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Research Paper

Intranasal C3a treatment ameliorates cognitive impairment in a mouse model of neonatal hypoxic–ischemic brain injury

Javier Morán [a], Anna Stokowska [a], Frederik R. Walker [b], Carina Mallard [c], Henrik Hagberg [c,d,e], Marcela Pekna [a,f,g,*]

[a] Center for Brain Repair and Rehabilitation, Department of Clinical Neuroscience, Institute of Neuroscience and Physiology, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden
[b] School of Biomedical Sciences and Pharmacy, University of Newcastle, New South Wales, Australia
[c] Perinatal Center, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
[d] Centre for the Developing Brain, King's College, London, UK
[e] Department of Obstetrics and Gynecology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
[f] Florey Institute of Neuroscience and Mental Health, Parkville, Victoria, Australia
[g] Hunter Medical Research Institute, University of Newcastle, New South Wales, Australia

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ABSTRACT

Perinatal asphyxia-induced brain injury is often associated with irreversible neurological complications such as intellectual disability and cerebral palsy but available therapies are limited. Novel neuroprotective therapies as well as approaches stimulating neural plasticity mechanism that can compensate for cell death after hypoxia–ischemia (HI) are urgently needed. We previously reported that single i.c.v. injection of complement-derived peptide C3a 1 h after HI induction prevented HI-induced cognitive impairment when mice were tested as adults. Here, we tested the effects of intranasal treatment with C3a on HI-induced cognitive deficit. Using the object recognition test, we found that intranasal C3a treated mice were protected from HI-induced impairment of memory function assessed 6 weeks after HI induction. C3a treatment ameliorated HI-induced reactive gliosis in the hippocampus, while it did not affect the extent of hippocampal tissue loss, neuronal cell density, expression of the pansynaptic marker synapsin I or the expression of growth associated protein 43. In conclusion, our results reveal that brief pharmacological treatment with C3a using a clinically feasible non-invasive mode of administration ameliorates HI-induced cognitive impairment. Intranasal administration is a plausible route to deliver C3a into the brain of asphyxiated infants at high risk of developing hypoxic–ischemic encephalopathy.

1. Introduction

Neonatal hypoxic–ischemic encephalopathy (HIE) due to perinatal asphyxia is the leading cause of neurological injury resulting from birth complications. It is caused by the disruption of blood flow and oxygen delivery to the brain prior to or during delivery and occurs in 1–3 of 1000 live term births (Kurinczuk et al., 2010). Recent advances in critical care have improved the survival of infants suffering from HIE, but approximately 50% of survivors will develop complications such as intellectual disability and cerebral palsy (Mwaniki et al., 2012). Therapeutic hypothermia of children with HIE is a clinically accepted therapy that reduces by 12% the number of children with disabilities at 2 years of age (Edwards et al., 2010). Inflammation is a critical contributor to both normal development and injury outcome in the immature brain; depending on the timing and context, inflammation can prime the brain for injury or be neuroprotective (Hagberg et al., 2015).

Although best known for its role in the elimination of pathogenic bacteria, complement has also other functions such as the initiation of inflammation and the regulation of antibody production. Research during the past 10 years has shown that complement is a major regulator of brain plasticity and function in the healthy as well as diseased brain. Complement regulates the number of synapses during CNS development (Perez-Alcazar et al., 2014; Schafer et al., 2012; Stevens et al., 2007), promotes neurogenesis in the adult mammalian CNS (Rahpeymai et al., 2006) and the complement activation-derived

Abbreviations: HI, hypoxia–ischemia; HIE, hypoxic–ischemic encephalopathy; C3aR, C3a receptor; EBM, Experimental Biomedicine; P, postnatal day; PBS, phosphate buffered saline; ORT, object recognition test; ISI, intersession interval; STM, short-term memory; LTM, long-term memory; PFA, paraformaldehyde; NeuN, neuronal nuclei; GAP-43, growth associated protein 43; SYN, synapsin I; VGLUT1, vesicular glutamate transporter 1; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; CA, cornu ammonis; DG, dentate gyrus; PBS-T, PBS with 0.05% Tween 20; DAB, diaminobenzidine; ANOVA, Analysis of Variance.

* Corresponding author at: Institute of Neuroscience and Physiology, Dept. of Clinical Neuroscience, The Sahlgrenska Academy at University of Gothenburg, Box 440, SE-405 30 Gothenburg, Sweden.
E-mail address: Marcela.Pekna@neuro.gu.se (M. Pekna).

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peptide C3a stimulates neurite outgrowth as well as neuronal differentiation of neural progenitor cells in vitro (Shinjyo et al., 2009). C3 is upregulated in sprouting neurons isolated from rat cortex after ischemic stroke (Li et al., 2010) and C3a promotes astrocyte survival in response to ischemia (Shinjyo et al., 2016). Using transgenic mice over-expressing C3a in the brain and injection of C3a into the brain ventricles of control and C3a receptor (C3aR) deficient mice 1 h after HI, we showed that C3a, acting through C3aR, is protective against neonatal HI-induced tissue loss and cognitive impairment (Järlestedt et al., 2013).

Intraventricular administration of drugs is not clinically feasible and systemic administration of C3a carries a risk of serious adverse reactions including anaphylaxis (Finkelman et al., 2016). In addition, the availability in the CNS of systemically administered C3a would be limited due its rapid inactivation by serum carboxypeptidases (Bokisch and Muller-Eberhard, 1970) as well as by the blood-brain barrier. Intranasal administration permits peptides to bypass the periphery and the blood-brain barrier, rapidly reaching the brain and entering the cerebrospinal fluid. Molecules delivered intranasally use extracellular bulk flow transport along olfactory and trigeminal perivascular channels and possibly also axonal transport (Bahadur and Pathak, 2012). Proteins with size of up to 20 kDa, including insulin-like growth factor 1, nerve growth factor and epidermal growth factor, have been successfully delivered to the brain using this method (De Rosa et al., 2005; Lin et al., 2009; Scafidi et al., 2014). Intranasal administration thus appears as an attractive, clinically highly relevant and non-invasive method of therapeutic delivery of C3a to the brain. We therefore sought to determine whether the HI-induced cognitive deficit could be reversed by brief intranasal treatment with C3a.

2. Materials and methods

2.1. Animals

Subjects were male C57BL6/CNr mice (Charles River Laboratories, Sultzfield, Germany). The local Animal Ethics Committee in Gothenburg (308-2012; 41-2015) approved all animal experiments and mice were housed at Experimental Biomedicine (EBM), Sahlgrenska Academy, University of Gothenburg. Mice were kept under standard conditions of temperature (20 °C), and relative humidity (45%) and on an artificial light–dark cycle of 12 h (lights on at 06:00). Food and water were available ad libitum.

2.2. Experimental design

Two groups of male mice were subjected to neonatal HI injury and intranasally treated for 3 days with either C3a (HI-C3a, $n = 18$) or PBS (HI-PBS, $n = 18$). A control group of sham-operated animals was treated with PBS (SHAM-PBS, $n = 19$). These mice were tested in an open field and object recognition test at P50–P54 and brains were collected at P55 (Fig. 1A).

2.3. HI injury induction

Neonatal HI injury was induced on postnatal day 9 (P9), as previously described with modifications for mice (Hedtjärn et al., 2002; Järlestedt et al., 2013; Rice et al., 1981; Sheldon et al., 1998). Mice were anesthetized with 3.5% isoflurane (Baxter Medical, Kista, Sweden) for induction and 1.5% thereafter, in 1:1 oxygen and nitrous oxide. The left common carotid artery was dissected and permanently ligated with a prolene suture. The incision was closed and infiltrated with lidocaine (Xylocain®, Astra Zeneca, Gothenburg, Sweden). Mice were returned to the dam for 1 h and then placed in a chamber with humidified air at 36 °C for 10 min, then exposed to humidified 10% oxygen in nitrogen for 30 min at 36 °C, and then kept in humidified air at 36 °C for 10 min before being returned to the dam. Sham animals were subjected to an incision in the neck on P9. These pups were also removed from the dam for the time duration that injured animals stayed in the chamber, but remained instead in a warming tray at 36 °C under normal oxygen conditions. At postnatal day 21 (P21) mice were weaned and group housed with same sex littermates. Given that this moderate HI injury affecting ipsilesional hippocampus and amygdala leads to persistent cognitive impairment without any apparent deficit motor function (Järlestedt et al., 2013), this model is particularly suitable for selective assessment of the effect of therapeutic interventions on learning and memory.

2.4. Intranasal C3a administration

Purified human C3a (Complement Technology Inc., Tyler, TX, USA) was diluted in sterile phosphate buffered saline (PBS) to a concentration of 200 nM, and a total of 8 μl, i.e. 1.6 pmol (4 μl/nostril; corresponding to ca. 2.56 μg/kg body weight) of peptide solution or PBS was given intranasally to awake and hand-restrained mice held in a supine position. Solutions were administered through a pipette tip, drop-wise in 2 μl-portions divided by 1 min intervals to allow for absorption. This method of administration to one nostril at a time does not affect breathing. C3a or PBS was given every 24 h for three days starting 1 h after HI induction, i.e. between P9 and P11, respectively. Mice in each litter were randomly assigned to C3a or PBS treatment. Sham animals received PBS. The investigators carrying out behavioral studies and analyzing data were blinded to treatment group.

2.5. Behavioral analysis

2.5.1. Object recognition test

The object recognition test (ORT) is based on the innate preference of mice to explore a novel object rather than a familiar one. Therefore, animals that remember the familiar object will spend more time exploring the novel object (Leger et al., 2013). From 3 days before starting the test, mice were daily handled for 2 min to minimize the possible stress due the researcher interaction. All experiments were performed at the same time of the day, between 9 am and 5 pm, and inside of a plastic box of 50 × 50 × 50 cm dimensions where the light intensity was dim and equal in all parts of the apparatus. Before being placed in the arena, mice had a 60 min habituation period in the behavioral room. Prior to the familiarization session, during which two identical objects were introduced, mice were habituated to the apparatus for 10 min during 3 consecutive days. Activity on the first habituation day was recorded as an open field test to assess locomotor and exploratory activities. During the familiarization session, two identical 250 ml bottles filled with shredded paper were placed at an equal distance from the arena walls (approximately 5 cm). Between mice, the apparatus and objects were cleaned with 50% ethanol to minimize olfactory cues. Animals were allowed to explore both objects for 10 min. A minimal exploration criterion of 20 s in total for both objects was used (Leger et al., 2013). Exploration was defined as directing the nose to the object at a distance <2 cm and/or touching the object with the nose or forepaws (Becerril-Ortega et al., 2014). Six hours after the familiarization session, intersession interval (ISI) = 6 h, animals were tested for short-term memory (STM). This involved replacing one familiar object with a novel object (T75 culture flask filled with sand) placed in the same position. Long-term memory (LTM) was tested 24 h after the familiarization (ISI = 24 h) by replacing the novel object used during the STM testing with another novel object (Lego tower). Animals were allowed to explore both objects for 10 min with a minimal exploration criterion of 20 s for both objects, during the STM and LTM testing. Animals that did not reach the 20 s criterion were excluded from the experiment. Mice were tracked by Viewer^3 video tracking system (Biobserve, Bonn, Germany), and the data were presented as time spent exploring individual objects and total exploration time (s).