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

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

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.

Keywords: Proteinase-activated receptor-2; LPS; sickness behaviour; locomotor activity; anxiety; sucrose preference.

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, 2011). 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.

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 ºC and 45-55% humidity, with a 12/12 hours 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 ± 0.4g; 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 x 40 x 40cm (lighting 45 lux) 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 x 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 lux) of a plus-shaped maze with two open (30 x 5cm, 60 lux) and two closed arms (30 x 5cm, 15 cm walls, 6 lux) made of IR-translucent Perspex with integral IR light sources elevated 70cm 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 21ºC with a transparent 10 cm diameter submerged platform, in a room (45 lux) 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 60s (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 72h 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^{o}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). 500ng 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 20µL reaction volume, which was subsequently diluted to 100µL 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 100nM of the relevant Universal Probe, 400nM of each primer, 2µl diluted cDNA and 5.0µl ABsolute QPCR ROX Mix (VWR International, Lutterworth, UK) in a final volume of 10µl. Reactions were run in triplicate, with the mean Ct value from the three reactions used for subsequent data analysis.

2.4. Statistics

All data are expressed as mean ± S.E.M. Data were compared by paired or unpaired Student's t-tests, one-way analysis of variance with Tukey's comparison or 2-way mixed-model ANOVA followed by Bonferroni *post-hoc* comparisons as appropriate. Differences were considered significant when $P<0.05$.

3. Results

3.1. PAR2 contributes to LPS-induced sickness behaviour

PAR2 is proposed to play a role in several CNS and peripheral diseases whose aetiology and pathology are closely linked to inflammation including Alzheimer's disease (AD), Multiple Sclerosis (MS) and rheumatoid arthritis (Ferrell et al., 2003; Noorbakhsh et al., 2006; Afkhami-Goli et al., 2007). Hence, we used the LPS-induced sickness behaviour model of systemic infection to examine whether PAR2 is involved in a number of the behavioural changes associated with activation of the immune system.

3.1.1. Open field test.

In the OFT, LPS injection (1mg kg^{-1}) resulted in a significant reduction in locomotor activity over the first 24 h after injection compared to vehicle-injected controls (2h post injection, $p<0.001$ for all 3 genotypes vs vehicle control), which returned to levels similar those observed in vehicle-injected controls by 72 h ($p>0.05$ for all 3 genotypes vs vehicle control, Figure 1A). However, $PAR2^{-/-}$ mice showed significantly increased locomotion at 24 h and 48 h post injection compared with $PAR2^{+/+}$ (24 h: $F_{(2,29)}=4.269$, $p=0.0237$, $PAR2^{+/+}$ versus $PAR2^{-/-}$ p=0.0217; 48h: $F_{(2,29)}=3.960$ $p=0.0301$, $PAR2^{+/+}$ versus $PAR2^{-/-}$ p=0.0217, Figure 1A). Furthermore, the number of entries into the centre square at 24 h post injection was increased in $PAR2^{-/-}$ mice when compared with $PAR2^{+/+}$ ($F_{(2,29)}=3.727$, $p=0.0362$, $PAR2^{+/+}$ versus $PAR2^{-/-}$ p=0.0282, Figure 1B). With regard to time spent in the centre square, following LPS injection, no differences were observed between genotypes at any time point investigated.

3.1.2. Sucrose preference test.

Another behavioural characteristic associated with sickness behaviour is the induction of anhedonia. We therefore used the sucrose preference test to investigate whether PAR2 contributes to the induction and maintenance of anhedonia under these experimental conditions. All mice showed a sucrose preference over the 3 days prior to LPS injection with no significant differences observed over the 3 days ($F_{(2,60)}$=0.327, p=0.723) or between genotypes ($F_{(2,30)}$=0.072, p=0.931, Figure 2A). Similarly, no effect of day ($F_{(2,58)}$=0.097, p=0.908) or genotype ($F_{(2,29)}$=1.948, p=0.161, Fig 2B) were seen in the total fluid drunk. These data indicate that PAR2 plays no role in sucrose preference or fluid intake in normal healthy mice and therefore we can use this model to investigate the role of PAR2 in LPS-induced anhedonia.

The effect of LPS injection on the % sucrose drunk was significantly reduced in the first 2 h post injection ($F_{(2,29)}$=3.751, p=0.035, $PAR2^{+/-}$ versus $PAR2^{-/-}$ p=0.035, $PAR2^{+/+}$ versus $PAR2^{-/-}$ p=0.119). Thus, the onset of anhedonia is delayed in the $PAR2^{-/-}$ mice when compared to $PAR2^{+/-}$ mice and shows a trend towards a delay when compared to $PAR2^{+/+}$ mice (Figure 2C).

3.1.3. Food intake and change in body weight.

We also examined whether PAR2 deletion contributes to the changes in food intake and body weight associated with sickness behaviour. No difference in initial body weight and food intake was observed between all three genotypes and the vehicle control group prior to LPS injection (p>0.05 for all 4 groups for both measures, data not shown) whereas food intake was significantly increased in $PAR2^{+/-}$ mice compared to $PAR2^{+/+}$ 48h post injection. At the same time point $PAR2^{-/-}$ mice also showed a trend towards increased food intake. ($F_{(2,29)}$=5.480, p=0.010, $PAR2^{+/-}$ versus $PAR2^{+/+}$ p=0.010, $PAR2^{-/-}$ versus $PAR2^{+/+}$ p=0.086,

Figure 3A). Furthermore, the % change in body weight was significantly less in $PAR2^{-/-}$ mice at both 48h and 72h post injection when compared to $PAR2^{+/+}$ mice (48 h: $F_{(2,29)}$=3.309, p=0.051, $PAR2^{-/-}$ versus $PAR2^{+/+}$ p=0.040; 72 h: $F_{(2,29)}$=3.806, p=0.034, $PAR2^{-/-}$ versus $PAR2^{+/+}$ p=0.027, Figure 3B). The effect of LPS on total fluid intake also differed between genotypes ($F_{(2,29)}$= 9.000, p=0.001). $PAR2^{-/-}$ mice drank significantly more total fluid than $PAR2^{+/+}$ (p=0.004) and $PAR2^{+/-}$ (p=0.001) in the first 2 h post injection (data not shown), which may be part of a more robust response to inflammation.

3.1.4. Habituation

In order to confirm that habituation does not contribute to the observed changes in behaviour, we conducted control experiments using the same time schedule as in the LPS experiment to examine the effect of habituation to repeated testing in the OFT. No significant effect of habituation on the OFT was observed, which indicates that effects seen in LPS-injected mice are those of LPS alone and not habituation.

3.2. PAR2 expression is unaltered under inflammatory CNS conditions.

Having established that PAR2 contributes to LPS-induced sickness behaviour and associated food intake and body weight changes, we examined whether CNS PAR2 expression was altered following LPS injection. PAR2 mRNA was detected in the three brain regions examined, the hypothalamus, hippocampus and the cerebellum obtained from control mice (PBS injection) and mice exposed to a single LPS injection (1mg kg^{-1}). Two hours post-injection, a time point at which behavioral changes were observed, no significant difference in PAR2 mRNA levels was observed in the hypothalamus (PBS ΔC_t=10.1±0.4 (n=5) vs LPS ΔC_t=9.9±0.2 (n=4); p=0.79, Figure 4A), the hippocampus (PBS ΔC_t=8.4±0.2 (n=5) vs LPS ΔC_t=8.3±0.4 (n=4); p=0.91, Figure 4A) or the cerebellum (PBS ΔC_t=9.4±0.2 (n=5) vs LPS ΔC_t=9.4±0.3 (n=4); p=0.91, Figure 4A). In contrast, mRNA levels for the inflammatory

cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α, are significantly increased in all three brain regions (p<0.01 for all regions, Figure 4A). Similarly, no significant difference in PAR2 mRNA levels was observed 24 hours post-injection between control mice and those exposed to a single LPS injection (1mg kg^{-1}) in the hypothalamus (PBS ΔC_t=10.7±0.2 vs. LPS ΔC_t=10.3±0.8; p=0.62, each group n=5, Figure 4B), the hippocampus (PBS ΔC_t=10.8±0.6 vs. LPS ΔC_t=11.1±0.4; p=0.75, each group n=5, Figure 6B) and the cerebellum (PBS ΔC_t=10.5±0.4 vs. LPS ΔC_t=9.5±0.1; p=0.08, each group n=5, Figure 4B). IL-1β (p=0.04) and TNF-α (p=0.03) mRNA levels were significantly increased in the hypothalamus 24 hours post LPS injection as was TNF-α (p=0.02) in the cerebellum but no significant changes were evident in all other samples 24 hours post LPS injection.

3.3. PAR2 deletion does not alter locomotor activity, anxiety-like behaviour or spatial memory.

3.3.1. Open field test.

General locomotor activity as measured by the distance moved over the testing period was unaffected ($F_{(2,45)}$=0.523, p=0.596, Figure 5A) as was the number of entries into the centre square ($F_{(2,45)}$=1.690, p=0.196, Figure 5B). However, $PAR2^{+/-}$ mice spent significantly more time in the centre square compared to both $PAR2^{+/+}$ and $PAR2^{-/-}$ mice ($F_{(2,45)}$=8.718, p=0.0006, $PAR2^{+/-}$ vs. $PAR2^{+/+}$ p=0.005, $PAR2^{+/-}$ vs. $PAR2^{-/-}$ p=0.001, $PAR2^{+/+}$ vs. $PAR2^{-/-}$ p=0.827, Figure 5C) indicating that $PAR2^{+/-}$ mice may be less anxious under these behavioural conditions.

3.3.2. Elevated Plus Maze.

PAR2 deletion did not affect anxiety-like behaviour in the EPM test as no significant differences were observed between genotypes in the % time spent in open arms ($F_{(2,46)}$=

0.261, p=0.771, Figure 5D) , % open arm entries ($F_{(2,47)}$=0.114, p=0.893, Figure 5E) and the number of total entries ($F_{(2,47)}$=0.763, p=0.472, Figure 1F) over the 10 min test period.

3.3.3. Morris water maze.

As we have previously shown that PAR2 activation induces a form of hippocampal synaptic plasticity *in vitro* (Gan et al., 2011), we examined whether PAR2 plays a role in spatial memory formation using the MWM. There was a significant effect of day in both latency to locate platform ($F_{(4,180)}$=50.036, p=0.0005, Figure 6A) and distance travelled to the platform ($F_{(4,180)}$= 50.1, p=0.0005, Figure 6B) over the 5 consecutive testing days. However, there was no significant effect of genotype and no interaction between day and genotype in both latency to locate platform (genotype: $F_{(2,42)}$=0.716, p=0.494,interaction: $F_{(8,180)}$=0.178, p=0.994, Figure 6A) and distance travelled (genotype: $F_{(2,42)}$=0.372, p=0.692, interaction: $F_{(8,180)}$=0.328, p= 0.954, Figure 6B). In addition, during a subsequent probe test mice spent significantly more time in the target quadrant compared to that spent in the opposite quadrant (p<0.001 for all genotypes) but there was no significant difference between genotypes ($F_{(2,44)}$=1.517; p=0.230, Figure 6C). These data indicate that PAR2 deletion has no deleterious effect on spatial reference memory.

4. Discussion

In the present study, we show for the first time that PAR2 contributes to the onset and maintenance of LPS-induced sickness behaviour whereas, in contrast, our data suggests that PAR2 does not contribute to locomotor activity and anhedonic-like behaviour under normal healthy conditions. In addition, we show that under neuroinflammatory conditions within the CNS, no change in PAR2 expression is observed indicating that PAR2 activation *per se* contributes to inflammation-induced changes in behaviour rather than altered expression.

4.1. PAR2 activation participates in the onset and maintenance of sickness behaviour.

PAR2 has been reported to be extensively expressed within the CNS from both rodent and human tissue, with a strong link to inflammation-related diseases (Bushell, 2007; Ramachandran et al., 2012). Hence, we examined the role of PAR2 in behavioural tests in which the conditions for its activation were favourable. It has been suggested that PARs may become activated under inflammatory conditions where the blood brain barrier (BBB) becomes leaky (Gingrich and Traynelis, 2000; Bushell, 2007), hence we utilised a well-established model of systemic infection that is used extensively to examine the role of inflammatory mediators on behaviour, namely LPS-induced sickness behaviour (Dantzer et al., 2008; McCusker and Kelly, 2013). Sickness behaviour can be measured using a number of parameters and we focused on locomotor activity and the induction of anhedonia as well as changes in food intake and body weight. Our data shows that sickness behaviour was induced post LPS injection in all genotypes tested but deficits in its induction and maintenance was apparent in $PAR2^{-/-}$ mice. Indeed in the SPT, the decrease in sucrose preference was delayed in $PAR2^{-/-}$ mice post LPS injection whereas in the OFT, an increased recovery in distance moved and entry into centre squares was observed in $PAR2^{-/-}$ mice post LPS injection. Furthermore, the decrease in body weight was significantly reduced 24 and 72

hours post injection. These data suggest for the first time that PAR2 contributes to the onset and maintenance of LPS-induced sickness behaviour when examined using these characteristic behavioural outcomes associated with sickness behaviour. So how is PAR2 involved? Our understanding of brain-immune interactions has developed significantly since the brain was thought to be an immune privileged site. It is suggested that peripheral infections and the inflammatory cytokines this induces cross the leaky BBB leading to the behavioural symptoms associated with sickness with recent evidence also indicating that these pro-inflammatory cytokines are also synthesised within the brain during systemic infections (Dantzer et al., 2008; Cunningham et al., 2009; Schedlowski et al., 2014). Some of the main protagonists suggested to underlie the observed behavioural changes include the pro-inflammatory cytokines IL-1β, IL-6 and TNFα with numerous studies confirming that these cytokines are increased following systemic infections as well as their direct application to the brain leading to changes in behaviour similar to those seen in sickness behaviour (Montkowski et al., 1997; Bluthé et al., 2000; Cunningham et al., 2009; McCusker and Kelly, 2013). Following this causal link between pro-inflammatory cytokines and sickness behaviour, a recent study revealed a link between LPS, the induction of the pro-inflammatory cytokine IL-32 and PAR2 activation (Nakayama et al., 2013). Indeed LPS significantly increased IL-32 production in THP-1 cells which in turn up-regulated proteinase-3 activity leading to PAR2 activation and TNFα production. In addition, there is evidence that LPS induces elevated levels of other potential endogenous PAR2 activators (Scarisbrick et al., 2006 and Kirshenbaum et al., 2008) but whether these contribute to the role of PAR2 in the onset and maintenance of sickness behaviour either by crossing the BBB or by induction of their production within the brain itself requires further investigation. In addition to LPS resulting in sickness-like behaviour, administration of LPS has also been shown to impair cognitive function as evidenced in tests of avoidance learning (Sparkman et al., 2005a),

contextual fear conditioning (Pugh et al., 1998; Kranjac et al., 2012), novel object recognition (Miwa et al., 2011) and spatial memory tests (Shaw et al., 2001; Sparkman et al., 2005b) as well as exacerbating memory deficits in a mouse model of delirium (Field et al., 2012; Griffin et al., 2013). Whilst not the focus of the present study, one could speculate that PAR2 deletion may also impair LPS-induced cognitive deficits and indeed this warrants further investigation.

4.2. Neuroinflammatory conditions do not result in increased PAR2 expression.

We have shown a role for PAR2 in LPS-induced sickness behaviour, hence we sought to determine whether PAR2 expression was up-regulated during LPS-induced sickness behaviour. Our data indicates that PAR2 mRNA expression remains unaltered following LPS injection both acutely and after 24 hrs thus suggesting that changes in PAR2 expression do not account for the changes in sickness behaviour observed in $PAR2^{-/-}$ mice. Our findings are in contrast to a number of studies that have shown that LPS as well as IL-1β and TNFα can lead to increased PAR2 expression in a variety of preparations (Hamilton et al., 2001; Morello et al., 2005; Ritchie et al., 2007). However, given increased PAR2 expression in these studies was observed in *in vitro* preparations rather than in vivo as per the current study, direct comparisons are difficult. Thus our data implies that activation of PAR2 *per se* accounts for the role of PAR2 in sickness behaviour but the exact identity of the PAR2 activator(s) remains to be determined with several potential candidates and mechanisms by which this may occur outlined above.

4.3. PAR2 does not contribute to behaviour under normal conditions.

We have recently shown that PAR2 activation indirectly modulates hippocampal neuronal excitability and synaptic transmission via astrocytic activation (Gan et al., 2011). However, whether PAR2 contributes to CNS function and behaviour under normal conditions is

unknown. Hence we utilised PAR2$^{-/-}$ mice to investigate its contribution to behaviour using standard tests for locomotor activity, anxiety-like behaviour, spatial memory and anhedonic-like behaviour. Our data reveals that PAR2 does not contribute to behavioural characteristics associated with these tests as behaviour in PAR2$^{-/-}$ mice was not altered in the OFT, EPM, MWM or SPT when compared to PAR2$^{+/+}$ controls. Whether an endogenous PAR2 activator released from resident cells present within the CNS remains to be elucidated with several candidates being proposed including mast cell tryptase, trypsinogen IV and kallikreins (Steinhoff et al., 2000; Noorbakhsh *et al*. 2005, 2006; Hollenberg et al., 2008) but our data indicates that PAR2 does not contribute to these behavioural paradigms in the normal healthy brain. Similar findings have been reported for another member of the PAR family, PAR1, with behaviour in PAR1$^{-/-}$ mice being unaltered in tests of locomotor activity and anxiety-like behaviour. However, PAR1$^{-/-}$ mice displayed deficits in emotionally motivated behavioural learning when examined using the passive avoidance task and in cued fear conditioning (Almonte et al., 2007). In addition, a recent study has revealed that a shift in G-protein signalling is a novel mechanism by which PAR1 modulates emotionally motivated behavioural learning in the amygdala (Bourgognon et al., 2013). The role of PAR1 has been further implicated in learning and memory with recent studies highlighting deficits in hippocampal-dependent learning and synaptic plasticity in PAR1$^{-/-}$ mice compared to their littermate controls (Almonte et al., 2013). These findings indicate that PAR1 and PAR2 are not involved in locomotor activity and anxiety-like behaviour but implicate PAR1 in learning and memory. No deficits were apparent in PAR2$^{-/-}$ mice in the MWM, suggesting no role in spatial memory but whether PAR2 contributes to emotionally motivated behavioural learning remains to be elucidated and is beyond the scope of this study.

4.4. Conclusions

In conclusion, we have found that PAR2 contributes to the onset and maintenance of LPS-induced sickness behaviour but does not contribute to locomotor, anxiety-like behaviour and spatial memory under normal healthy conditions. We suggest that a change in PAR2 expression within the CNS does not underlie our observations in sickness behaviour and is not evident under chronic inflammatory conditions such as obesity and AD. Further work is required to fully elucidate the mechanisms and signalling pathways underlying the role of PAR2 in sickness behaviour and to determine whether PAR2 plays a significant role in other conditions where changes in the inflammatory environment are observed.

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Competing Interests.

The authors declare that they have no competing interests.

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Figure legends

Figure 1. PAR2 contributes to the onset and maintenance of LPS-induced sickness behaviour. $PAR2^{-/-}$ mice recovered significantly more quickly from LPS-induced behavioural deficits compared to both $PAR2^{+/+}$ as shown by **A)** an increased distance moved at 24 and 48 hrs post LPS injection and **B)** increased entries into the centre square 24 hrs post LPS injection. # = $P<0.05$ vs vehicle, * = $P<0.05$ vs $PAR2^{+/+}$. $N\geq7$ for all mice.

Figure 2. Induction of anhedonia is delayed in $PAR2^{-/-}$ mice following LPS injection. A) Sucrose preference and **B)** fluid intake are similar in all 3 genotypes tested in the SPT. **C)** Anhedonia induction is delayed in $PAR2^{-/-}$ mice compared to $PAR2^{+/+}$ and $PAR2^{+/-}$ mice as shown by sucrose preference being maintained in the first 2 hrs post LPS injection. * = $P<0.05$ vs $PAR2^{+/-}$. $N\geq7$ for all mice.

Figure 3. Food intake and body weight changes are reduced following LPS injection. A) Food intake recovers more quickly in $PAR2^{+/-}$ mice compared to $PAR2^{+/+}$ controls at 48h post LPS injection with a trend towards a recovery seen in $PAR2^{-/-}$ mice. **B)** Body weight recovers more quickly in $PAR2^{-/-}$ mice compared to $PAR2^{+/+}$ controls at 48 and 72h post LPS injection. # = $P<0.05$ vs vehicle, * = $P<0.05$ vs $PAR2^{+/+}$. $N\geq7$ for all mice.

Figure 4. PAR2 mRNA expression remains unchanged in conditions associated with neuroinflammation. A) PAR2 mRNA levels are unaltered but IL-1β and TNF-α mRNA levels are increased 2 hours post injection in all 3 brain regions examined. **B)** PAR2 mRNA levels are unchanged 24 hours post LPS injection but elevated IL-1β and TNF-α mRNA levels are maintained in the hypothalamus. * = $P<0.05$ vs control, ** = $P<0.01$ vs control, *** = $P<0.001$ vs control, $n\geq4$ for all tissue tested.

Figure 5. PAR2 does not contribute to locomotor activity and anxiety-like behaviour. **A-C)** No significant differences were observed on the total distance moved and entries into the centre square between all 3 PAR2 genotypes tested in the OFT. However, $PAR2^{+/-}$ mice spent significantly more time in the centre square compared to both $PAR2^{+/+}$ and $PAR2^{-/-}$ mice. **D-F)** No changes in performance were observed for time in open arms, open arm entries or total arm entries for all 3 genotypes tested in the EPM. ** = P<0.01 vs both $PAR2^{+/+}$ and $PAR2^{-/-}$. N=16 for all genotypes.

Figure 6. PAR2 deletion does not affect performance in a spatial reference memory task. No difference in performance was observed for all 3 genotypes in the MWM as gaged by **A)** latency to locate the platform, **B)** distance travelled to locate the platform and **C)** time spent in the target vs opposite quadrant. *** = P<0.001 vs target quadrant. N=16 for all genotypes.

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

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

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.

Keywords: Proteinase-activated receptor-2; LPS; sickness behaviour; locomotor activity; anxiety; sucrose preference.

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, 2011). 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.

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 their 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 ºC and 45-55% humidity, with a 12/12 hours light/dark cycle (lights on at 0600, off at 1800). Mice were group-housed (3-10 per cage), 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), the Morris water maze (MWM) and the sucrose preference test (SPT) was carried out on 48 males (26.0 ± 0.4g; 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 x 40 x 40cm (lighting 45 lux) 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 x 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 lux) of a plus-shaped maze with two open (30 x 5cm, 60 lux) and two closed arms (30 x 5cm, 15 cm walls, 6 lux) made of IR-translucent Perspex with integral IR light sources elevated 70cm 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 21ºC with a transparent 10 cm diameter submerged platform, in a room (45 lux) 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 60s (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 72h 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^{o}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). 500ng 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 20µL reaction volume, which was subsequently diluted to 100µL 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 100nM of the relevant Universal Probe, 400nM of each primer, 2µl diluted cDNA and 5.0µl ABsolute QPCR ROX Mix (VWR International, Lutterworth, UK) in a final volume of 10µl. Reactions were run in triplicate, with the mean Ct value from the three reactions used for subsequent data analysis.

2.4. Statistics

All data are expressed as mean ± S.E.M. Data were compared by paired or unpaired Student's t-tests, one-way analysis of variance with Tukey's comparison or 2-way mixed-model

ANOVA followed by Bonferroni *post-hoc* comparisons as appropriate. Differences were considered significant when $P<0.05$.

3. Results

3.1. PAR2 contributes to LPS-induced sickness behaviour

With PAR2 proposed to play a role in several CNS and peripheral diseases whose aetiology and pathology are closely linked to inflammation including Alzheimer's disease (AD), Multiple Sclerosis (MS) and rheumatoid arthritis (Ferrell et al., 2003; Noorbakhsh et al., 2006; Afkhami-Goli et al., 2007), we used the LPS-induced sickness behaviour model of systemic infection to examine whether PAR2 is involved in the behavioural changes associated with activation of the immune system, which include reduction in locomotor activity, anorexia with associated loss of body weight and anhedonia.

3.1.1. Open field test.

In the OFT, LPS injection (1mg kg^{-1}) resulted in a significant reduction in locomotor activity over the first 24 h after injection compared to vehicle-injected controls (2h post injection, $p<0.001$ for all 3 genotypes vs vehicle control), which returned to levels similar those observed in vehicle-injected controls by 72 h ($p>0.05$ for all 3 genotypes vs vehicle control, Figure 1A). However, $PAR2^{-/-}$ mice showed significantly increased locomotion at 24 h and 48 h post injection compared with $PAR2^{+/+}$ (24 h: $F_{(2,29)}=4.269$, $p=0.0237$, $PAR2^{+/+}$ versus $PAR2^{-/-}$ p=0.0217; 48h: $F_{(2,29)}=3.960$ $p=0.0301$, $PAR2^{+/+}$ versus $PAR2^{-/-}$ p=0.0217, Figure 1A). Furthermore, the number of entries into the centre square at 24 h post injection was increased in $PAR2^{-/-}$ mice when compared with $PAR2^{+/+}$ ($F_{(2,29)}=3.727$, $p=0.0362$, $PAR2^{+/+}$ versus $PAR2^{-/-}$ p=0.0282, Figure 1B). With regard to time spent in the centre square, following LPS injection, no differences were observed between genotypes at any time point investigated.

3.1.2. Sucrose preference test.

Another behavioural characteristic associated with sickness behaviour is the induction of anhedonia. We therefore used the sucrose preference test to investigate whether PAR2 contributes to the induction and maintenance of anhedonia under these experimental conditions. All mice showed a sucrose preference over the 3 days prior to LPS injection with no significant differences observed over the 3 days ($F_{(2,60)}$=0.327, p=0.723) or between genotypes ($F_{(2,30)}$=0.072, p=0.931, Figure 2A). Similarly, no effect of day ($F_{(2,58)}$=0.097, p=0.908) or genotype ($F_{(2,29)}$=1.948, p=0.161, Fig 2B) were seen in the total fluid drunk. These data indicate that PAR2 plays no role in sucrose preference or fluid intake in normal healthy mice and therefore we can use this model to investigate the role of PAR2 in LPS-induced anhedonia.

The effect of LPS injection on the % sucrose drunk was significantly reduced in the first 2 h post injection ($F_{(2,29)}$=3.751, p=0.035, $PAR2^{+/-}$ versus $PAR2^{-/-}$ p=0.035, $PAR2^{+/+}$ versus $PAR2^{-/-}$ p=0.119). Thus, the onset of anhedonia is delayed in the $PAR2^{-/-}$ mice when compared to $PAR2^{+/-}$ mice and shows a trend towards a delay when compared to $PAR2^{+/+}$ mice (Figure 2C).

3.1.3. Food intake and change in body weight.

We also examined whether PAR2 deletion contributes to the changes in food intake and body weight associated with sickness behaviour. No difference in initial body weight and food intake was observed between all three genotypes and the vehicle control group prior to LPS injection (p>0.05 for all 4 groups for both measures, data not shown) whereas food intake was significantly increased in $PAR2^{+/-}$ mice compared to $PAR2^{+/+}$ 48h post injection. At the same time point $PAR2^{-/-}$ mice also showed a trend towards increased food intake. ($F_{(2,29)}$=5.480, p=0.010, $PAR2^{+/-}$ versus $PAR2^{+/+}$ p=0.010, $PAR2^{-/-}$ versus $PAR2^{+/+}$ p=0.086,

Figure 3A). Furthermore, the % change in body weight was significantly less in $PAR2^{-/-}$ mice at both 48h and 72h post injection when compared to $PAR2^{+/+}$ mice (48 h: $F_{(2,29)}$=3.309, p=0.051, $PAR2^{-/-}$ versus $PAR2^{+/+}$ p=0.040; 72 h: $F_{(2,29)}$=3.806, p=0.034, $PAR2^{-/-}$ versus $PAR2^{+/+}$ p=0.027, Figure 3B). The effect of LPS on total fluid intake also differed between genotypes ($F_{(2,29)}$= 9.000, p=0.001). $PAR2^{-/-}$ mice drank significantly more total fluid than $PAR2^{+/+}$ (p=0.004) and $PAR2^{+/-}$ (p=0.001) in the first 2 h post injection (data not shown), which may be part of a more robust response to inflammation.

3.1.4. Habituation

In order to confirm that habituation does not contribute to the observed changes in behaviour, we conducted control experiments using the same time schedule as in the LPS experiment to examine the effect of habituation to repeated testing in the OFT. No significant effect of habituation on the OFT was observed, which indicates that effects seen in LPS-injected mice are those of LPS alone and not habituation.

3.2. PAR2 expression is unaltered under inflammatory CNS conditions.

Having established that PAR2 contributes to LPS-induced sickness behaviour and associated food intake and body weight changes, we examined whether CNS PAR2 expression was altered following LPS injection. PAR2 mRNA was detected in the three brain regions examined, the hypothalamus, hippocampus and the cerebellum obtained from control mice (PBS injection) and mice exposed to a single LPS injection (1mg kg^{-1}). Two hours post-injection, a time point at which behavioral changes were observed, no significant difference in PAR2 mRNA levels was observed in the hypothalamus (PBS ΔC_t=10.1±0.4 (n=5) vs LPS ΔC_t=9.9±0.2 (n=4); p=0.79, Figure 4A), the hippocampus (PBS ΔC_t=8.4±0.2 (n=5) vs LPS ΔC_t=8.3±0.4 (n=4); p=0.91, Figure 4A) or the cerebellum (PBS ΔC_t=9.4±0.2 (n=5) vs LPS ΔC_t=9.4±0.3 (n=4); p=0.91, Figure 4A). In contrast, mRNA levels for the inflammatory

cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α, are significantly increased in all three brain regions (p<0.01 for all regions, Figure 4A). Similarly, no significant difference in PAR2 mRNA levels was observed 24 hours post-injection between control mice and those exposed to a single LPS injection (1mg kg^{-1}) in the hypothalamus (PBS ΔC_t=10.7±0.2 vs. LPS ΔC_t=10.3±0.8; p=0.62, each group n=5, Figure 4B), the hippocampus (PBS ΔC_t=10.8±0.6 vs. LPS ΔC_t=11.1±0.4; p=0.75, each group n=5, Figure 6B) and the cerebellum (PBS ΔC_t=10.5±0.4 vs. LPS ΔC_t=9.5±0.1; p=0.08, each group n=5, Figure 4B). IL-1β (p=0.04) and TNF-α (p=0.03) mRNA levels were significantly increased in the hypothalamus 24 hours post LPS injection as was TNF-α (p=0.02) in the cerebellum but no significant changes were evident in all other samples 24 hours post LPS injection.

3.3. PAR2 deletion does not alter locomotor activity, anxiety-like behaviour or spatial memory.

3.3.1. Open field test.

General locomotor activity as measured by the distance moved over the testing period was unaffected ($F_{(2,45)}$=0.523, p=0.596, Figure 5A) as was the number of entries into the centre square ($F_{(2,45)}$=1.690, p=0.196, Figure 5B). However, $PAR2^{+/-}$ mice spent significantly more time in the centre square compared to both $PAR2^{+/+}$ and $PAR2^{-/-}$ mice ($F_{(2,45)}$=8.718, p=0.0006, $PAR2^{+/-}$ vs. $PAR2^{+/+}$ p=0.005, $PAR2^{+/-}$ vs. $PAR2^{-/-}$ p=0.001, $PAR2^{+/+}$ vs. $PAR2^{-/-}$ p=0.827, Figure 5C) indicating that $PAR2^{+/-}$ mice may be less anxious under these behavioural conditions.

3.3.2. Elevated Plus Maze.

PAR2 deletion did not affect anxiety-like behaviour in the EPM test as no significant differences were observed between genotypes in the % time spent in open arms ($F_{(2,46)}$=

0.261, p=0.771, Figure 5D) , % open arm entries ($F_{(2,47)}$=0.114, p=0.893, Figure 5E) and the number of total entries ($F_{(2,47)}$=0.763, p=0.472, Figure 1F) over the 10 min test period.

3.3.3. Morris water maze.

As we have previously shown that PAR2 activation induces a form of hippocampal synaptic plasticity *in vitro* (Gan et al., 2011), we examined whether PAR2 plays a role in spatial memory formation using the MWM. There was a significant effect of day in both latency to locate platform ($F_{(4,180)}$=50.036, p=0.0005, Figure 6A) and distance travelled to the platform ($F_{(4,180)}$= 50.1, p=0.0005, Figure 6B) over the 5 consecutive testing days. However, there was no significant effect of genotype and no interaction between day and genotype in both latency to locate platform (genotype: $F_{(2,42)}$=0.716, p=0.494,interaction: $F_{(8,180)}$=0.178, p=0.994, Figure 6A) and distance travelled (genotype: $F_{(2,42)}$=0.372, p=0.692, interaction: $F_{(8,180)}$=0.328, p= 0.954, Figure 6B). In addition, during a subsequent probe test mice spent significantly more time in the target quadrant compared to that spent in the opposite quadrant (p<0.001 for all genotypes) but there was no significant difference between genotypes ($F_{(2,44)}$=1.517; p=0.230, Figure 6C). These data indicate that PAR2 deletion has no deleterious effect on spatial reference memory.

4. Discussion

In the present study, we show for the first time that PAR2 contributes to the onset and maintenance of LPS-induced sickness behaviour whereas, in contrast, our data suggests that PAR2 does not contribute to locomotor activity and anhedonic-like behaviour under normal healthy conditions. In addition, we show that under neuroinflammatory conditions within the CNS, no change in PAR2 expression is observed indicating that PAR2 activation *per se* contributes to inflammation-induced changes in behaviour rather than altered expression.

4.1. PAR2 activation participates in the onset and maintenance of sickness behaviour.

PAR2 has been reported to be extensively expressed within the CNS from both rodent and human tissue, with a strong link to inflammation-related diseases (Bushell, 2007; Ramachandran et al., 2012). Hence, we examined the role of PAR2 in behavioural tests in which the conditions for its activation were favourable. It has been suggested that PARs may become activated under inflammatory conditions where the blood brain barrier (BBB) becomes leaky (Gingrich and Traynelis, 2000; Bushell, 2007), hence we utilised a well-established model of systemic infection that is used extensively to examine the role of inflammatory mediators on behaviour, namely LPS-induced sickness behaviour (Dantzer et al., 2008; McCusker and Kelly, 2013). Sickness behaviour can be measured using a number of parameters and we focused on locomotor activity and the induction of anhedonia as well as changes in food intake and body weight. Our data shows that sickness behaviour was induced post LPS injection in all genotypes tested but deficits in its induction and maintenance was apparent in $PAR2^{-/-}$ mice. Indeed in the SPT, the decrease in sucrose preference was delayed in $PAR2^{-/-}$ mice post LPS injection whereas in the OFT, an increased recovery in distance moved and entry into centre squares was observed in $PAR2^{-/-}$ mice post LPS injection. Furthermore, the decrease in body weight was significantly reduced 24 and 72

hours post injection. These data suggest for the first time that PAR2 contributes to the onset and maintenance of LPS-induced sickness behaviour when examined using these characteristic behavioural outcomes associated with sickness behaviour. So how is PAR2 involved? Our understanding of brain-immune interactions has developed significantly since the brain was thought to be an immune privileged site. It is suggested that peripheral infections and the inflammatory cytokines this induces cross the leaky BBB leading to the behavioural symptoms associated with sickness with recent evidence also indicating that these pro-inflammatory cytokines are also synthesised within the brain during systemic infections (Dantzer et al., 2008; Cunningham et al., 2009; Schedlowski et al., 2014). Some of the main protagonists suggested to underlie the observed behavioural changes include the pro-inflammatory cytokines IL-1β, IL-6 and TNFα with numerous studies confirming that these cytokines are increased following systemic infections as well as their direct application to the brain leading to changes in behaviour similar to those seen in sickness behaviour (Montkowski et al., 1997; Bluthé et al., 2000; Cunningham et al., 2009; McCusker and Kelly, 2013). Following this causal link between pro-inflammatory cytokines and sickness behaviour, a recent study revealed a link between LPS, the induction of the pro-inflammatory cytokine IL-32 and PAR2 activation (Nakayama et al., 2013). Indeed LPS significantly increased IL-32 production in THP-1 cells which in turn up-regulated proteinase-3 activity leading to PAR2 activation and TNFα production. In addition, there is evidence that LPS induces elevated levels of other potential endogenous PAR2 activators (Scarisbrick et al., 2006 and Kirshenbaum et al., 2008) but whether these contribute to the role of PAR2 in the onset and maintenance of sickness behaviour either by crossing the BBB or by induction of their production within the brain itself requires further investigation. In addition to LPS resulting in sickness-like behaviour, administration of LPS has also been shown to impair cognitive function as evidenced in tests of avoidance learning (Sparkman et al., 2005a),

contextual fear conditioning (Pugh et al., 1998; Kranjac et al., 2012), novel object recognition (Miwa et al., 2011) and spatial memory tests (Shaw et al., 2001; Sparkman et al., 2005b) as well as exacerbating memory deficits in a mouse model of delirium (Field et al., 2012; Griffin et al., 2013). Whilst not the focus of the present study, one could speculate that PAR2 deletion may also impair LPS-induced cognitive deficits and indeed this warrants further investigation.

4.2. Neuroinflammatory conditions do not result in increased PAR2 expression.

We have shown a role for PAR2 in LPS-induced sickness behaviour, hence we sought to determine whether PAR2 expression was up-regulated during LPS-induced sickness behaviour. Our data indicates that PAR2 mRNA expression remains unaltered following LPS injection both acutely and after 24 hrs thus suggesting that changes in PAR2 expression do not account for the changes in sickness behaviour observed in $PAR2^{-/-}$ mice. Our findings are in contrast to a number of studies that have shown that LPS as well as IL-1β and TNFα can lead to increased PAR2 expression in a variety of preparations (Hamilton et al., 2001; Morello et al., 2005; Ritchie et al., 2007). Thus our data implies that activation of PAR2 *per se* accounts for the role of PAR2 in sickness behaviour but the exact identity of the PAR2 activator(s) remains to be determined with several potential candidates and mechanisms by which this may occur outlined above.

4.3. PAR2 does not contribute to behaviour under normal conditions.

We have recently shown that PAR2 activation indirectly modulates hippocampal neuronal excitability and synaptic transmission via astrocytic activation (Gan et al., 2011). However, whether PAR2 contributes to CNS function and behaviour under normal conditions is unknown. Hence we utilised $PAR2^{-/-}$ mice to investigate its contribution to behaviour using standard tests for locomotor activity, anxiety-like behaviour, spatial memory and anhedonic-

like behaviour. Our data reveals that PAR2 does not contribute to behavioural characteristics associated with these tests as behaviour in $PAR2^{-/-}$ mice was not altered in the OFT, EPM, MWM or SPT when compared to $PAR2^{+/+}$ controls. Whether an endogenous activator of PAR2 is present within the CNS remains to be elucidated with several candidates being proposed including mast cell tryptase, trypsinogen IV and kallikreins (Steinhoff et al., 2000; Noorbakhsh *et al*. 2005, 2006; Hollenberg et al., 2008) but our data indicates that PAR2 does not contribute to these behavioural paradigms in the normal healthy brain. Similar findings have been reported for another member of the PAR family, PAR1, with behaviour in $PAR1^{-/-}$ mice being unaltered in tests of locomotor activity and anxiety-like behaviour. However, $PAR1^{-/-}$ mice displayed deficits in emotionally motivated behavioural learning when examined using the passive avoidance task and in cued fear conditioning (Almonte et al., 2007). In addition, a recent study has revealed that a shift in G-protein signalling is a novel mechanism by which PAR1 modulates emotionally motivated behavioural learning in the amygdala (Bourgognon et al., 2013). The role of PAR1 has been further implicated in learning and memory with recent studies highlighting deficits in hippocampal-dependent learning and synaptic plasticity in $PAR1^{-/-}$ mice compared to their littermate controls (Almonte et al., 2013). These findings indicate that PAR1 and PAR2 are not involved in locomotor activity and anxiety-like behaviour but implicate PAR1 in learning and memory. No deficits were apparent in $PAR2^{-/-}$ mice in the MWM, suggesting no role in spatial memory but whether PAR2 contributes to emotionally motivated behavioural learning remains to be elucidated and is beyond the scope of this study.

4.4. Conclusions

In conclusion, we have found that PAR2 contributes to the onset and maintenance of LPS-induced sickness behaviour but does not contribute to locomotor, anxiety-like behaviour and spatial memory under normal healthy conditions. We suggest that a change in PAR2

expression within the CNS does not underlie our observations in sickness behaviour and is not evident under chronic inflammatory conditions such as obesity and AD. Further work is required to fully elucidate the mechanisms and signalling pathways underlying the role of PAR2 in sickness behaviour and to determine whether PAR2 plays a significant role in other conditions where changes in the inflammatory environment are observed.

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Competing Interests.

The authors declare that they have no competing interests.

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Figure legends

Figure 1. PAR2 contributes to the onset and maintenance of LPS-induced sickness behaviour. $PAR2^{-/-}$ mice recovered significantly more quickly from LPS-induced behavioural deficits compared to both $PAR2^{+/+}$ as shown by **A)** an increased distance moved at 24 and 48 hrs post LPS injection and **B)** increased entries into the centre square 24 hrs post LPS injection. # = $P<0.05$ vs vehicle, * = $P<0.05$ vs $PAR2^{+/+}$. $N\geq7$ for all mice.

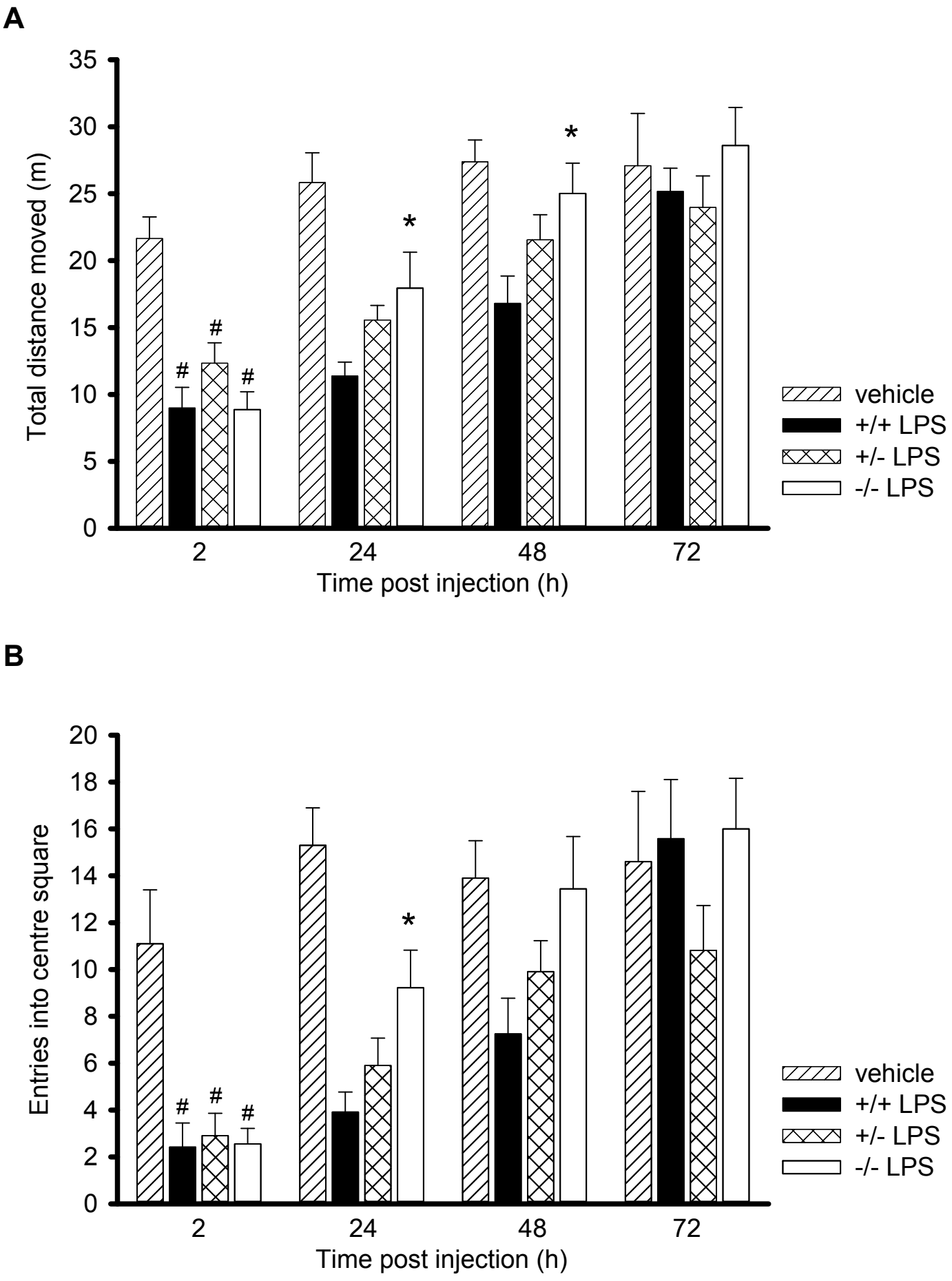
Figure 2. Induction of anhedonia is delayed in $PAR2^{-/-}$ mice following LPS injection. A) Sucrose preference and **B)** fluid intake are similar in all 3 genotypes tested in the SPT. **C)** Anhedonia induction is delayed in $PAR2^{-/-}$ mice compared to $PAR2^{+/+}$ and $PAR2^{+/-}$ mice as shown by sucrose preference being maintained in the first 2 hrs post LPS injection. * = $P<0.05$ vs $PAR2^{+/-}$. $N\geq7$ for all mice.

Figure 3. Food intake and body weight changes are reduced following LPS injection. A) Food intake recovers more quickly in $PAR2^{+/-}$ mice compared to $PAR2^{+/+}$ controls at 48h post LPS injection with a trend towards a recovery seen in $PAR2^{-/-}$ mice. **B)** Body weight recovers more quickly in $PAR2^{-/-}$ mice compared to $PAR2^{+/+}$ controls at 48 and 72h post LPS injection. # = $P<0.05$ vs vehicle, * = $P<0.05$ vs $PAR2^{+/+}$. $N\geq7$ for all mice.

Figure 4. PAR2 mRNA expression remains unchanged in conditions associated with neuroinflammation. A) PAR2 mRNA levels are unaltered but IL-1β and TNF-α mRNA levels are increased 2 hours post injection in all 3 brain regions examined. **B)** PAR2 mRNA levels are unchanged 24 hours post LPS injection but elevated IL-1β and TNF-α mRNA levels are maintained in the hypothalamus. * = $P<0.05$ vs control, ** = $P<0.01$ vs control, *** = $P<0.001$ vs control, $n\geq4$ for all tissue tested.

Figure 5. PAR2 does not contribute to locomotor activity and anxiety-like behaviour. **A-C)** No significant differences were observed on the total distance moved and entries into the centre square between all 3 PAR2 genotypes tested in the OFT. However, $PAR2^{+/-}$ mice spent significantly more time in the centre square compared to both $PAR2^{+/+}$ and $PAR2^{-/-}$ mice. **D-F)** No changes in performance were observed for time in open arms, open arm entries or total arm entries for all 3 genotypes tested in the EPM. ** = P<0.01 vs both $PAR2^{+/+}$ and $PAR2^{-/-}$. N=16 for all genotypes.

Figure 6. PAR2 deletion does not affect performance in a spatial reference memory task. No difference in performance was observed for all 3 genotypes in the MWM as gaged by **A)** latency to locate the platform, **B)** distance travelled to locate the platform and **C)** time spent in the target vs opposite quadrant. *** = P<0.001 vs target quadrant. N=16 for all genotypes.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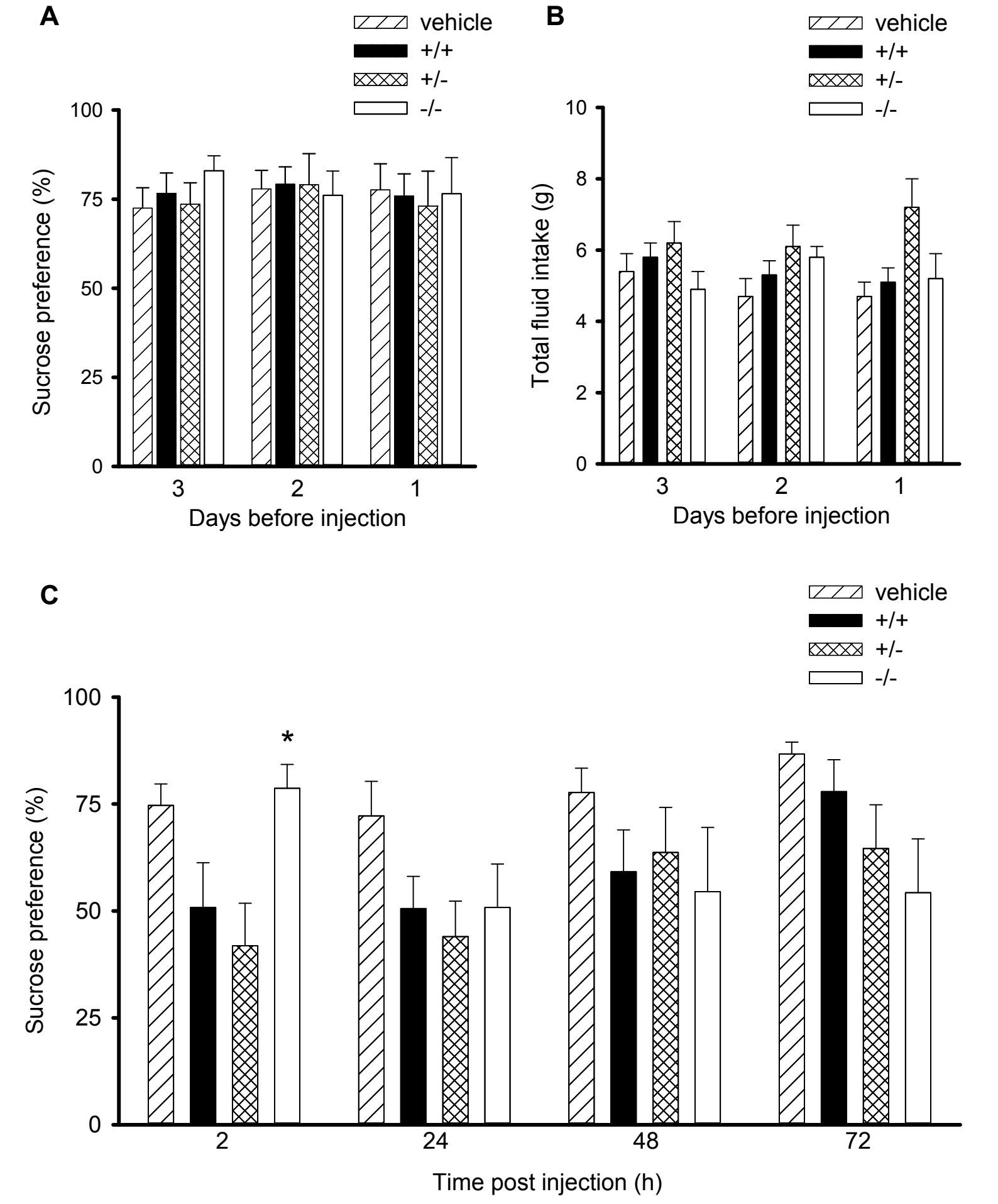
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Total fluid intake (g)
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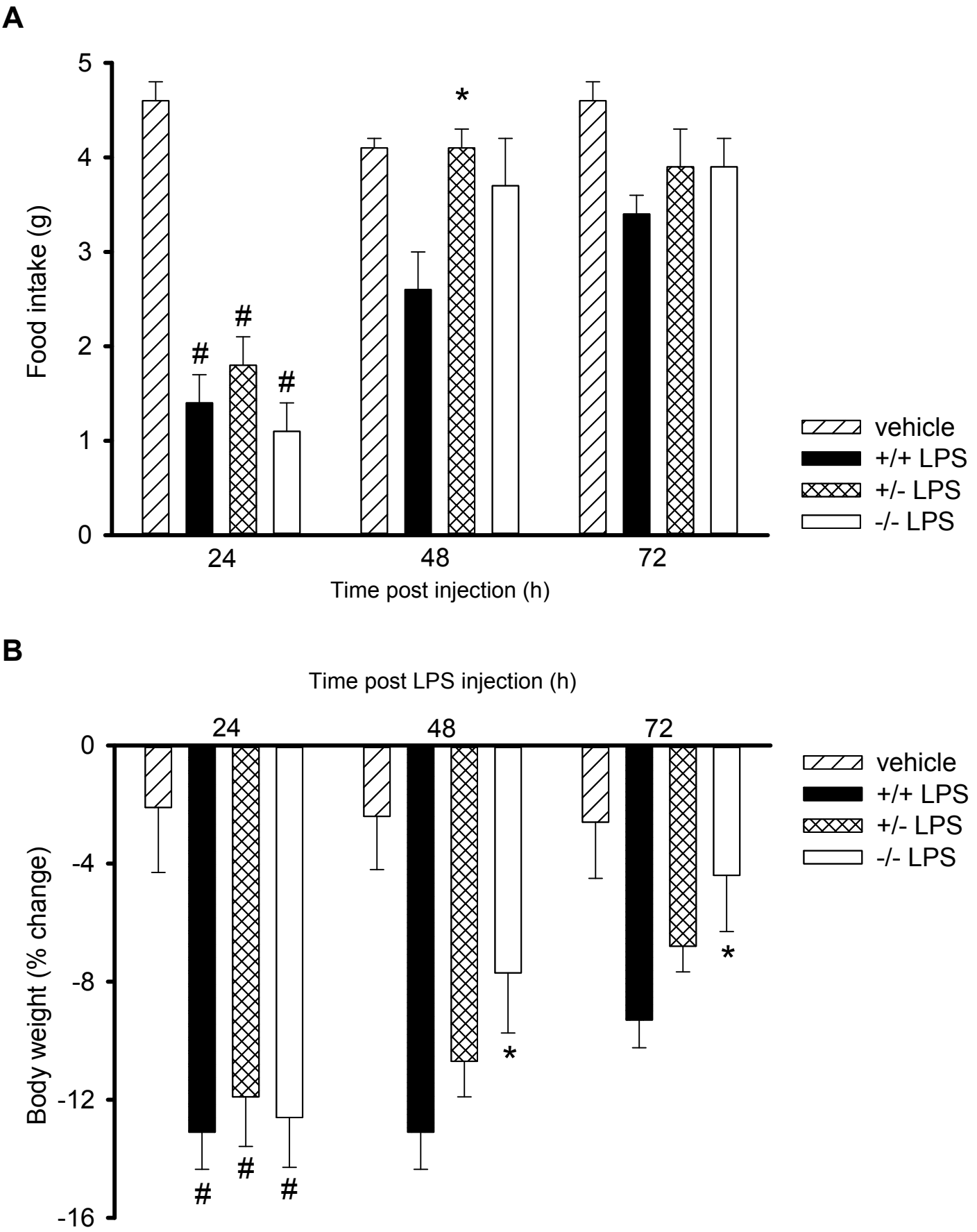
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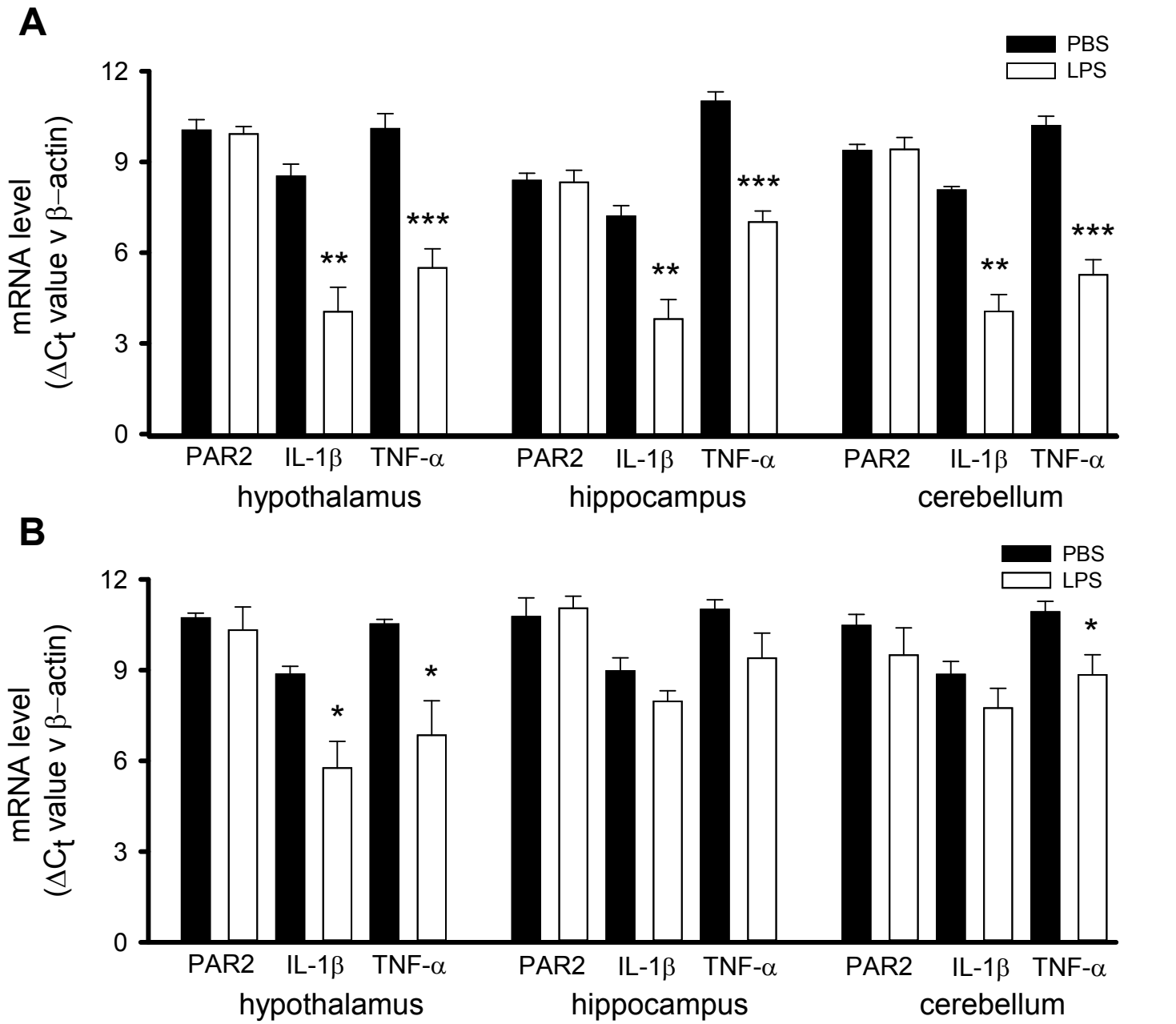
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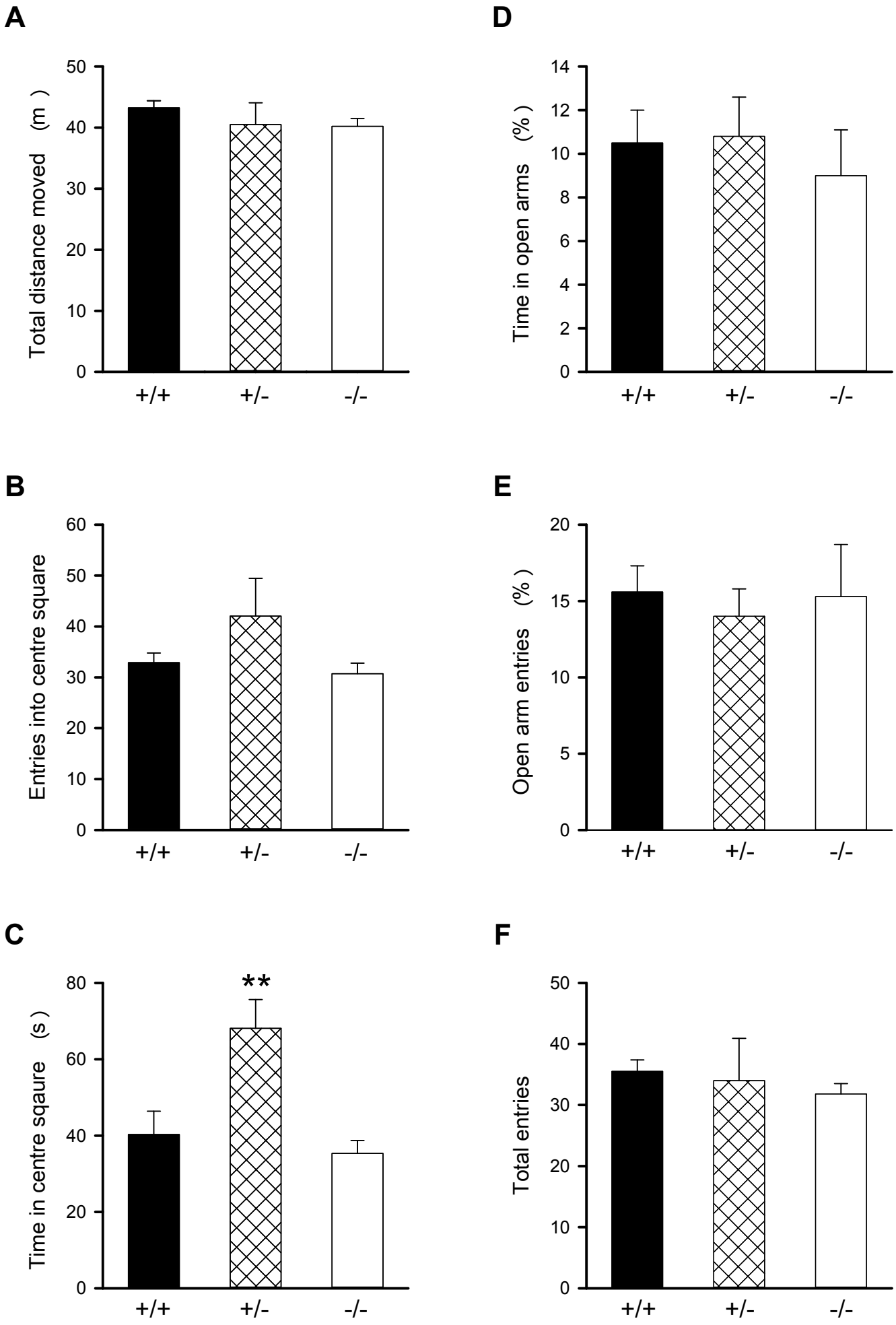
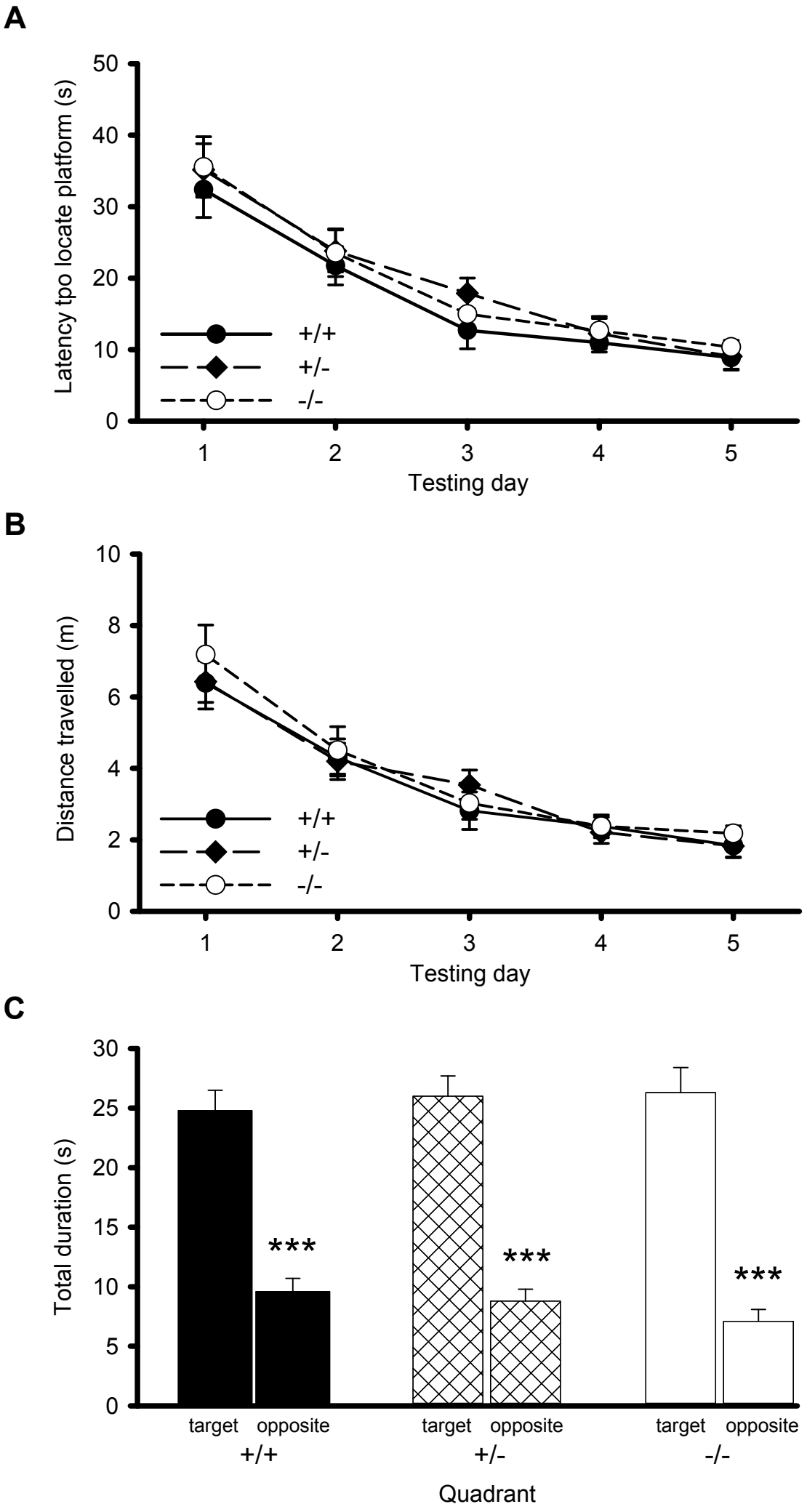
TNF-α
hypothalamus
hippocampus
cerebellum


A
Total distance moved (m)
50
40
30
20
10
0
+/+
+/-
-/-
D
Time in open arms (%)
14
12
10
8
6
4
2
0
+/+
+/-
-/-
B
Entries into centre square
60
50
40
30
20
10
0
+/+
+/-
-/-
E
Open arm entries (%)
20
15
10
5
0
+/+
+/-
-/-
C
Time in centre sqaure (s)
**
80
60
40
20
0
+/+
+/-
-/-
F
Total entries
50
40
30
20
10
0
+/+
+/-
-/-


A
Latency tpo locate platform (s)
50
40
30
20
10
0
+/+
+/-
-/-
1
2
3
4
5
Testing day
B
Distance travelled (m)
10
8
6
4
2
0
+/+
+/-
-/-
1
2
3
4
5
Testing day
C
Total duration (s)
30
25
20
15
10
5
0
***
***
***
target
opposite
target
opposite
target
opposite
+/+
+/-
-/-
Quadrant