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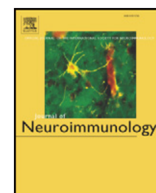

Proteinase-activated receptor 2 is involved in the behavioural changes associated with sickness behaviour

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ABSTRACT

Proteinase-activated receptor-2 (PAR2) is widely expressed in the CNS but whether it plays a key role in inflammation-related behavioural changes remains unknown. Hence, in the present study we have examined whether PAR2 contributes to behaviour associated with systemic inflammation using PAR2 transgenic mice. The onset of sickness behaviour was delayed and the recovery accelerated in $PAR2^{-/-}$ mice in the LPS-induced model of sickness behaviour. In contrast, PAR2 does not contribute to behaviour under normal conditions. In conclusion, these data suggest that PAR2 does not contribute to behaviour in the normal healthy brain but it plays a role in inflammation-related behavioural changes.

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1. Introduction

Proteinase-activated receptors (PARs) are a novel class of GPCRs that are unique in their activation, whereby the cleavage of the N-terminus by a serine proteinase unveils a sequence that acts as a "tethered-ligand". The "tethered-ligand" binds to the second extracellular loop of the receptor, leading to the activation of the receptor. To date, four members of the PAR family have been cloned, namely PAR 1–4. Of these, PAR1, 3 & 4 are preferentially activated by thrombin, whereas trypsin and trypsin-like proteinases are proposed to preferentially activate PAR2 (Macfarlane et al., 2001; Adams et al., 2011; Ramachandran et al., 2012), although within the central nervous system (CNS), the endogenous activators for PARs remain speculative. Several selective PAR-activating peptides have been developed to probe the distinct functions of each receptor, although evidence suggests diligence is required when using such agonists and their use for CNS investigations in vivo is limited due to poor bioavailability (Ramachandran et al., 2012). To overcome these issues, novel non-peptidic agonists, including AC-264613 and GB110, with high potency and good stability have been developed (Gardell et al., 2008; Barry et al., 2010).

Despite PAR2 being expressed in neurones, astrocytes, microglia and oligodendrocytes within both the human and rodent CNS (Noorbakhsh et al., 2003; Bushell, 2007), there remains a large void in our knowledge as to the functional role of PAR2 in the brain. Evidence from both human and experimental models has implicated PAR2 in CNS disorders including Alzheimer's disease (AD), HIV dementia, multiple sclerosis and stroke (Jin et al., 2005; Noorbakhsh et al., 2005; 2006). However, much of this is indirect in the form of observed alterations in PAR2 expression rather than evidence of an active role in disease pathogenesis per se. Indeed, data suggest that PAR2 activation can be protective or pro-degenerative depending on the cell type (neurones or astrocytes) where increased expression is observed (Bushell, 2007; Jin et al., 2005; Noorbakhsh et al., 2005; 2006). Recent studies, including those from our laboratory, have provided direct functional evidence that PAR2 activation is neuroprotective, an effect mediated indirectly via astrocytic activation, chemokine release and inhibition of MAPK signalling (Wang et al., 2007; Greenwood and Bushell, 2010). Furthermore, we and others have previously shown in primary hippocampal cultures that PAR2 activation evokes physiological elevations in intracellular calcium (Ca^{2+}) through the G_q/PLC pathway (Wang et al., 2002; Bushell et al., 2006) and we were recently the first to report that PAR2 activation modulates hippocampal neuronal excitability and synaptic transmission in vitro (Gan et al., 2011). Interestingly, despite the presence of PAR2 on neuronal populations, this modulation of neuronal excitability and synaptic transmission appears indirect and mediated via astrocytic activation. This mirrors the neuroprotective role of PAR2 which is also primarily mediated via astrocytic activation.

Abbreviations: EPM, elevated plus maze; LPS, lipopolysaccharide; MWM, Morris water maze; OFT, open field test; PAR2, proteinase-activated receptor-2.

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However, despite PAR2 activation modulating synaptic activity and being neuroprotective in in vitro preparations, whether PAR2 plays a key role in behaviour examined in the normal brain under healthy conditions or under conditions favourable for its activation remains unknown. Hence, in the present study, we have utilised *F2RL1* genetically modified mice to examine the contribution of PAR2 to inflammation-related behavioural changes and to locomotor activity, anxiety- and anhedonic-like behaviour and spatial reference memory under normal conditions. Our novel findings indicate that PAR2 contributes to inflammation-related changes in behaviour and that the role of PAR2 in inflammation-related CNS disorders should be examined further to fully elucidate its therapeutic potential.

2. Materials and methods

2.1. Animals

F2RL1 genetically modified mice (PAR2$^{+/+}$, PAR2$^{+/-}$ and PAR2$^{-/-}$), which are bred on a C57BL/6J background, were obtained from multiple crossings of 14 pairs of PAR2$^{+/-}$ mice which were supplied by Professor R. Plevin, University of Strathclyde (Ferrell et al., 2003). C57BL/6J mice used for vehicle control experiments were obtained from in house colonies from the Biological Procedures Unit, University of Strathclyde. All mice were 12 weeks old at the commencement of behavioural testing. They were housed at 21 ± 2 °C and 45–55% humidity, with a 12/12 h light/dark cycle (lights on at 0600, off at 1800). Mice were group-housed according to genotype (housed 3–10 per cage depending on availability but testing was performed on a minimum of 6 mice per genotype per run) except where required to be singly housed for the purposes of the experiment, provided with environmental enrichment in the form of plastic huts and nesting material, and given ad libitum food and water. Procedures were in compliance with the requirements of the UK Animals (Scientific Procedures) Act 1986. In all experiments, mice were handled on the day prior to the beginning of testing to habituate the animals to the tester and all data is generated from repeated experiments of at least two cohorts of mice.

2.2. Behavioural testing

Testing in the open field test (OFT), elevated plus-maze (EPM) and the Morris water maze (MWM) was carried out on 48 males (26.0 ± 0.4 g; n = 16 for all 3 genotypes), during the light cycle.

2.2.1. Open field test

Mice were placed in the centre of an open field 40 × 40 × 40 cm (lighting 45 lx) made from black infrared light (IR)-translucent Perspex placed on an in IR light box (Tracksys, Nottingham, UK). Total distance moved and entries into and time spent in a 14 × 14 cm centre square were recorded for 10 min by tracking software (Ethovision, Noldus, Netherlands).

2.2.2. Elevated plus-maze

Mice were placed in the centre (45 lx) of a plus-shaped maze with two open (30 × 5 cm, 60 lx) and two closed arms (30 × 5 cm, 15 cm walls, 6 lx) made of IR-translucent Perspex with integral IR light sources elevated 70 cm from the floor (Tracksys, Nottingham, UK). Entries into each type of arm were recorded for 10 min by Ethovision software (Noldus, Netherlands) and the total number of entries, % open arm entries and % open arm time calculated.

2.2.3. Morris water maze

Mice were tested in a 98 cm diameter maze containing water at 21 °C with a transparent 10 cm diameter submerged platform, in a room (45 lx) with extra-maze cues. 3–4 times daily for 5 days, mice were released at one of 4 randomly varied points, and swam until they located the platform. Platform location remained constant for each mouse. On the final trial of day 5, the platform was removed and mice allowed to swim for 60 s (probe test). Time spent in the quadrant of the previous location of the platform (target quadrant) and the opposite quadrant was recorded.

2.2.4. Sucrose preference test

Prior to the test, each mouse was singly housed and each cage was supplied with two bottles of tap water. The amount of water drunk was measured daily by weight for two consecutive days in order to determine which bottle position, either left or right, was preferred. The sucrose preference test was started by replacing the water bottle in the non-preferred position with an identical bottle containing 1% sucrose solution. On subsequent days the position of the two bottles was randomly determined to avoid a place preference. The amount of water and sucrose consumed was measured daily over the whole experimental period and the % sucrose drunk calculated. Daily food intake and body weight was also measured over the whole period.

2.2.5. LPS-induced sickness behaviour

LPS-induced sickness behaviour was investigated in 39 male mice (27.7 ± 0.6g; PAR2$^{+/+}$ n = 12, PAR2$^{+/-}$ n = 11, PAR2$^{-/-}$ n = 9, vehicle controls n = 7). On day 1, mice were handled and on day 2 they were singly housed with two water bottles. Sucrose preference testing (SPT) was then carried out over days 4–7. On day 8, baseline parameters of the OFT, SPT, food intake and body weight were measured. On day 9, mice were injected with either LPS (1 mg kg^{-1} in PBS; extracted from *S. Enteritidis*, Sigma-Aldrich, UK, Cat No. L6011) or PBS alone (vehicle) and OFT, SPT, food intake and body weight parameters measured at baseline prior to injection and 0, 2, 24, 48 and 72 h post LPS injection. Baseline measurements were taken in the morning at 10.00.

2.3. Quantitative RT-PCR

2.3.1. LPS injection

C57BL/6J male mice (10–12 weeks old) were given a single injection of either LPS (1 mg kg^{-1} in PBS) or PBS alone and the cerebellum, hippocampus and hypothalamus collected either 2 hours (PBS n = 5, LPS n = 4) or 24 hours (n = 5 per group) post injection and stored in RNAlater tissue storage solution (Life Technologies, Paisley, UK) at <4 °C until processing.

2.3.2. RNA isolation and reverse transcription

Total RNA was isolated from rodent brain regions using the RNeasy Lipid Tissue Mini Kit (QIAGEN Ltd., Manchester, UK) standard protocol, then DNased using the TURBO DNA-free kit (Life Technologies Ltd., Paisley, UK). 500 ng RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega UK, Southampton, UK) with random primers (0.5 mg; 6.0 mM $MgCl_2$) in a 20 μL reaction volume, which was subsequently diluted to 100 μL with water to provide cDNA template for realtime PCR. Negative water blank and -RT controls were used throughout.

2.3.3. Real-time PCR

Cycle threshold (C_t) values were generated on an ABI 7900 HT Prism Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the Universal ProbeLibrary (UPL) System (Roche Applied Science) and ABsolute QPCR ROX Mix (Abgene). qRT-PCR results were analysed using the relative quantification method of comparative Ct (ΔΔCt), with β-Actin acting as the calibrator or 'housekeeping' gene for mouse and rat samples and GAPDH for human samples. Expression of the calibrator genes was stable and did not differ significantly between control and treatment groups (data not shown). All assays were designed using the online ProbeFinder software (lifescience.roche.com) to generate primer sequences (Table 1), except for mouse β-actin, which used the commercially-available Universal Probe Library Mouse ACTB Gene Assay (Roche). Each reaction contained 100 nM of the relevant

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