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Comparison of different quantification methods to determine hippocampal damage after cerebral ischemia

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HIGHLIGHTS

- The number of NeuN and Fluoro-Jade B positive cells in CA1 is dependent on localization within CA1.
- Mean cell number does not change significantly over time from day 3 to 28.
- Variations within groups often become smaller resulting in lower SD and higher effect size at 28 days post insult.
- Number of Fluoro-Jade B positive cells does not decrease over time between 3 and 28 days post insult, suggesting that this staining is not labeling dying cells.
- Placement of counting windows has a major influence on extent of differences between moderate and severe ischemic brain damage.

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ABSTRACT

Background: Experimental stroke studies use multiple techniques to evaluate histopathological damage. Unfortunately, sensitivity and reproducibility of these techniques are poorly characterized despite pivotal influence on results.
Method: The present study compared several quantification methods to differentiate between two severities of global cerebral ischemia and reperfusion. Male Sprague-Dawley rats were randomized to moderate (10 min) or severe (14 min) ischemia by bilateral carotid occlusion (BCAO) with hemorrhagic hypotension. Neuronal cell count was determined in hippocampus at bregma −3.14 mm and −3.8 mm on day 3 and 28 post insult by counting neurons in the whole CA1 or in one to three defined regions of interest (ROI) placed in NeuN and Fluoro-Jade B stained sections.
Results: In healthy rats hippocampal neurons were arranged uniformly, while distribution became inhomogeneous after ischemia. The number of NeuN and Fluoro-Jade B positive cells was dependent on localization. Differences between ischemia severities became more prominent at 28 days compared to 3 days. Fluoro-Jade B positive cell count increased at 28 days, staining rather injured not dying neurons.
Comparison with existing methods: Placement of counting windows has a major influence on extent of differences between degree of neuronal injury and variations within groups.
Conclusions: The investigated quantification methods result in inconsistent information on the degree of damage. To obtain consistent and reliable results observation period should be extended beyond 3 days. Due to inhomogeneous distribution of viable neurons in CA1 after ischemia neuronal counting should not be performed in a single ROI window, but should be performed in multiple ROIs or the whole CA1 band.

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Abbreviations: BCAO, bilateral common carotid artery occlusion; CA, cornu ammonis; DG, dentate gyrus; FJB, Fluro-Jade B staining; MABP, mean arterial blood pressure; NeuN, neuron nuclei protein; ROI, region of interest.

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1. Introduction

Brain damage after ischemic stroke is a major cause of disabilities in adults (Perel et al., 2008). In order to investigate pathophysiology of cerebral damage, mechanism of neuronal cell death, and strategies to protect brain tissue, multiple experimental models have been established in rodents to generate reproducible brain damage. Ischemic brain injury undergoes a well-known pattern of regionally selective cell death (Pulsinelli et al., 1982; Kirino and Sano, 1984). In the hippocampus formation, the CA1 region is highly vulnerable to brief ischemic episodes, whereas CA3 region or dentate gyrus are more resistant (Muñoz and Grossman, 1981). Therefore, most studies on ischemic brain damage focus on CA1 region of hippocampus. Although CA1 damage is the primary end-point in most neuroprotection studies investigating global cerebral ischemia, quantification of histopathological damage is not standardized. Different techniques are used to visualize histopathological damage and even counting techniques vary between studies, e.g. counting all neurons (dead or viable) in the whole CA1 region (Engelhard et al., 2007; Domoráková et al., 2009; Kauppinen et al., 2009; Sun et al., 2009; Wei and Doré, 2010) or counting of neurons within a defined single region of interest (ROI) (Sasaoka et al., 2009) or multiple ROIs (Corbett et al., 2008; Traub et al., 2009; Lebesgue et al., 2010) placed inside CA1. So far, differences between the sensitivity and reproducibility of quantification methods have not been exactly characterized although this may strongly influence study results.

Therefore, in the present study effect estimates and statistical conclusions to identify a difference between moderate and severe damage in hippocampus after global cerebral ischemia and reperfusion in rats were compared between several methods to determine neuronal injury.

2. Methods

2.1. Animals

Twenty-five male adult Sprague-Dawley rats (Charles River Laboratories, Sulzfeld, Germany) weighing 336 ± 14 g (age: 3 months) were kept under a constant 12 h light/dark cycle and at a room temperature of 20–23 °C. Food (Purina rodent chow) and water were available ad libitum. All experiments were approved by the local governmental authorities (Landesuntersuchungsamt Koblenz, Rhineland-Palatinate) and were performed in accordance with German animal protection law.

2.2. Animal preparation

The animals were fasted overnight and anesthetized in a bell jar saturated with 8 vol% sevoflurane (Sevorane®, Abbott GmbH, Wiesbaden, Germany), then were intubated and mechanically ventilated with 3.5 vol% sevoflurane in oxygen and air (FiO_2 = 0.33). After placing the animal in supine position on a thermostatically controlled heating blanket (TCAT-2DF Controller, Physitemp Instruments Inc., Clifton, NJ, USA) a rectal temperature probe was inserted to maintain the animals' core temperature at 37.0 °C. A second temperature probe (Hypodermic needle probe, Omega Technologies Co., Standford, CT, USA) was inserted into the right temporal muscle to monitor and maintain the pericranial temperature at 37.0 °C with an infrared overhead heating lamp. The groin area was shaved and cleaned with a disinfection solution. After a skin incision the right femoral artery and vein were exposed and catheters (Cavafix Certo, 18 G, 5 cm, B. Braun Melsungen AG, Melsungen, Germany) were inserted into the vessels for measuring mean arterial blood pressure (MABP), blood gas analyses and withdrawing blood for induction of hypotension. After shaving the neck, disinfection and performing a skin incision in the jugular area, the sternohyoid muscles were exposed and divided without cutting. The carotid arteries were exposed and loose ligatures were placed around both vessels for later clamping. The right jugular vein was exposed and cannulated with a catheter for drug administration. All wounds were infiltrated with local anesthesia (bupivacaine 0.5%, 0.1–0.2 ml, AstraZeneca GmbH, Wedel, Germany). Respiratory variables, arterial blood gases, arterial pH, and plasma glucose concentration were monitored during the experiment by blood gas analysis (BGA; Radiometer Medical ApS, Kopenhagen, Denmark).

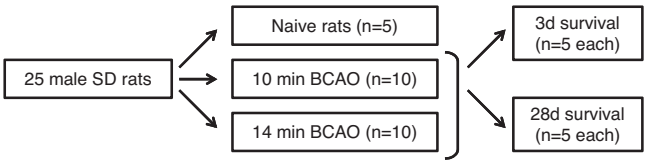

Fig. 1. Study design. Schematic presentation of the allocation protocol used to randomize animals into treatment groups.

2.3. Cerebral ischemia and reperfusion

Global cerebral ischemia was induced by bilateral carotid occlusion (BCAO) as previously described (Lasarzik et al., 2009, 2011). After surgical preparation a bolus of 10 μg/kg sufentanil (Sufenta mite®, Janssen-Cilag GmbH, Neuss, Germany) was administrated through the jugular vein followed by continuous infusion with 3.3 $\mu g \times kg^{-1} \times h^{-1}$. Sevoflurane was adjusted to 2 vol% to maintain balanced anesthesia (FiO_2 = 0.3) for 30 min. After a 30 min equilibration period, forebrain ischemia was induced by clipping both common carotid arteries in combination with hemorrhagic hypotension to a MABP of 35 mmHg for 10 min or 14 min, respectively. The withdrawn blood was kept on a 37 °C heating pad in heparinized and sterile syringes (2 × 5 ml) with intermittent gentle shaking. At the end of ischemia, clips were removed and the withdrawn blood was reinfused slowly over a period of 15 min. The physiological variables were recorded at four time points: before hemorrhagic hypotension (baseline), at the end of hemorrhagic hypotension, at the end of cerebral ischemia (ischemia), and 15 min after the end of reperfusion (recovery). At the end of the recovery period, catheters were removed and wounds were closed. All animals were relocated in individual cages and kept in an incubator with temperature controlled at 35 °C for 24 h after ischemia.

2.4. Experimental design

25 animals were assigned to following study groups (Fig. 1): 20 animals were randomly distributed into 10 and 14 min BCAO ischemia groups and were sacrificed after 3 or 28 days survival (n = 5 each). Additional 5 healthy rats were investigated as naïve controls. Hypotension was confirmed by continuous arterial blood pressure measurement. All animals survived the observation period and were included in the data analysis.

2.5. Tissue preparation

After 3 or 28 days of survival, the animals were anesthetized in a bell jar saturated with 8 vol% sevoflurane. In deep anesthesia animals were decapitated, brains were rapidly removed and immersed in methylbutane (Sigma–Aldrich, Steinheim, Germany) cooled with dry ice to −30 °C for quick fixation. Afterwards brain samples were stored at −24 °C until use. Frozen sections of 7 μm thickness out of three levels (3.14 and 3.8 mm posterior to bregma according the "The Rat Brain in Stereotaxic Coordinates" by George Paxinos

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