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Short communication

Complement C3a receptor modulates embryonic neural progenitor cell proliferation and cognitive performance

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ABSTRACT

The complement system of innate immunity is emerging as a novel player in neurodevelopmental processes. The receptor for C3a, C3aR, shares a close evolutionary and functional relationship with C5a receptors. Whilst the C5a receptor, C5aR1, has been demonstrated to promote embryonic neural stem cell proliferation, little is known about the role of C3aR in this process. Here we show that C3aR is expressed in a similar manner to C5aR1 in mice, at the apical pole of the embryonic ventricular zone, though it has an opposing function. Using *in utero* delivery of C3aR agonist and antagonist compounds to the embryonic ventricle, we demonstrate that C3aR functions to decrease proliferation of apical neural progenitor cells (NPC). Intriguingly, C3aR$^{-/-}$ animals also have altered NPC proliferation, but demonstrate an opposing phenotype to animals subjected to pharmacological blockade of C3aR. Finally, despite a grossly normal development of C3aR$^{-/-}$ animals, cognitive behavioural testing of adult mice showed subtle deficits in recall memory. These data demonstrate that in addition to C5a, C3a also has a critical role in the normal development of the mammalian brain.

1. Introduction

The complement activation fragment, C3a, acting through its primary receptor, C3aR, is a potent conductor of inflammation and innate immunity (Coulthard and Woodruff, 2015). Recent work has shown that in addition to traditionally ascribed immune functions, complement and C3aR can act in a number of systems to drive non-immune processes. In particular, there has been strong evidence of a pivotal role for complement in the development of the central nervous system (CNS) (Hawksworth et al., 2017).

C3aR is expressed within the adult CNS, on both neurons and glia, and is a key player in the response to neuropathology (Beek, 2000; Davoust et al., 1999; Gasque et al., 1998). Interestingly, given the traditional role of this receptor in the immune response, expression of C3aR in neural progenitor cells (NPC) of the adult brain has also been reported. In culture, NPCs derived from adult brains show altered signalling to SDF-1α in the presence of C3a that lead to a modulation of migratory activity, perhaps suggesting a role in the stem cell response to neuronal injury (Shinjyo et al., 2009). This is supported *in vivo*, by studies demonstrating that interruption of C3aR signalling also results in reduced adult basal neurogenesis, and worsened outcomes after ischaemic insult (Rahpeymai et al., 2006). Consequently, there is interest in the therapeutic potential of C3aR agonists in stimulating neurogenesis after ischaemic injury (Järlestedt et al., 2013; Morán et al., 2017; Stokowska et al., 2017).

Disordered cortical layering in both the neocortex and cerebellum resulting from interruption of C3a-C3aR signalling has been observed during development (Bénard et al., 2008; Gorelik et al., 2017). We have also previously shown expression of C3aR in mouse embryos throughout the early neurulation period (Jeanes et al., 2015). This expression is strikingly similar to expression of C5aR1, a close relation of C3aR. C5aR1 is also expressed at later development ages, at the apical pole of the ventricular zone and, in this context, is a regulator of progenitor proliferation in a polarity-dependent manner (Coulthard et al., 2017).

Despite these previous reports there has been no study of the

Abbreviations: C3ar, complement factor 3a receptor; BrdU, bromodeoxyuridine; CNS, central nervous system; NPC, neural progenitor cell; E(n), embryonic day (n); MWM, Morris water maze

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expression and potential function of C3aR on embryonic NPCs. Thus, here we investigated C3aR expression and function in the developing ventricular zone, and the effect of C3aR genetic deletion on adult cognitive function. We demonstrate that endogenous C3a-C3aR signalling plays a crucial role in inhibiting NPC proliferation during neurodevelopment, and knockout of C3aR results in impaired memory recall in adult mice.

2. Methods

2.1. Animal use

Wild-type and C3aR knockout ($C3aR^{-/-}$) animals (Kildsgaard et al., 2000) on a C57BL/6J congenic background were housed in the University of Queensland Biological Resources SPF facility. Animal experiments were conducted with prior approval from the University of Queensland Animal Ethics Committee. Time-mated embryos were generated within the animal facility and presence of a vaginal plug post-mating was ascribed embryonic day (E) 0.5.

2.2. Neurosphere culture

Neurospheres were cultured as previously described (Coulthard et al., 2017). Briefly, the neocortex of E14.5 embryos was dissected using sterile technique in Hanks balanced salt solution (HBSS; Sigma Aldrich, USA). Trypsin-EDTA solution (Sigma Aldrich, USA) supplemented with 2 kU Bovine pancreas DNase (Sigma Aldrich, USA) was used to dissociate the tissue, and a single cell suspension was achieved by passing the dissociate through a 40 µm cell filter (Biologix, USA). Cells were cultured to form neurospheres for one week in DMEM/F12 (Sigma Aldrich, USA), 1x B27 supplement (Life Technologies, USA), 1x Penicillin/Streptomycin (Sigma Aldrich, USA), 20 ng/mL FGFb (Millipore, Germany) and 20 ng/mL EGF (Millipore, Germany) at 37 °C, 5% CO2. Passaging of neurospheres was achieved with Trypsin-EDTA/DNase solution after centrifugation.

2.3. Immunofluorescence

Tissues and neurosphere cultures used for immunofluorescence were fixed in 4% paraformaldehyde (4% PFA) before the generation of coronal cryostat sections at 12 µm. Sections were blocked in 5% goat serum/0.1% Triton X-100 and incubated with primary antibodies (Neurosphere cultures; 1:250 Chicken anti-mouse C3aR BMA Biomedical, Switzerland; 1:300 Rabbit anti-mouse CD133, Abcam, UK. Tissue sections; 1:250 Chicken anti-mouse C3aR BMA Biomedicals, Switzerland, 1:500 Rat anti-mouse C3aR Hycult Biotech, or 1:500 Rabbit anti-mouse Pax6 Cell Signalling) overnight at 4 °C. Sections were probed with a complementary secondary antibody (1:1000, AlexaFluor secondary antibodies, Life Technologies, USA). Nuclei were stained with 1:25,000 dilution of 4′,6-diamidino-2-phenylindole (DAPI) before mounting and imaging using an Olympus BX51 confocal microscopy system or Diskovery Spinning Disk confocal microscopy system.

2.4. PCR

RNA was extracted from cultured cells using a RNeasy kit (QIAGEN, The Netherlands) according to the manufacturer's instructions. RNA was treated with DNase-I (New England Biolabs, USA) to removed genomic DNA contamination and 1 µg RNA reverse transcribed with random hexamer priming (Tecto kit, Bioline, UK). Primers for C3ar1 (F –CCCCCAGCCTCTTCTTTATC; R – AGCCTAAGGCCCTTCTCTTG), C3 (F –ATGCTGATGCTGGATGCTAGGCTGA; R – TAGGCTGATCGGATGCTG AGCTAGCT) and ActB (F – GTGGGCCGCCCTAGGCACCAG; R – CTCT TTGATGTCACGCACGATTTC) were used to amplify transcript using MyTaq Redmix (Bioline, UK). cDNA from the immortalised microglial cell line, BV-2, was used as positive control. PCR products were visualized under UV-light, post-agarose gel electrophoresis and staining in 0.5 µg/mL EtBr solution.

2.5. In utero injections

E13.5 embryos from 6 dams were subjected to surgical delivery of C3aR modulators as previously described (Coulthard et al., 2017). 1 µL of a selective C3aR agonist (WWGKKYRASLGLAR, 100 nM dissolved in dH_2O; Ember et al., 1991; Wu et al., 2013; Wuxi Peptides, China; n = 12); 1 u L of a C3aR antagonist (SB290157, 10 µM dissolved in dH_2O/0.05% DMSO/0.95% ethanol, Calbiochem, USA; n = 8), or appropriate vehicle controls (n = 11 and 8, respectively), and supplemented with DNA loading buffer (New England Biolabs, USA) and delivered to the embryonic ventricle. The DNA loading buffer was used to confirm correct delivery to the ventricular system.

2.6. BrdU assay

BrdU (3 µg/mL) and DMEM/F12 (vehicle control) or 10 nM C3aR agonist, was added to neurospheres three days post-plating (1000/cells per well in 200 µL media, n = 6 per group). Cells were incubated for 48 h before collection. Proliferation was assessed using a BrdU proliferation kit (EMD Millipore, Germany) according to manufacturer's instructions. BrdU incorporation relative to control wells were assayed through the use of a spectrophotometer. Statistical significance was determined using Student's *t*-test (Prism 7 software, Graphpad, USA).

2.7. Determining M-phase cell density at E14.5

M-phase cell density in the embryonic ventricle was assessed as previously described (Coulthard et al., 2017). Sections were stained with anti-phosphohistone H3 antibody (1:1000 CST, USA) and probed with AlexaFluor anti-mouse 555 (Molecular Probes, USA). PHH3 positive cells were counted and grouped as mean positive cells per 100 µm. Significance was determined by one-way ANOVA with Dunnett's post-test, Prism 7 software (GraphPad, USA).

2.8. Behavioural testing

Adult wild type (wt) (n = 9) and $C3aR^{-/-}$ male (n = 8) mice at approximately 6 months of age were assessed in behavioural testing. For the Morris water maze, a 105 cm radius tank with sides of 51 cm was filled with ambient temperature water supplemented with opacifier 631 (Morton SA, France). A platform of 5 cm radius was placed 1 cm beneath the water surface in the centre of the North-East quadrant. Distal visual cues, temperature and ambient light were constant throughout the training and test periods. Mice were released into the area at one of the North, South, East and West points of the maze in a sequence randomised throughout the five days of the training protocol and four trials per day. Each animal was tracked in the arena using Ethovision software (Noldus, The Netherlands) for a period of five minutes or until the platform was reached. Parameters assessed were distance moved and time to reach platform. After five days of training, mice were subjected to a test of memory on the seventh day by removing the platform from the arena and tracking passages through the platform zone. Mice were released from the South release point and tracked for 5 min within the arena. Data was assessed using Grubbs' test for outliers and Student's *t*-test between treatment groups with Holm-Sidak correction for multiple comparisons (Prism 7 software, GraphPad, USA). For the passive avoidance test, a light/dark chamber with separating trapdoor was used (Gemini Avoidance System, USA). For the training phase animals were acclimatised for 15 s in the light compartment before the dividing door opened. Upon passage to the dark compartment, mice received an inescapable single electric shock of 0.5 mA/1 s. The latency to enter the dark compartment was recorded. The memory-retention test, recording time to passage to the dark compartment, was