PB, mounted on precoated glass slides and dried at room temperature overnight. Samples were dehydrated by ethanol immersion, and de-fatting was performed overnight (chloroform:methyl-alcohol = 1:1). Samples were rehydrated, washed with distilled water (DW) and transferred to thionin 0.15% (w/v) solution for 30–60 s. Samples were washed again, and thionin excess was removed using ethanol. Finally, samples were cleared using xylene, and cover-slipped using Entellan-neu (Merck).

To assess WM, myelin staining (Larsen et al., 2003) was performed with some modifications. One in every four sections were incubated with 0.01 M PB containing 0.005% (v/v) Triton X-100 for 30 min at room temperature, washed with 0.05 M PB, mounted on precoated glass slides and dried at room temperature for 24 h. Samples were fixed with 4% (w/v) paraformaldehyde for 5 min, and then washed and blocked in 10% (w/v) citrate buffer twice for 2 min each. Sections were transferred to autometallographic (AMG) developer solution composed of gum Arabic (0.5 kg/ml) 270 ml, citrate buffer 45 ml, hydroquinone (0.006 g/ml) 67.5 ml and silver nitrate (0.007 g/ml) 67.5 ml (Larsen et al., 2003) in a dark chamber for 115 min, followed by the developer fixative
(fresh AMG developer solution mixed with sodium thiosulfate anhydrous 5% (w/v)) for 10 min. All reagents used were purchased from Nacalai Tesque Inc., Kyoto, Japan. After washing with DW, samples were dehydrated in ethanol, cleared with xylene and cover-slipped with DPX (Merck Millipore, Darmstadt, Germany). Pictures were taken using bright field microscopy (Keyence BZ 8000).

Image analysis

Lesion volume calculation. Injured areas were evaluated from stained slices using UTHSCSA Image Tool for windows software version 3.0 (University of Texas Health Science Center, San Antonio, TX, USA). Nissl-stained samples were used to measure the total infarcted area of each slice (infarct volume) and Myelin slices to measure the infarct areas compromising only the IC (IC infarct volume). The infarct volume and IC infarct volume were derived from the sum of the areas and slice thickness. Additionally, the left IC ratio was calculated using the myelin-stained slices (stereotactic reference: interaural +5.6 mm to +11.3 mm. Hardman and Ashwell, 2012) by measuring the left and right IC area, and calculating the volumes from sum of the left and right IC areas and slice thickness; data were expressed as a percentage by comparison of the left (infarcted) IC with the contralateral (nonimpaired) IC as described before (Puentes et al., 2012).

Infarct topographical analysis. A frequency map was constructed by hand drawing each infarct area (from Nissl staining) over myelin-stained templates (one sample set from a sham-operated animal). The original color was modified to improve the contrast with the infarcted areas (Adobe Illustrator CS 5.1., Adobe Systems Inc. CA, USA). Each marmoset infarct map was given a different color. White areas indicate overlapping of three different colors. Thinned color areas indicate the overlapping of two different colors.

Statistical analysis

Statistical analyses were performed using R software (version 3.1.0). Error bars are expressed as the standard deviation of the data. Dixon test type 10 was used to determine the outliers; later, the relation between the parameters was calculated by using a linear mixed model (Latin square test). Subsequently, an analysis of variance (ANOVA) was applied followed by the pairwise t-test with Bonferroni correction for group analysis. Finally, a Tukey honestly significant difference (HSD) simultaneous test was conducted to evaluate significant differences among groups in a time-dependent manner.

RESULTS

Marmoset vascular distribution resembles the human anatomy

In all marmosets, the vascular pattern resembled the human anatomy finding a complete Willis circle (Fig. 1A). In 10 animals, the AChA sprouted from the ICA between the posterior communicating artery (PcomA) and the ICA bifurcation (Fig. 1B), running over the optic tract (AChA-ICA bifurcation: 1.4 mm ± 0.2 mm). Four animals exhibited some anatomical variations (Fig. 1C) where duplicated or triplicated AChA origins converged in a single main artery or an aberrant AChA was found. In two cases (Fig. 1C, left column), one of the additional origins emerged from the PcomA instead of the ICA. The distance from the main branch of the AChA to the bifurcation of the ICA was 1.6 mm (±0.18). In humans, it is well known that the AChA is a main feeder artery of the IC (Hupperts et al., 1994; Ois et al., 2009). Therefore, if the anatomical distribution of this artery is close between humans and marmosets, its occlusion might lead to infarction of the IC.

Development of the surgical protocol

A large craniotomy was required to expose the deep structures of the brain (Fig. 2), and the left AChA was found behind the temporal lobe being the last branch before the ICA bifurcation (Figs. 2B, 3A, B). When two arteries were found emerging at the expected place of...
the AChA, both were coagulated and sectioned. Latex injection performed after AChAO confirmed the complete section of the AChA (Fig. 3C) in 92% (11/12) of the marmosets. One marmoset had an additional branch bypassing the AChA to the PcomA, so the occlusion of the branch visible during the surgery was insufficient to cut the bloodstream to the AChA.

AChAO induced motor behavioral changes

Before surgery, all animals (n = 8) showed top scores for both FS (total = 25 points, hemilateral = 10 points per each side) and MNS (total = 40 points, hemilateral = 14 points per each side). After AChAO (n = 5), we noticed heterogeneous behavior of the operated animals. After individual evaluation, three marmosets manifested right sided neurologic deficits, as measured by the applied scores (FS and MNS), and required longer nursing and hand feeding during the observation period. Conversely, the remaining two marmosets recovered faster after surgery, and by day 11 their behavior was comparable to the status before surgery when the scores were applied. To group the animals that underwent AChAO with respect to their behavior, a Dixon test type 10 for outliers was applied to the total MNS score, finding the animals with higher scores as outliers (P < 0.01 for days 7 and 10). Operated animals were then divided into two groups: AChAO with neurologic deficits (AChAO + ND: n = 3) and AChAO without neurologic deficits (AChAO – ND: n = 2). For FS and MNS, the AChAO + ND group showed a reduction in total and right side scores in comparison with AChAO – ND and sham-operated animals, which was more pronounced for MNS (Fig. 4; AChAO + ND, Tukey HSD simultaneous tests: **P < 0.01). These animals also required longer periods to eat by themselves, requiring hand feeding until day 5–7. Additionally, they shifted the hand preference to the left side when attempting to eat by themselves. The AChAO – ND group showed a slight reduction in scores, which quickly improved over time (Fig. 4, AChAO – ND). This group also required less nursing and 2–3 days after surgery started to eat by themselves. One marmoset kept using his right hand as the preferred one, and the other marmoset increased use of his left hand without neglecting the right one during feeding. Left side scores did not change after surgery for any group. Sham-operated animals recovered fast after surgery and nursing requirement was minimal. Hand preference did not change after surgery. There was no statistical difference between AChAO – ND and sham groups (Tukey HSD simultaneous tests: P > 0.05).

AChAO induced damage to the IC

MRI performed 4 days after surgery showed injury extending from the genu to the posterior limb of the IC in the AChAO + ND group (Fig. 5A); extension of the infarction to surrounding structures differed between animals, but the posterolateral expansion was common in the three marmosets (Fig. 5A, D). The findings of AChAO – ND group were different: one animal did not show IC compromise, and the other showed a small infarction located medially in the genu of the IC without posterolateral expansion as observed in the AChAO + ND group (Fig. 5B, E). Animals from the sham group did not show relevant changes. Histology showed IC impairment congruent with MRI findings. For the AChAO + ND group, myelin staining showed important demyelination of the IC at the infarct level and Nissl staining showed dense infiltrates affecting the IC and expanding briefly to surrounding structures (Fig. 6A). The AChAO – ND group showed small demyelinated zones accompanied by cell infiltration in the optic tract and basal ganglia, and one marmoset showed a small demyelinated zone in the genu of the IC (Fig. 6B). Sham-operated animals did not show relevant changes. The left IC ratio was briefly reduced for the AChAO + ND (85.67% ± 5.62) and AChAO – ND (94.76%) groups because WM was lost at the IC for the AChAO-operated animals in comparison to the sham group (99.74% ± 0.66). However, the infarct volume...
was markedly larger for the AChAO + ND than
AChAO – ND group (AChAO + ND: 18.41 mm³ ± 9.75
AChAO – ND: 2.97 mm³) as the IC infarct volume
(AChAO + ND: 3.06 mm³ ± 0.40. AChAO – ND:
0.14 mm³). Infarct frequency maps revealed concomitant
damage to the IC in AChAO + ND group (Fig. 6 C) in
contrast to the smaller infarct in AChAO – ND group
(Fig. 6 D).

**DISCUSSION**

In this study, we found that the marmoset vascular
distribution was generally similar to the pattern found in
the human brain (Fig. 1) with a complete circle of Willis
and an AChA running over the optic tract (Wiesmann
et al., 2001; Uz et al., 2005). However, there was evi-
dence of anatomical variations (Fig. 1 C), which were
expected given their presence in humans. An intraopera-
tive anatomical study performed in humans by Akar et al.
(2009) found that 86.4% of the evaluated patients had a
single AChA emerging from the ICA; the remaining
patients showed different branching patterns and in some
cases duplicated or triplicated AChAs were found. This
anatomical variability broadens the spectrum of clinical
conditions for AChA stroke patients. In our study, marmo-
sets that underwent AChAO showed different behavior
during the observation period. It is likely that a nonvisible
AChA branch during the surgical procedure bypassed the
blood flow to the distal AChA reducing the impact of the
artery occlusion in two of the operated animals that did
not show neurologic impairment (Fig. 6 D, AChAO – ND).
Although this feature reduces the reproducibility of the
model, conditions are similar to the human reproducing
also the anatomical variations at some level. Additionally,
the medial lenticulostriate arteries from the MCA and
some perforating branches from the ICA can provide
blood flow to the posterior limb of the IC (Ghika et al.,
1990). This condition may influence infarct size. Visualiza-
tion of the IC irrigation state before surgery may provide
relevant information for future research.

MRI images and histological preparations (chronic
experiments) showed that the AChAO was able to
induce a small infarction affecting the IC, and other
subcortical structures in animals with evident neurologic
deficits (Figs. 5 and 6; AChAO + ND). Despite the
smaller size in infarct in comparison with previous
MCAO studies performed in marmosets (Marshall and
Ridley, 2003; Freret et al., 2008), the neurological deficit
was still evident 10 days after surgery (Fig. 4). Overall,
these results clearly suggest that the AChAO is a feasible
technique able to induce an infarction affecting the mar-
moset IC with consequent motor deficits, and is the first
described WM stroke model in the marmoset monkey.

**Other studies implementing the AChAO**

As far as we know, the AChAO model has only been
proposed in one previous study made by Tanaka et al.
(2008) in miniature pigs; they reported a high rate of suc-
cess for brain infarct induction (91.4%) when an aneurism
clip was placed or electrocoagulation was performed in
the proximal AChA. As an advantage, their study was
generated in an animal species with a gyrencephalic brain
and the AChAO was able to induce motor impairment.
However, recovery occurred within 10 days even though
a clear IC injury was observed 4 weeks after surgery.
By contrast, our marmosets exhibited motor impairment even at day 10 (Fig. 4, AChAO + ND). This discrepancy could be ascribed to the vascular anatomical differences between species that may affect the functional recovery of the animals. In the miniature pig, triplicated MCAs emerge from the ICA (Imai et al., 2006), suggesting the existence of a higher number of lenticulostriatal arteries. This condition may improve collateral blood flow to the IC after AChAO, thus attenuating the ischemic impact and allowing fast neurological recovery. By contrast, the brain vasculature of the marmoset resembles the human’s (Fig. 1A), with a single MCA and an AChA with a similar anatomical pattern in most cases. Because blood flow supported by the lenticulostriatal arteries is likely to be similar in species with a unique MCA, we can infer that the motor deficit evidenced in the AChAO may not be reversible as seen in AChA stroke patients.

Animal species selection for stroke research

Stroke research has been conducted mainly in rodents owing to their handling and reproduction rate advantages (Macrae, 2011; Canazza et al., 2014). However, owing to translational research failure from several therapeutic approaches tested in these species (Xu and Pan, 2013), the development of novel stroke models in different animal species is required. Owing to their phylogenetic similarities to humans, the NHP has drawn attention for the generation of new stroke models (Fukuda and del Zoppo, 2003). However, the use of gyrencephalic NHPs, such as the baboon or macaque, is restricted owing to their size, care and breeding requirements. Nevertheless, the common marmoset is a NHP species relatively easy to house and handle owing to their small size (∼300 g) and high reproduction ratio (Abbott et al., 2003; Okano et al., 2012). Therefore, marmosets offer a rich scenario for stroke research balancing resemblance to human features, closer ergonomics and smaller GM/WM ratio in an animal that is easier to handle than a larger NHP.

Clinical relevance

AChA territory infarctions account for 2.9–11% of all patients with acute ischemic stroke (Hamoir et al., 2004; Ols et al., 2009) and are frequently associated with motor deficits (Palomeras et al., 2008), where IC involvement correlates importantly with motor outcome (Nelles et al., 2008). Induced hemiparesis can be severe and progressive (Steinke and Ley, 2002). Owing to the catastrophic
conditions of AChA stroke patients, where human studies directly correlate WM damage to motor outcome after stroke (Puig et al., 2011), we consider studies focusing on damage to the IC will provide more relevant information and aid in the search for new strategies to improve recovery from motor function deficits.

Limitations of the model

This model was developed in a common marmoset aiming to generate a NHP model that could be easily comparable to the human condition. Although phylogenetically humans and marmosets are closer than rodents or other inferior mammals, the marmoset brain is still lissencephalic. Despite the increased WM ratio in comparison with rodents, the cortical distribution is quite different to the human. It is important to remember such differences across species for translational research.

A second limitation refers to the possibility to design a reperfusion model by occluding the AChA that may allow the evaluation of pharmacological interventions targeting the reperfusion phase following ischemia (Macrae, 2011). Our initial design was to occlude the AChA using an aneurism clip. However, owing to space restrictions it was not possible to use this approach. Instead we decided to perform a permanent occlusion of the vessel. These conditions did not allow us to evaluate the reperfusion state after stroke. Further surgical procedures need to be developed to overcome this difficulty.

Third, we found neurologic impairment only in 60% of operated marmosets, which is a low rate of success in comparison with the established stroke models for rodents (Tamura et al., 1981; Kohno et al., 1995) and marmosets (Marshall and Ridley, 2003; Freret et al., 2008). This lower success rate could be a disadvantage for applying this model for translational research in the development of neuroprotective drugs as well as cell therapies due to the requirement of a large number of animals.

Fourth, our model may not be directly applicable to the majority of human stroke studies because AChA stroke in humans is relatively rare in comparison with major infarctions such as MCA stroke (Rordorf et al., 1998). However, by establishing the AChA stroke model, we wanted to offer an opportunity to study the WM ischemia process without impairing the cortex. Therefore, we believe this model may be comparable to any human stroke that is accompanied by WM ischemia.

Future directions

The AChAO method in marmoset monkeys established in this study will allow us to perform a detailed examination of motor dysfunction and recovery using previously reported behavioral evaluations (Marshall and Ridley,
and additional evaluations such as gait pattern, pressure distribution and muscular synergy alterations after WM stroke. Additionally, showing the physiological mechanism for damaged IC compensation by other descending or cortical and subcortical networks will have a crucial implication on the establishment of novel rehabilitation strategies in human stroke patients.

CONCLUSIONS

The occlusion of the AChA in marmosets was able to induce a focal infarction that compromised the IC, and resulted in neurologic deficits, which were evident during natural behavior and that were sustained to day 10. This model offers a new approach to understand the pathological process of WM ischemia, as well as stroke treatment, including pharmacological therapies and physiotherapy routines, allowing the development of new strategies that focus on improving motor function after WM impairment.

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DISCLOSURES
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