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RESEARCH ARTICLE

Improved assessment of outcomes following transient global cerebral ischemia in mice

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Abstract Mouse models of global cerebral ischemia (GCI) allow experimental examination of cerebral pathophysiology in genetically modified mice and fast screening of new treatment strategies. Various surgical protocols of GCI-induction in mice have been published; however, many of these studies are hindered by limited neurological assessment protocols and present insufficient reporting of the cumulative survival rate. Therefore, we aim at developing a reproducible and easily implementable model of transient GCI in mice with minimal impact on normal mouse behavior. GCI was induced in male C57BL/6 mice by bilateral occlusion of the common carotid arteries for 10 min combined with isoflurane-induced hypotension which resulted in severe reduction in the cerebral blood flow of the forebrain. Sham operation served as a control. Exploratory behavior was evaluated in a home-cage environment the day before and again daily for up to 7 days after GCI or sham operation and was found to be significantly decreased 1–7 days after GCI compared to sham. Furthermore, we found delayed neuronal cell death in the frontal cortex and hippocampus 5 and 7 days after GCI but not at day 3 or after sham operation. The survival rate at day 7 was 100 % after sham operation and 42 % after GCI. The model of GCI in mice presented in this study compromises the exploratory behavior and resembles the cerebral damage and mortality rate seen after cardiac arrest and/or GCI in man, and is therefore a good model to use for studies of GCI pathophysiology.

Keywords Global cerebral ischemia · Mouse model · Exploratory behavior assessment · Delayed neuronal cell death · Delayed mortality

Introduction

Global cerebral ischemia (GCI) is most often caused by cardiac arrest as a consequence of the transient failure of the cardiovascular system. Patients who are successfully resuscitated are still at great risk of dying or developing severe neurological deficits within the first month after cardiac arrest due to delayed neuronal cell death (Mangus et al. 2014; Schneider et al. 2009). There are currently no treatments available to prevent post-GCI delayed neuronal death in the brain. Although hypothermia has been shown to have neuroprotective effects, additional treatment strategies are needed in order to improve patient outcome (Bro-Jeppesen et al. 2009; Friberg and Nielsen 2009).

Rodent models are often used when studying GCI pathophysiology since they are inexpensive and allow us to study the evolving pathology after GCI and the cerebral pathophysiology in wild-type and various genetically altered (GA) mouse strains. GA mice are powerful tools in the search of new mechanistic targets for the prevention of delayed neuronal death after GCI.

In mouse models of GCI, cerebral ischemia is most often induced by bilateral clamping of the common carotid arteries either alone or in combination with hypotension (two-vessel occlusion; 2VO) or additional clamping of the vertebral arteries (four-vessel occlusion, 4VO) (Kristian

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and Hu 2013). Mouse models of cardiac arrest have been published, and although more clinically relevant the models of cardiac arrest have disadvantages such as increased mortality due to failure of resuscitation and variable outcome (Deng et al. 2014; Kofler et al. 2004; Kristian and Hu 2013).

We initially aimed to find a simple model with which to examine the cerebral pathology of GCI in mice. The choice of model was based upon a systematic review of the literature from which an overview was established of the advantages and disadvantages, and common practices, of the model as used in a total of 31 previous studies (see flowchart in Supplementary Table 1). Based upon this review, we chose to induce transient GCI in mice by two-vessel occlusion combined with isoflurane-induced hypotension, and optimized the surgical procedure for our laboratory.

Anesthesia-induced hypotension was previously shown in rat and mouse to be an alternative to inducing hypotension mechanically by blood withdrawal from a jugular catheter (Onken et al. 2012; Bendel et al. 2005). To validate the model, we assessed mean arterial blood pressure (MABP) during surgery and delayed neuronal cell death up to 7 days after GCI-induction. Furthermore, we report the cumulative survival rate up to 7 days after GCI-induction. Most importantly, we developed a new method to assess early neurological deficits which is an important parameter to quantify especially when evaluating new treatment protocols. The need for new alternatives to evaluate neurological deficits in mouse models of GCI is vital since existing tests give mixed results (see supplementary Table 1).

We present a clinically relevant, reproducible method of GCI in mice coupled with a novel outcome assessment procedure that quantifies exploratory behavior before the operation and daily for up to 7 days after the surgery in an enriched home-cage environment.

Methods

Animals

A total of 41 animals were included in this study, eight underwent sham surgery, and 33 underwent GCI-induction. The animal experiments were carried out in accordance with the European Communities Directive 2010/63/EU on the protection of animals used for scientific purposes, and the surgery protocol was approved by the Danish Animal Inspectorate (license no. 2012-15-2934-00281). We used male C57BL/6NTac mice (10–12 weeks old) from Taconic, Denmark. The mice were housed in the animal facility at Glostrup Research Institute at a constant temperature of 23 °C and a diurnal cycle of 12-h light/dark (lights on from 7.00 till 19.00). The mice were supplied with standard chow (Altromin #1314, Brogaarden, Denmark) and water ad libitum.

Experimental global cerebral ischemia

Prior to surgery, the mice were fasted overnight. Anesthesia was induced with 5 % isoflurane in 70:30 N_2O and O_2 followed by intubation (PE30 tubing on an 18-G stump needle), and the mouse was kept on 1.5 % isoflurane in 70 % N_2O and 30 % O_2 throughout the surgical procedure. The mouse was placed on a homeothermic heating pad coupled to a rectal thermometer to ensure a constant body temperature of 37 ± 0.5 °C. Likewise, a constant head temperature of 37 ± 0.5 °C was ensured by a needle thermometer inserted next to the skull and by adjusting the height of a heating lamp throughout the procedures. The tail artery was cannulated (20 μL Eppendorf tip 5242 956.003) for continuous MABP recordings with LabChart7 (ADInstruments, UK), and the carotid arteries carefully isolated. Hypotension was induced 2 min prior to clamping the carotid arteries by increasing the isoflurane concentration to 5 %. The common carotid arteries were clamped for 10 min, and the concentration of isoflurane was regulated within this period to maintain a MABP of around 40 mmHg. The isoflurane concentration was lowered to 0 %, 2–3 min before declamping the arteries which allowed the MABP to recover to preclamp levels. When the clamps were removed, the isoflurane concentration was increased to 1.5 % isoflurane for an additional 30 min to allow recovery from the cerebral insult, the incisions were sutured, and analgesic was applied (Rimadyl®, Carprofen 5 mg/kg). Next anesthesia was discontinued and the mouse left to regain spontaneous consciousness followed by extubation. Sham-operated mice served as control and went through the same surgical procedures as described above except for lowering MABP with isoflurane and clamping of the common carotid arteries. Postoperatively, the mice were kept in pairs in a heating cabinet at 33 °C (type 48-VS-III, Scanbur Technology, Denmark) for the first 24 h and then kept at standard housing conditions at a temperature of 23 °C until termination. Mice were placed in a heating cabinet for the first 24 h to avoid hypothermia since this has been shown to be neuroprotective (Tsuchiya et al. 2002; Webster et al. 2009). Every day until termination, the mice were weighted, supplemented with 0.9 % saline solution intravenously if dehydrated, supplied with fresh liquid high caloric food (DietGel Recovery/boost, Brogaarden, Denmark) and analgesic was applied (Rimadyl®, Carprofen 5 mg/kg). If a mouse at any point showed severe distress as seizure activity or severe weight loss (greater than 20 % of pre-surgery weight), the animal was euthanized and not included in subsequent experiments.

The cortical cerebral blood flow of the forebrain was measured in a couple of mice by laser Doppler flowmetry before, during, and after clamping of the common carotid arteries to verify the drop in CBF. The laser Doppler probe was placed above the skull 3 mm anteriorly of the bregma above the right hemisphere.

Exploratory behavior assessment

The exploratory behavior of the mice was recorded on film the day before the operation and then again on a daily basis up till 7 days after GCI or sham operation in order to evaluate neurological deficits. Recording individual baseline behavior of each mouse, the day before the operation enables one to map recovery or maturing deficits. Each mouse was filmed individually each day in a new cage (390 × 225 × 145 mm) with standard chow, water ad libitum, and a hide (mouse igloo, product no. 32467, Brogaarden, Denmark). This cage was placed in a room without disturbances and filmed behind a closed door for 10 min to investigate the behavioral patterns. All films were recorded at the same time of day (between 8 and 11 a.m.) in order to avoid diurnal variations in the behavioral pattern of the mice. The assessor was blinded to the identity of the mice in the films. The behaviors assessed were locomotion, rearing, eating, grooming, and activities as crawling on cage bars in the wire lid or food hopper, crawling into hide, crawling on top of hide, and digging in the litter. We determined both the total time spent doing each of the activities and the number of times the mouse engaged in a certain activity, and each behavior was noted as a binary event. We found that the pattern of decreased exploratory behavior was seen whether the behavior was depicted as number of times engaging in an activity or total time spent doing an activity and we chose to show the total time spent on the different exploratory behaviors in Fig. 3. We analyzed data by one-way ANOVA combined with Bonferroni's posttest by comparing the respective days after GCI and sham operation separately.

TUNEL staining

The mice were euthanized by sedation in a mixture of 70 % CO_2 in 30 % O_2 followed by decapitation and immediate, careful removal of the brain. Brains were fixed overnight in formalin buffer with 4 % paraformaldehyde followed by saturation in 10 %, and then 25 % sucrose solutions made with physiological buffered saline (PBS) followed by embedding in gelatin medium (30 % egg albumin, 3 % gelatin in distilled water). The brains were sectioned coronally (LeicaCM3050S cryostate), and 20-μm sections collected from the forebrain (bregma 2.10) and the midbrain (bregma −2.20 mm), respectively. We analyzed the sections for neuronal cell death with a TUNEL kit according to the manufactures instructions (DeadEnd™ Fluorometric TUNEL System, Promega, USA), which detects and binds fragmented DNA. In brief, slides were rehydrated by washing in PBS for 5 min followed by cell permeabilization with 0.2 % Triton-x PBS (PBS-T) for 15 min and again washed 2 × 5 min with PBS. Next slides were incubated for 8 min with proteinase-K for protein digestion and washed 2 × 5 min. Positive control slides were incubated with DNaseI for 10 min (Promega, USA). The slides were immersed in equilibrium buffer for 10 min followed by incubation with rTdT incubation buffer for 60 min at 37 °C. Negative control slides were incubated without rTdT. Lastly, slides were immersed in 2 × SSC (sodium chloride and sodium citrate buffer) for 15 min, washed 2 × 5 min, and cover glasses mounted with mounting media (Vectashield, Vector Laboratories, Burlingame, CA, USA) containing 4′,6-diamino-2-phenylindole (DAPI) for nucleus staining. Sections were analyzed on a NikonEclipse80i microscope, and images captured with a 20× objective. TUNEL stainings were performed three times of fore- and midbrain sections of sham-operated ($n = 2$) and GCI ($n = 4$–5) mice. The TUNEL stainings were blinded to the assessor, and the brain areas with positive TUNEL staining were identified and noted.

Data presentation

Data are presented and given as mean ± standard error mean (SEM). Figures 1, 2, and 3 were prepared with GraphPad Prism5 and Adobe illustrator. Figure 4 was prepared with Adobe photoshop.

Results

Verification of GCI-induction in the mouse

In this study, we used a mouse model of GCI where we clamped the common carotid arteries in combination with isoflurane-induced hypotension. As seen in Fig. 1a, the high concentration of isoflurane (5 %) lowered the MABP from 84.1 ± 9.9 to 49.6 ± 14.8 mmHg within 2 min, followed by clamping of the isolated common carotid arteries for 10 min as marked by the vertical lines in Fig. 1a. During the occlusion, the isoflurane concentration was regulated continuously to maintain a MABP around 40 mmHg the first 8 of the 10 min. The isoflurane supply was terminated the last 2 of the 10 min of occlusion to allow the MABP to recover before declamping the common carotid arteries. When the clamps were removed, the MABP was 81.1 ± 32.1 mmHg whereupon the MABP became hyperemic for 5–10 min with a maximum of 96.7 ± 18.5 mmHg

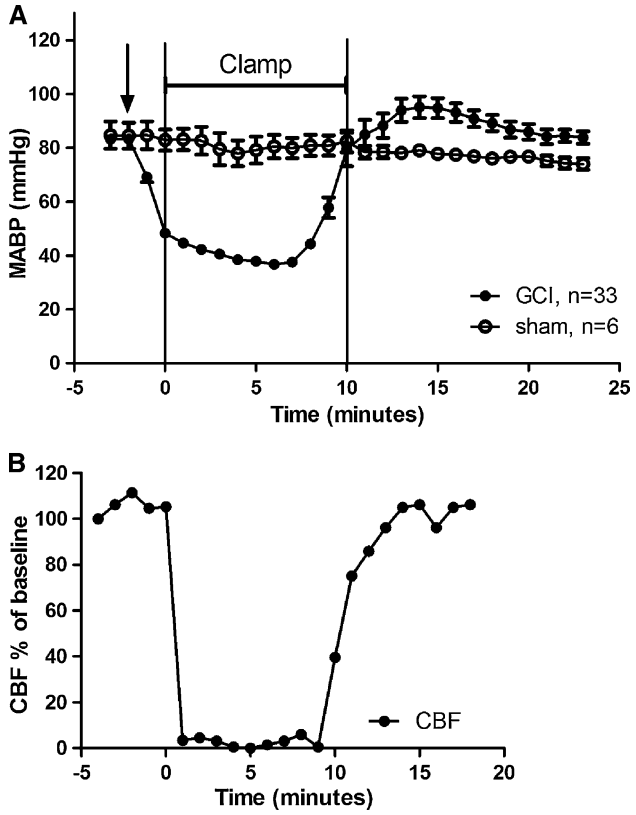

Fig. 1 MABP and CBF before, during, and after sham operation and GCI in mice. **a** The MABP was lowered with a high concentration of isoflurane (marked by the *arrow*) followed by clamping of the common carotid arteries for 10 min (marked by the *vertical lines*) in GCI mice (*filled circle*). MABP of sham-operated mice is also shown and given by (*open circle*). **b** Laser Doppler flowmetry revealed that the CBF in the frontal cortex was completely abolished by bilateral carotid artery clamping combined with isoflurane-induced hypotension

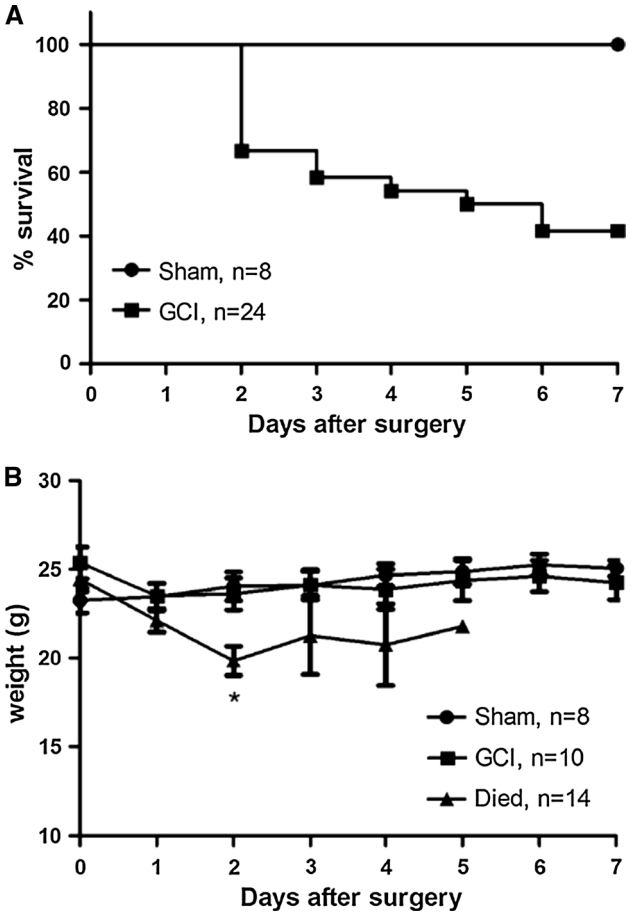

Fig. 2 Survival rate and weight of the 32 animals designated to be terminated 7 days after GCI or sham operation. **a** Survival rate at day 7 after GCI was found to be 42 % (*filled square*), whereas the survival rate was 100 % in sham-operated mice (*filled circle*). **b** The weight of mice dying at day 2 or later (*filled triangle*) was significantly decreased compared to GCI mice surviving until day 7 (*filled square*) or sham-operated mice (*filled circle*). The drop in weight reflected poor general health and indicated a fatal outcome for the mouse

4 min after reperfusion compared to sham (Fig. 1a). Bilateral common carotid artery occlusion resulted in complete elimination of cortical forebrain cerebral blood flow (CBF) as seen by a representative laser Doppler flowmetry trace in Fig. 1b. Furthermore, hypotension alone was not found to affect CBF and there was no evidence of reactive hyperemia of the CBF after the clamps were removed (Fig. 1b).

Survival rate in the mouse model of GCI

The survival rate of sham-operated mice was 100 % which contrasted with 42 % survival at 7 days after GCI (Fig. 2a). In the initial experimental design, a total of 24 mice were designated to survive until day 7 and 14 of the 24 mice died prior to termination at day 7. The mice that died prior to the designated termination point died primarily at day 2 (8 out of 14 mice), and all mice that died within the 7 days were excluded from subsequent analysis. Additionally, we found that mice dying before day 7 experienced a significant drop in weight at day 2 compared to sham-operated mice or GCI mice that survive until day 7, which reflected the decreased overall wellbeing and that the mouse was either dead or was likely to die soon (Fig. 2b). The drop in weight of the mice that died at day 2 showed a severe weight loss at around 20 %.

Exploratory behavior assessment in an enriched novel environment

We analyzed eight different behavioral activities of the mice the day before operation and again daily for up to 7 days after GCI or sham operation. Before the surgical procedures, the mice were constantly active when placed in the novel environment exhibiting each of the identifiable exploratory behaviors; climbing in the cage bars (Fig. 3a), climbing on top of

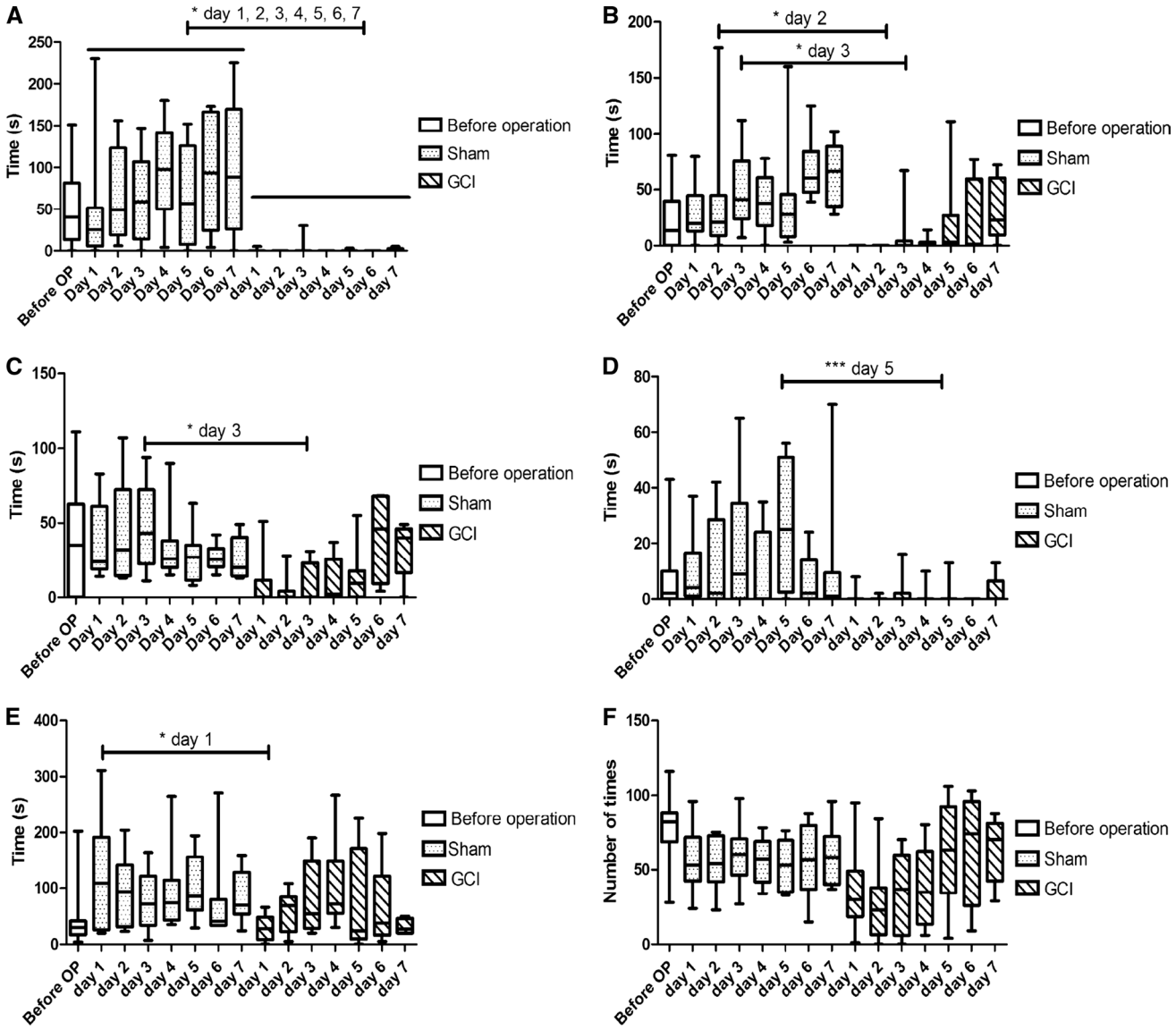

Fig. 3 Changes in the behavioral pattern of mice reflected neurological deficits in the first 7 days after GCI. Mouse behaviors as **a** time spent on cage bars, **b** time spent on top of hide, **c** time spent in hide, **d** time spent digging, **e** time spent grooming, and **f** rearing were recorded and analyzed in mice before (*clear bars*) and again daily for up to 7 days after GCI (*crossed bars*) or sham operation (*dotted bars*). Before operation, $n = 32$; sham and ischemia, $n = 5$–9

the hide (Fig. 3b), entering and staying in the hide (Fig. 3c), digging in the litter (Fig. 3d), grooming (Fig. 3e), and rearing (Fig. 3f). Operation in itself was not found to significantly alter any of the exploratory behaviors investigated, although the exploratory behavior was generally found to be decreased after GCI compared to sham-operated mice (Fig. 3).

On each of the 7 days after GCI, the mice spent significantly less time than sham-operated animals climbing in the cage bars (Fig. 3a), while the time spent on top of the hide was significantly reduced only on days 2 and 3 (Fig. 3b) and the time spent in the hide was significantly reduced only on day 2 (Fig. 3c), although there were similar trends toward reduced occurrence of these behaviors on other days as well (Fig. 3). In mice surviving until day 7, the exploratory behaviors of spending time on top of and in hide seemed to recover at days 6 and 7 (Fig. 3b, c); however, the time spent on cage bars was still significantly decreased 6–7 days after GCI compared to sham (Fig. 3a). After GCI mice tended to spend less time digging in the litter of the cage (Fig. 3d), this behavior was found to be significantly decreased day 5 after GCI compared to sham (Fig. 3d). However, not all mice spent time digging, and therefore, this might not be a good parameter to assess in order to evaluate behavioral outcome.

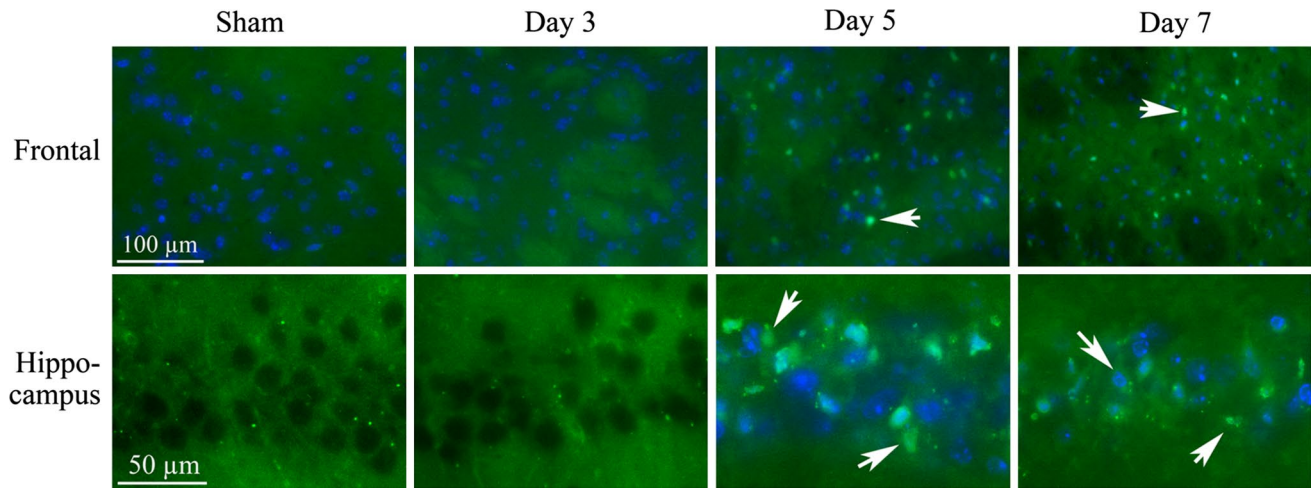

Fig. 4 Delayed neuronal cell death was found in the frontal cortex and hippocampal CA1 region 5 and 7 days after GCI (*white arrows*). No cell death was observed in the frontal cortex or hippocampus 3 days after GCI or in sham-operated animals terminated at day 7. TUNEL stainings were performed three times, sham ($n = 2$) and GCI ($n = 4$–5)

Additionally, we found that sham-operated mice spent significantly more time grooming at day 1 compared to GCI mice (Fig. 3e) which might reflect a general decreased well-being of GCI mice since grooming is a natural behavior of animals after surgery. At days 2–7 after GCI or sham operation, there was no differences in the time spent grooming. The first 2 days after operation both sham and GCI mice tended to rear less (Fig. 3f), a tendency that increased after GCI compared to sham. The decreased tendency to rearing might be due to postoperative discomfort from the incision at the front of the throat even though the animals were given analgesics every day after the surgery. Rearing reached preoperative levels at days 5–7 after GCI (Fig. 3f).

We found no differences in locomotion and the time spent eating and drinking between GCI and sham-operated mice and concluded that this was not good outcome assessment parameters (data not shown).

Verification of delayed neuronal cell death by TUNEL staining

We found scattered delayed neuronal cell death in the frontal cortex and consistent neuronal cell death in the CA1 region of the hippocampus at days 5 and 7 (white arrows in Fig. 4), whereas no cell death was found in the frontal cortex or CA1 region of the hippocampus at day 3 after GCI or in sham-operated mice terminated day 7 (Fig. 4). At day 5 after GCI, the TUNEL-positive cells had a defined rounded shape, and at day 7 after GCI, we found that the TUNEL-positive cells were pyknotic corresponding to a more diffuse staining.

Discussion

In this study, we present novel aspects of a mouse model of GCI with reproducible delayed neurological damage and delayed mortality, ideal for assessing behavioral outcome and studying GCI pathophysiology.

Methodological considerations before setting up a mouse model of GCI

Previously published methods of GCI-induction in mice are presented in the flowchart (supplementary Table 1) which provides an easily accessible overview of the literature of GCI models in mice for established researchers in the field as well as those new to the field. The literature search presented in the flowchart in supplementary Table 1 made us aware of methodological aspects to consider before setting up a mouse model of GCI. In the flowchart, we have singled out 31 different articles with mouse models of GCI illustrating the variations of how to induce GCI in mice. Models of cardiac arrest in mice have also been published previously (Deng et al. 2014; Kofler et al. 2004) but were not included in the flowchart. We found that GCI is most often induced by bilateral occlusion of the common carotid arteries for 10 up to 75 min depending on the strain of mouse (Fujii et al. 1997; Wellons et al. 2000). The occlusion time necessary to obtain reproducible neurological damage in mice differs among strains due to differences in the competence of the circle of Willis (Kristian and Hu 2013).

Many GA mice are produced on a C57BL/6 background, and this strain is therefore often used for GCI models in

mice (Kristian and Hu 2013). The posterior communicating arteries are often absent or poorly developed in C57BL/6 mice (Fujii et al. 1997; Wellons et al. 2000). Bilateral occlusion of the common carotid arteries in mice without the posterior communicating artery results in GCI-induced neurological damage within 10–30 min since the posterior communicating arteries cannot compensate for the reduction in CBF via the vertebral-basilar arteries upon carotid occlusion (Olsson et al. 2004). Although often absent or poorly developed, the posterior communicating arteries are still intact in some C57BL/6 mice and mice with intact, posterior communicating arteries will develop less ischemic damage upon 2VO alone.

A way to overcome this problem and to get a model of GCI with a reproducible neurological damage, as presented in this study, is to simultaneously clamp the carotid arteries and lower the systemic MABP below the autoregulatory threshold (50 mmHg). Thereby the posterior communicating arteries are unable to compensate for the CBF reduction obtained when clamping the common carotid arteries. In our group, we induce GCI in rats by simultaneous bilateral clamping of the common carotid arteries and lowering of the MABP mechanically by withdrawing blood from a catheter in the jugular vein (Johansson et al. 2014). In mice, previous studies have utilized GCI models combining bilateral carotid occlusion and MABP lowing by withdrawing blood from the jugular vein (Homi et al. 2003; Sheng et al. 1999). Thus, a recent study in C57BL/6 mice showed that lowering MABP with isoflurane combined with bilateral carotid occlusion for 10 min resulted in reproducible delayed neuronal cell death similar to the delayed neuronal damage seen when clamping the carotid arteries combined with mechanical lowing of the MABP (Homi et al. 2003; Onken et al. 2012; Sheng et al. 1999).

To limit invasive surgery, we chose to lower the MABP with isoflurane in the model of GCI presented here. We found great variability in isoflurane sensitivity among the mice and therefore chose to continuously record MABP in all mice of the study by cannulating the tail artery. Even though the isoflurane concentration is increased to induce hypotension, the total amount of isoflurane experienced by the mice will be the same as when inserting a jugular catheter for mechanical hypotension induction due to the extended length of the surgical procedures. Isoflurane is a centrally acting drug and has been shown to decrease the activity of the serotonergic system in vivo (Massey et al. 2015; Mukaida et al. 2007; Whittington and Virag 2006). Furthermore, isoflurane has been shown to cause endothelium-independent relaxation of middle cerebral arteries in vitro, where the relaxing effect was seen to disappear when isoflurane was removed (Flynn et al. 1992). It is unknown whether the central decrease in serotonergic activity has long-term effects on brain pharmacology after discontinuation of isoflurane, it might just have acute effects like the relaxing effect on cerebral arteries in vitro, which will not affect investigation of brain injury in the days after GCI. Thus, isoflurane has been shown to have neuroprotective effects by an unknown mechanism 3 days after GCI in mice that were independent of mechanically induced hypotension during GCI (Homi et al. 2003). However, long-term outcome has been shown to be independent on the choice of anaethetic after GCI (Elsersy et al. 2004).

As seen in the flowchart (supplementary Table 1), mice were fasted prior to surgery in all studies where bilateral common carotid artery occlusion was combined with systemic hypotension. Hyperglycemia is known to exacerbate neurological damage which results in worsened outcome after cardiac arrest and/or GCI (Sheng et al. 1999; Wass and Lanier 1996). If mice are allowed free access to food prior to the surgery, the glucose concentration in their blood will depend on the amount of food consumed as well as the time of food intake. To avoid this variability in our experiments, we chose to fast all mice overnight prior to surgery.

The mouse model of GCI induced by two-vessel occlusion and isoflurane-induced hypotension with continuous MABP recording during surgery

We here present a mouse model of GCI where we occlude the common carotid arteries bilaterally combined with isoflurane-induced hypotension as previously reported by Onken et al. (2012). We refined the method by cannulating the tail artery for continuous MABP recordings during the procedure. The MABP was lowered by a high concentration of isoflurane (5 %) followed by clamping of the carotid arteries which resulted in total elimination of forebrain CBF (Fig. 1).

We found that the relationship between the dose of isoflurane and the hypotensive response was very variable between individual mice and so the level of hypotension could only be controlled by titrating the dose against absolute measurement of real-time blood pressure. In order to do that, we chose to cannulate the tail artery since this was a more gentle surgical intervention than cannulating a femoral artery. When an artery is cannulated during surgery, it must be occluded at the end of surgery and occluding the femoral artery alter the behavior of the mouse since the function of the leg supplied by that femoral artery will be severely affected following the operation. Occluding the tail artery on the other hand was not seen to affect the exploratory behavior investigated in this study, of the hide and the cage bars (Fig. 3e–g), in sham-operated mice compared to mice assessed before operation. Consequently, the decreased exploratory behavior of GCI mice was concluded to be due to neurological deficits not the surgery in itself.

We found a delayed mortality of 58 % within the first 7 days after GCI-induction (Fig. 2) which is in line with mortality rates of 30–50 % at day 7 after 10 min of GCI in C57BL/6 mice (Otsuka et al. 2009; Wei et al. 2008). Similar mortality rates have been reported for other strains (Murakami et al. 1998) and found to increase with increasing occlusion time (Tsuchiya et al. 2002). The fact that our data show a secondary delayed mortality in mice receiving GCI is well-worth highlighting since the mortality of successfully resuscitated cardiac arrest patients is around 50 % within the first month due to delayed neuronal cell death (Bro-Jeppesen et al. 2009). We found delayed neurological cell death at days 5 and 7 after GCI in the hippocampal CA1 region and frontal cortex (Fig. 4). Delayed neurological cell death is a hallmark of GCI pathophysiology and is responsible for the development of cognitive deficiencies and in worst-case scenario death of patients who are successfully resuscitated after cardiac arrest (Mangus et al. 2014; Schneider et al. 2009). Delayed mortality and delayed neuronal cell death in the mouse model of GCI presented here resemble the clinical observations after GCI and/or cardiac arrest and are therefore an excellent model allowing us to study GCI pathophysiology.

Assessment of outcome after GCI in mice

It is challenging to find reproducible and inexpensive neurological and behavioral tests well suited to evaluate cerebral ischemic damage in mice. Most studies using behavioral testing to evaluate neurological damage in mouse models of GCI utilize motor tests (see supplementary Table 1). Motor tests evaluate motor deficiencies due to neuronal cell death of the cerebral sensory-motor cortex after GCI. Many studies did not find GCI-induced motor impairments in mice (Fatehi-Hassanabad and Tasker 2011; Onken et al. 2012; Sheng et al. 1999; Wellons et al. 2000). However, the choice of anesthetic used when inducing GCI in mice has been shown to affect motor functions 3 days after GCI-induction. Isoflurane utilization resulted in intact motor functions of GCI mice, whereas fentanyl/N_2O utilization resulted in motor impairments of GCI mice at day 3 (Homi et al. 2003). This is supported by a study using chloral hydrate anesthesia which found a difference in motor related behavior between sham-operated and GCI mice with the rota rod test (Rehni et al. 2010). However, overall motor tests seem to be insufficient in the evaluation of neurological deficits after GCI in mice.

Another alternative in evaluating neurological deficits after GCI in mice is to observe locomotion, general health assessment, and exploratory behavior assessment (Wahlsten et al. 2003). We found that the three exploratory behaviors: time spent on cage bars, on top of hide, and in hide, were strong parameters when evaluating differences in early neurological deficits in mice the first 7 days after GCI and that some of these functions seemed to recover at days 6 and 7. On the other hand, time spent digging seemed to be sporadic and it may not be a suitable parameter to assess for the determination of neurological deficits and worsened outcome in GCI mice. Likewise, rearing tended to decrease the first 2 days after GCI compared to sham, but no statistical significance was obtained. Grooming was neither found to be a suitable parameter reflecting the neurological deficits after GCI.

We also assessed locomotion by recording the total time the mice were active in the observational period, but since most mice were constantly active in the observational period, this method may not be a suitable outcome assessment factor. However, it would have been interesting to determine the total distance travelled in the recording period instead of simply noting whether the animal was active or not, since GCI mice were found to be either more slow/careful or hyperactive (own observations). With the open field test, you are able to record general locomotion (total distance travelled), activity along the edges of or in the middle of the cage, and rearing in a designated time frame (Neumann et al. 2013). General locomotion (total distance travelled during the recording period) has been shown to be decreased at day 5 after GCI in mice (Onken et al. 2012), whereas another study found no change in locomotion 4 days after GCI in mice (Gaur and Kumar 2010). The open field test is also used to determine anxiety-related behavior by calculating the ratio of time spent in the middle or at the edges of the cage in the designated time frame. However, in the open field test normal exploratory behavior of the mice cannot take place to the same extend as when placed in a novel environment similar to their home cage with cage bars in the lid, a hide, standard chow, and water ad libitum. The laboratory animal behavior observation registration and analysis system (LABORAS, Metris, the Netherlands) enables one to determine behavior as locomotion, rearing, grooming, drinking, and eating, and recently, they launched an additional data package which makes it possible to register climbing on cage bars. If it becomes possible to detect movements on and into enrichment objects as the hide in our setup, it would be less time-consuming and generate more detailed data of the exploratory behavior using the LABORAS system instead of manual evaluation. However, filming the mice and evaluating the behavior manually are an inexpensive alternative to the LABORAS system.

We found deficits in the exploratory behavior in a novel enriched home-cage environment 1–7 days after GCI, whereas delayed neuronal cell death of the hippocampus and frontal cortex was found at days 5 and 7 after GCI. Furthermore, exploratory behavior of the cage bars in the wire lid and food hopper was found to be significantly decreased all 7 days after GCI; however, the exploratory behavior on

top and time spent in the hide seemed to recover at days 6 and 7 after GCI. This indicates that the behavioral deficits found after GCI in this study does not reflect the maturing damage of the hippocampus and frontal cortex. Memory test like the T-maze and Morris water maze are good tests to evaluate delayed neuronal cell death of the hippocampus after GCI but are very time-consuming, require pre-training of the animals, and can be affected by variable stress responses of individual animals (Hanell and Marklund 2014; Rehni et al. 2010; Vorhees and Williams 2014).

Even though this decreased exploratory behavior does not correlate with the timing of neuronal cell death after GCI in this study, the novel exploratory behavioral assessment protocol does reflect early neurological impairments after GCI. To the best of our knowledge, early neurological deficits after GCI are sparsely investigated since most studies have focused on impairments in memory and spatial learning reflecting hippocampal damage from day 5 and onwards after GCI (Neumann et al. 2013). A previous study in rats have shown that locomotion and anxiety were increased 1 day after GCI followed by decreased anxiety at day 5 after GCI although locomotion was still increased (Milot and Plamondon 2009). Furthermore, sensori-motor deficits were previously shown in rats 1–7 days after GCI by rota rod and tape removal tests (Albertsmeier et al. 2007; Johansson et al. 2014). These studies and ours illustrate that early neurological deficits is found after GCI in rodents and are most likely a consequence of the initial brain injury; however, the cerebral molecular changes behind these behavioral changes in the early phase after GCI are still unknown. These early neurological deficits deserve more attention in future studies.

In conclusion, even though successful resuscitation is achieved in cardiac arrest patients, the first weeks are characterized by delayed morbidity (due to cerebral damage) and mortality and the animal models used to study this disease pathology must resemble the clinical situation after cardiac arrest in patients. We have presented a model of GCI in mouse resulting in delayed neuronal cell death and mortality. Moreover, we present a novel exploratory behavior assessment protocol and found a decreased exploratory behavior after GCI compared to sham reflecting neurological impairments which are most likely a consequence of the initial cerebral damage after GCI.

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