Neuroscience Letters 505 (2011) 140–145

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Positive allosteric modulation of metabotropic glutamate receptor 5 down-regulates fibrinogen-activated microglia providing neuronal protection

Thomas M. Piers[a], Simon J. Heales[b], Jennifer M. Pocock[a,*]

[a] Cell Signalling Laboratory, Department of Neuroinflammation, Institute of Neurology, University College London, London WC1N 1PJ, UK
[b] Molecular and Genetics Unit, Institute of Child Health, University College London, London WC1N 1EH, UK

ARTICLE INFO

Article history:
Received 8 September 2011
Received in revised form
30 September 2011
Accepted 3 October 2011

Keywords:
Fibrinogen
mGluR5
Microglia
Neurotoxicity

ABSTRACT

Microglial activation and blood brain barrier dysfunction are significant hallmarks in an array of neurodegenerative disorders. A leaky blood brain barrier potentially allows infiltration of blood-borne proteins into the CNS parenchyma, and previous studies have shown that the blood borne protein fibrinogen (FG) can activate microglia to produce a neurotoxic phenotype. Here we show that FG-mediated neurotoxicity and ERK1/2 phosphorylation in neuronal cultures is significantly attenuated by activation of metabotropic glutamate receptor 5 (mGluR5) but not mGluR2. Furthermore, FG-mediated microglial activation was down-regulated by direct mGluR5 activation on these cells but not by mGluR2, suggesting that targeting microglial mGluR5 provides neuronal protection against blood protein-triggered innate inflammatory responses.

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1. Introduction

Microglia activation and blood brain barrier (BBB) dysfunction are now widely considered as prominent hallmarks of a vast array of neurodegenerative disorders (NDDs) [4,32]. Infiltration due to BBB dysfunction of the blood borne protein, fibrinogen (FG), is associated with microglial activation and cognitive impairment in animal models as well as in the human form of Alzheimer's disease (AD) [14,24]. FG interacts with microglia *in vitro*, inducing a phagocytic phenotype [1,26], and promoting pro-inflammatory cytokine release from mononuclear cells [15,25,26]. Taken together these data suggest a possible role for microglia in FG-mediated cognitive decline.

MGluR5 activation can reduce β-amyloid-induced cell death in primary neuronal cultures [23], suggesting a role for this receptor in neuronal integrity and neuroprotection. Furthermore, modulation of mGluR5 can down-regulate the activated microglial phenotype [6,19]. Activation of mGluR2 on microglia, however, promotes neurotoxicity and may underlie other activation cascades in microglia [29,30]. Therefore, modulation of mGluR5 was investigated as a possible protective strategy to control FG-mediated neurotoxicity coupled with mGluR2 investigations used as negative, neurotoxic controls.

2. Materials and methods

2.1. Animals and materials

Sprague Dawley rats were bred and reared in house from stock animals from Charles River UK Ltd. (Kent, UK). Culture components were from Invitrogen (Paisley, UK). Fibrinogen was from Sigma (Dorset, UK) and verified endotoxin-free. CDPPB was from Merck Chemicals (Darmstadt, Germany). Other mGluR agonists and antagonists were from Tocris Bioscience (Bristol, UK). FAM-DEVD-FMK and anti-NeuN was from Millipore (Watford, UK). TNFα ELISA systems were from R&D systems (Oxon, UK). Anti-iNOS was from BD Biosciences (Oxford, UK), anti-ED-1 from AbD Serotec (Oxford, UK), anti-phospho-ERK1/2, anti-total ERK1/2, and anti-cleaved Caspase-3 from Cell Signalling Labs (Herts, UK), anti-TNFα from Santa Cruz (Heidelberg, Germany), and anti-β-actin and all other reagents from Sigma (Dorset, UK).

Abbreviations: BBB, blood brain barrier; CDPPB, 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide; CGC, cerebellar granule cells; CNS, central nervous system; DCGIV, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine; ERK1/2, extracellular signal-regulated kinase1/2; FBS, foetal bovine serum; FG, fibrinogen; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; LME, leucine-methyl-ester; LPS, lipopolysaccharide; MEM, minimum essential medium with Earle's salts; mGluR, metabotropic glutamate receptor; MGCM, microglial-conditioned-medium; MTEP, 3-((2-methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride; NAAG, N-acetylaspartylglutamate; NDDs, neurodegenerative disorders; PFA, paraformaldehyde; PI, propidium iodide; PMX, polymyxin B; STS, staurosporine; TNFα, tumour necrosis factor-α.

* Corresponding author at: Cell Signalling Lab, Department of Neuroinflammation, UCL Institute of Neurology, 1 Wakefield St, London WC1N 1PJ, UK.
Tel.: +44 20 7679 4031; fax: +44 20 7278 6572.
E-mail address: j.pocock@ucl.ac.uk (J.M. Pocock).

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doi:10.1016/j.neulet.2011.10.007

2.2. Cell culture preparation and treatment

Primary cultured rat microglia and cerebellar granule cell (CGCs) cultures were isolated and cultured from 5-day-old Sprague Dawley rats essentially as previously described [13,17], in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986. Where indicated in figure legend, microglia or CGCs were treated directly with mGluR agonists and antagonists (100 nM–100 μM), FG (1 mg/mL) or LPS (1 μg/mL) for 24 h, or with staurosporine (0.5 μM) for 8 h. Where indicated, polymyxin B (PMX; 100 nM) was added to MG cultures 1 h prior to main treatment to inhibit any possible contaminating endotoxin. Alternatively, and where indicated in figure legend, primary microglia were treated directly as above and then microglial-conditioned medium (MGCM) was collected and subsequently added at a 1:1 ratio with CGC medium to CGCs previously depleted of microglia, and incubated for a further 24 h before analysis.

2.3. Specific depletion of microglia from neuronal cultures

Microglia present in CGC cultures were removed by treatment with 25 mM leucine-methyl-ester (LME), as previously described [12,22,26]. Briefly, cells were exposed to LME for 1 h followed by washing, replacement of original medium, and resting for 24 h before further treatment. Control cultures were identically treated except for the omission of LME. Cultures where microglia were still present were denoted (+) and cultures depleted of microglia were denoted (−).

2.4. Assessment of apoptotic morphology

Treated CGCs were fixed in 4% paraformaldehyde (PFA) and incubated for 20 min with 5 μg/mL 2′-[epoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazol Hoechst 33342 (Hoechst 33342) as previously described [27]. Apoptotic cells (with brightly stained pyknotic nuclei) or healthy cells (with nuclei staining less bright and less condensed) were observed with a Zeiss Axioskop 2 fluorescence microscope plus 40× Neofluar objective (Zeiss, Oberkochen, Germany). Cell counts were performed on at least three fields per coverslip, three coverslips per treatment from three independent experiments.

2.5. Lactate dehydrogenase assay

Neuronal culture viability after MGCM exposure was analysed by lactate dehydrogenase (LDH) release assay as previously described [10]. The percentage of LDH released into culture medium was calculated from three separate preparations as follows: LDH activity in medium/total LDH in medium after cell lysis with Triton-X100.

2.6. Active caspase-3/7 live cell staining

Treated primary microglia were assessed for caspase-3/7 induction by incubation at 37 °C for 1 h with the specific fluorescently tagged peptide FAM-DEVD-FMK (1 μg/mL). Coverslips were mounted in basic medium (153 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM TES, 5 mM $NaHCO_3$, 1.2 mM Na_2SO_4, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5 mM glucose) and observed immediately by fluorescence microscopy. Cultures were counterstained with Hoechst and propidium iodide (PI; 5 μg/mL) for assessment of death as previously described [9].

2.7. Immunocytochemistry

Cells were fixed with 4% PFA, permeabilized with 100% methanol, or 0.1% Triton-X100, and non-specific binding blocked with PBS containing 1% BSA or 4% NGS and assessed by standard antibody isotype controls. Cultures were incubated overnight at 4 °C with primary antibodies; anti-iNOS (1:500) and anti-ED-1 (1:250), followed by incubation at room temperature with appropriate secondary antibodies for 2 h. Finally cells were incubated with DAPI, mounted with Vectashield, and visualized by fluorescence microscopy.

2.8. Western blot analysis

Proteins were resolved using standard techniques. Primary antibodies, incubated overnight at 4 °C, were anti-phospho-ERK1/2 (1:1000), anti-total ERK1/2 (1:1000), anti-TNFα (1:500), anti-cleaved Caspase-3 (1:1000) or anti-β-actin (1:2000), followed by incubation with appropriate secondary antibodies at room temperature for 1 h and visualization by ECL. Immuno-blots shown are representative of three independent experiments.

2.9. Statistical analysis

To directly compare two treatments, two-tailed paired Student's *t*-tests were performed. P values < 0.05 were considered statistically significant, and $^{\#}P < 0.05$, $^{\#\#}P < 0.005$, $^{\#\#\#}P < 0.001$. To compare two or more treatments with a control group a one-way analysis of variance (ANOVA) was used followed by a Dunnett's post test. P values < 0.05 were considered statistically significant, and $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$. Experiments were performed at least three times, independently. Unless otherwise indicated by connecting lines, statistical comparisons were made with non-treated controls in all figures.

3. Results

3.1. mGluR5 modulation inhibits FG-induced neuronal toxicity and ERK signalling

Consistent with previous findings, the positive activator of apoptosis staurosporine induced significant apoptotic morphology in CGCs (Fig. 1Ai). Furthermore, as previously described [30], treatment with the group II (mGluR2 and 3) mGluR agonist, DCGIV, at previously published concentrations [29,30] induced significant apoptosis in neuronal cultures whilst the specific mGluR3 agonist NAAG, at concentrations previously administered [30], did not induce significant toxicity (Fig. 1Ai). FG induced significant apoptotic morphology in CGCs, which was not attenuated by pre-treatment DCGIV but was by co-treatment with NAAG. Furthermore FG-induced apoptosis was not attenuated by pre-treatment with group I (mGluR1 and 5) agonist DHPG, at concentrations previously described (100 μM) [20]. Conversely, co-treatment with the group I mGluR5 positive allosteric modulator CDPPB, using an EC_{50} concentration (100 nM) calculated for this receptor in rat [28], significantly attenuated FG-mediated apoptosis (Fig. 1Ai). Apoptotic nuclei in FG-treated cultures were associated with the neuronal marker NeuN (Fig. 1Aii). FG treatment of CGCs significantly induced cleavage of executioner caspase-3, which was dependent on the presence of microglia in the cultures. DHPG and CDPPB treatment of CGCs significantly inhibited FG-induced caspase-3 cleavage, irrespective of the presence of microglia (Fig. 1B). Furthermore, immuno-localisation suggested cleaved caspase-3 expression was neuronal (Fig. 1Biii). FG treated CGCs expressed significant ERK1/2 phosphorylation to levels similar to those in DCGIV-treated cultures (Fig. 1Ci and Cii), and independent of microglia in CGC

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