**FAAH inhibition attenuates TLR3-mediated hyperthermia, nociceptive- and anxiety-like behaviour in female rats**

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**Abbreviations**

AEA: anandamide; ADT: Acetone drop test; $CB_1$: cannabinoid receptor 1; $CB_2$: cannabinoid receptor 2; CD11b: cluster of differentiation molecule 11b: CD68, cluster of differentiation 68; CNS: central nervous system; COX-2: cyclooxygenase 2; EPM: Elevated plus maze; FAAH: Fatty acid amide hydrolase; FST: Forced swim test; GFAP: glial fibrillary acidic protein; IFN: interferon; IL: interleukin; i.p: intraperitoneal; IP-10: Interferon gamma-induced protein 10; IRF: interferon regulatory factor; LMA: Locomotor activity; LPS: lipopolysaccharide; MRC2: Mannose receptor C type 2; NFκB: Nuclear factor kappa B; OEA: *N*-oleoylethanolamide; OFT; Open field test; PEA: *N*-palmitoylethanolamide; $PGE_2$: prostaglandin E2; Poly I:C: Polyinosinic: polycytidylic acid; SPT: Sucrose preference test; TLR: Toll-like receptor; TNF: tumour necrosis factor; URB597: [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate; VFT: Von Frey test.

**Abstract**

Aberrant activation of toll-like receptor (TLR)s results in persistent and prolonged neuroinflammation and has been implicated in the pathogenesis and exacerbation of psychiatric and neurodegenerative disorders. TLR3 coordinates the innate immune response to viral infection and recent data have demonstrated that inhibiting fatty acid amide hydrolase (FAAH), the enzyme that primarily metabolizes anandamide, modulates TLR3-mediated neuroinflammation. However, the physiological and behavioural consequences of such modulation are unknown. The present study examined the effect of URB597, a selective FAAH inhibitor, on neuroinflammation, physiological and behavioural alterations following administration of the TLR3 agonist and viral mimetic poly I:C to female rats. URB597 attenuated TLR3-mediated fever, mechanical and cold allodynia, and anxiety-like behaviour in the elevated plus maze and open field arena. There was no effect of URB597 on TLR3-mediated decreases in body weight and no effect in the sucrose preference or forced swim tests. URB597 attenuated the TLR3-mediated increase in the expression of CD11b and CD68, markers of microglia/macrophage activation. In summary, these data demonstrate that enhancing FAAH substrate levels suppresses TLR3-mediated microglia/macrophage activation and associated changes in fever, nociceptive responding and anxiety-related behaviour. These data provide further support for FAAH as a novel therapeutic target for neuroinflammatory disorders.

**Keywords:** endocannabinoid, anandamide, neuroinflammation, viral, brain, behavior

## 1. Introduction

The endocannabinoid system has been shown to exhibit potent immunomodulatory effects and represents a potential therapeutic target for peripheral and central inflammatory disorders [1-5]. *N*-arachidonoylethanolamine (anandamide; AEA), the most studied endocannabinoid to date, mediates its effects via cannabinoid ($CB_1$ and $CB_2$) and non-cannabinoid (TRPV1, PPARs and GPR55) receptors and is primarily broken down by fatty acid amide hydrolase (FAAH) [6]. *In vitro* and *in vivo* evidence have demonstrated that FAAH inhibition, and associated increases in AEA and the related *N*-acylethanolamines *N*-oleoylethanolamide (OEA) and *N*-palmitoylethanolamide (PEA), result in the modulation of inflammatory responses induced following the activation of the pattern recognition receptors, toll-like receptor (TLR)s [for review see [7]. (TLR)3 activation results in the induction of type 1 interferon (IFN-α and IFN-β) and NFκB-inducible (e.g. IL-1β, IL-6 and TNF-α) inflammatory cascades which are responsible for coordinating the innate immune response to viral infection. Recent data has highlighted that FAAH inhibition attenuates the TLR3-mediated increase in the expression of IFN-inducible genes and pro-inflammatory cytokines in brain regions such as the hippocampus and hypothalamus, without altering peripheral immune responses [8, 9]. The behavioural and physiological consequences of TLR3 activation include the induction of sickness behaviours such as fever/hypothermia, hypoactivity and anorexia [8, 10-13] and enhanced pain sensitivity [14] which represents a highly adaptive coping mechanism by the CNS to fight viral infection. However, aberrant activation of TLR3 can elicit adverse effects on the CNS including increased neuronal excitability and seizure susceptibility [15, 16], impaired contextual and working memory [16], anxiety- and depressive-like behaviour [17] and exacerbation of underlying neurodegenerative processes [18, 19]. However, it is unknown if FAAH-induced modulation of TLR3-mediated inflammatory responses result in associated physiological and behavioural changes.

Several studies have demonstrated that FAAH inhibition alters anxiety- [20-25] and depressive-like behaviour [21, 26] and elicits analgesic effects [22]. However, few studies have evaluated if similar effects occur in the presence of heightened inflammatory tone. The FAAH substrates AEA, OEA and PEA have been shown to modulate TLR4-induced thermoregulatory changes and hypophagia [27-29], most likely mediated via modulation of hypothalamic cytokine expression [28]. A recent study from our group demonstrated that FAAH inhibition modulated TLR4-mediated neuroinflammatory responses in the hippocampus and frontal cortex, an effect which was accompanied by an attenuation of TLR4-mediated anhedonia, but not sickness behaviour [30]. Furthermore, FAAH inhibition has been demonstrated to reverse TLR4-mediated mechanical allodynia [31], thermal hyperalgesia and paw oedema [32]. Collectively, these results demonstrate a role for FAAH substrates in the modulation of behavioural responses following TLR4 activation, although there are no studies to date examining if similar responses occur following activation of other TLRs such as TLR3. Thus, the aim of the present study was to examine the effect of enhancing FAAH substrate levels on TLR3-mediated neuroimmune activation and resulting physiological and behavioural responses.

## 2. Experimental Procedures

### *2.1 Animals*

Experiments were carried out on female Sprague-Dawley rats (weight, 180-250g; NUI Galway breeding facility), housed singly for at least 3 days prior to the experiment in transparent plastic bottomed cages (48cm × 20cm × 27cm) containing wood shavings as bedding. The animals were maintained at a constant temperature (21 ± 2ºC) under standard light-dark cycle conditions (12: 12 h light-dark, lights on from 0700 to 1900 h). All experiments were carried out during the light phase between 0800 h and 1800 h. Food and water were available *ad libitum.* Animals were habituated to handling and received an intraperitoneal (i.p.) injection of sterile saline (0.89% NaCl) for 2-3 days before experimentation in order to minimise the influence of the injection procedure on behaviour and biological endpoints and the minimum number of animals used. The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland Galway under licence from the Irish Department of Health and Children in compliance with the European Communities Council directive 2010/63/EU and ARRIVE guidelines.

### *2.2 Experimental Design*

#### *2.2.1 Experiment 1: The effect of FAAH inhibition on poly I:C-induced sickness behaviour, nociceptive responding, anxiety- and depressive-like behaviour*

Rats were randomly assigned to one of three treatment groups: Vehicle-saline (n = 6-8), Vehicle-poly I:C (n = 9) and URB597-poly I:C (n = 9). The TLR3 agonist poly I:C (3mg/kg i.p., GE Healthcare, Ireland) or saline vehicle (0.89% NaCl, i.p.) were administered in an injection volume of 1.5ml/kg 30 minutes following systemic i.p. administration of the FAAH inhibitor URB597 (1mg/kg, Cayman Chemicals, Estonia) or vehicle (ethanol: cremophor: saline; 1:1:18) in an injection volume of 2ml/kg. The dose and timing of URB597 and poly I:C administration were chosen based on previous published work [8, 9, 33]. Behavioural responding was assessed over a 24hr period. Sickness behaviour was assessed by recording rectal temperature, home cage locomotor activity and body weight. Nociceptive responding to

mechanical and cold stimuli was assessed using the von Frey and acetone drop tests, respectively. Anxiety-like behaviour was assessed in the open field and elevated plus maze and depressive-like behaviour was assessed using the sucrose preference test (anhedonia) and forced swim test (behavioural despair/stress coping behaviour). Separate cohorts of animals were used for behavioural testing. Cohort 1: Temperature, Homecage activity (HCA), Body weight and Open Field Test (OFT). Cohort 2: Sucrose Preference (SPT). Cohort 3: Von Frey test (VFT) and Acetone drop test (ADT), Elevated plus maze (EPM) and Forced Swim test (FST). At the end of behaviour testing, animals from cohort 3 were sacrificed, brain removed and hypothalamus excised and frozen at -80$^{o}$C until expression of markers for microglia/macrophage and astrocyte activation.

*2.2.2 Experiment 2: The effect of FAAH inhibition on temperature, nociceptive responding and anxiety-like behaviour in the absence of TLR3 stimulation*

Rats were randomly assigned to one of two treatment groups: Vehicle-saline (n = 6) and URB597-saline (n = 7). The FAAH inhibitor URB597 (1mg/kg) or vehicle (ethanol: cremophor: saline; 1:1:18) were administered i.p. in an injection volume of 2ml/kg followed 30min later by an i.p, injection of sterile saline (0.89% NaCl) administered in an injection volume of 1.5ml/kg. Animals were analysed for temperature, nociceptive responding and anxiety behaviour in the EPM.

***2.3 Temperature recording and body weight***

Body temperature was measured using a rectal probe (Omron EcoTemp Smart Digital Thermometer) prior to any experimental manipulation and 4, 8 and 24hrs post poly I:C/saline

injection. Body weight was recorded prior to any experimental manipulation and 24hrs following poly I:C/saline injection and used to calculate body weight gain over the 24hr period.

### *2.4 Behavioural testing*

#### *2.4.1 Homecage locomotor activity monitoring*

Home cage locomotor activity was assessed using the Opto-M3 Dual Axis system (Columbus Instruments, Columbus, OH) as previously described [34, 35]. Following poly I:C/saline injection, animals were returned to their home cage and horizontal activity (total beam breaks) was recorded and presented as activity during the light phase (0-8 h post poly I:C/saline) and the dark phase (nocturnal activity: 14–22 h post poly I:C/saline).

#### *2.4.2.Nociceptive responding to mechanical and cold stimuli*

The arena used for the von Frey test (VFT) and acetone drop test (ADT) consisted of a six-compartment Perspex arena (11cm × 20cm × 15cm) with wire mesh flooring as previously described [36-38]. A modified von Frey behavioural testing was performed to assess mechanical allodynia as previously described [14]. In brief, rats were habituated to the arena for at least 15min after which time an 8g von Frey filament (Touch-Test® Sensory Evaluators, North Coast Medical, Inc., Gilroy, CA, USA) was applied perpendicular to the mid-plantar surface of the hindpaw, for up to a maximum of 5 seconds or until flinching, licking or withdrawal of the paw occurred.

Testing occurred on both right and left hindpaws five times (alternating between paws for a total of ten withdrawals). Results were expressed as the percent response frequency of paw withdrawals (number of withdrawals/10 × 100). Immediately following VFT, animals were assessed for cold allodynia in the ADT. In brief, 0.2ml of acetone (Sigma-Aldrich, Dublin, Ireland) was applied to the plantar surface of the hindpaw and latency to respond within 60

seconds was recorded. A positive response was considered as a flinch, lick or withdrawal of the hindpaw. If the animal did not respond within 60 seconds, this value was taken as the latency. The average of the 3 trials was calculated for each hind-paw.

Von Frey and acetone drop testing were carried out 24hrs prior to any experimental manipulation (baseline) and 2, 4, 8 and 24hrs post poly I:C/saline injection by an experimenter blind to the treatment procedure.

*2.4.3 Open field test*

As previously described [36, 39, 40], 24hrs post saline/poly I:C administration animals were removed from the home cage placed into the centre of a brightly-lit (220 lux) novel open field arena (diameter 75cm) for 5min. The distance moved (cm) and number of transitions between the inner zone and the outer zone was assessed using a video tracking system (EthoVision® XT11.5, Noldus, Netherlands).

*2.4.4 Elevated plus maze*

The elevated plus maze (EPM) test was carried out as previously described [40]. In brief, 24hrs post poly I:C/saline administration animals were placed on the EPM for 5min and distance moved (cm), number of open and closed arm entries and the duration of time spent in the open arm(s) assessed using a video tracking system (EthoVision® XT11.5, Noldus, Netherlands).

*2.4.5 Sucrose preference test*

As previously described [30], animals were singly housed and presented with two drinking bottles in their home cage, one containing tap water and the other containing a 1% (w/v) sucrose solution for 3 days prior to the experiment and for 24hrs following poly I:C/saline injection. The bottle position was alternated each day and bottles were weighed before and after testing. Preference scores were calculated by dividing the amount of sucrose consumed by the total

amount of fluid consumed (water + sucrose) during the 24hr period and comparing against the average sucrose preference during the 3 day training period.

*2.4.5 Forced swim test*

Rats were placed into glass cylinders (height: 45cm; diameter: 20cm) containing water (30cm depth, 23-25$^{o}$C) for 15min at 26hrs post poly I:C/saline injection. Time spent immobile was assessed with the aid of a video tracking system (EthoVision® XT11.5, Noldus, Netherlands) by an experimenter blind to the treatment procedure.

### *2.5 Expression of markers of glial activation using quantitative real-time PCR*

In order to evaluate the effect of URB597 on TLR3-induced neuroinflammation, the expression of *CD11b* and *GFAP* were examined in the hypothalamus 24hrs post poly I:C, as markers of microglia/macrophage and astrocyte activation [17, 30]. The hypothalamus was selected as it is a key brain region involved in the inflammatory and sickness response to infection, including the viral TLR3-mediated response. Furthermore, the hypothalamus is an important region in the modulation of the stress response, emotional responding and nociceptive processing. The expression of CD68 and MRC2, genes up-regulated in activated microglia/macrophages with bias towards a pro-inflammatory M1 vs. a restorative/anti-inflammatory M2 phenotype respectively, were also analysed. In brief, RNA was extracted from the hypothalamic tissue using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Germany) and reverse transcribed into cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, UK). Taqman gene expression assays (Applied Biosystems, UK) were used to quantify the gene of interest and real-time PCR was performed using an ABI Prism 7500 instrument (Applied Biosystems, UK), as. Assay IDs for the genes were *CD11b* (Rn00709342_m1), *CD68* (Rn01495634_g1), *MRC2* (Rn01456616_m1) and *GFAP* (Rn00566603_m1) and β-actin was

used as an endogenous control. Relative gene expression was calculated using the ΔΔCT method and expressed as % change from vehicle-saline-treated groups.

### *2.6 Statistical Analysis*

SPSS statistical package (IBM SPSS v20.0 for Microsoft Windows; SPSS Inc., Chicago, IL, USA) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk and Levene's test, respectively. Data were analysed using repeated measures ANOVA for more than two groups over various time points or one-way ANOVA's for more than two groups on one factor. *Post-hoc* analysis was performed using Fisher's *LSD* test where appropriate. Data were analysed using unpaired t-test for two groups. The level of significance was set at $p < 0.05$. All graphs were constructed using Graphpad Prism and results are expressed as group means ± SEM.

## 3. Results

### *3.1 FAAH inhibition attenuates TLR3-mediated fever but not sickness behaviour*

In order to evaluate if enhancing FAAH substrate levels modulates TLR3-mediated acute physiological and sickness responses, the effect of systemic URB597 administration on body temperature, locomotor activity and body weight over a 24hr period following TLR3 activation using the viral mimetic poly I:C was examined. There was no significant difference in temperature at baseline between groups [one-way ANOVA; $p > 0.05$ (vehicle-saline 36.7ºC ± 0.3; vehicle-poly I:C 36.4ºC ± 0.4; URB597-poly I:C 36.8ºC ± 0.4)]. Repeated measures ANOVA revealed a significant effect of group [$F_{(1,18)} = 4.023$, $p < 0.05$] but not time or a time × group interaction on change in temperature from baseline. *Post hoc* analysis revealed that poly I:C significantly increased temperature 4hrs post administration compared to saline-treated counterparts (Fig 1a). URB597-treated animals exhibited a significant decrease in temperature 4 and 8hrs post poly I:C administration when compared to vehicle-treated counterparts (Fig 1a). Although poly I:C tended to reduce homecage locomotor activity (LMA) (0-8hrs post administration) and nocturnal homecage LMA (14-22hrs post administration) (Fig 1b,c), these effects did not reach statistical significance (one-way ANOVA; $p > 0.05$). In addition, a one-way ANOVA revealed that poly I:C significantly reduced body weight [$F_{(2,23)} = 12.756$, $p < 0.01$] over the 24hr period post administration compared to saline-treated counterparts, an effect not altered by prior URB597 administration (Fig 1d).

### *3.2 FAAH inhibition attenuates the increase in nociceptive responding to mechanical and cold stimuli following TLR3 activation*

Repeated measures ANOVA revealed a significant effect of time [$F_{(4)} = 3.564$, $p < 0.05$] and group [$F_{(1,20)} = 8.250$, $p < 0.01$] on frequency of withdrawals in the von Frey test. Subsequent *post hoc* analysis revealed that animals treated with poly I:C displayed mechanical allodynia,

expressed as an increase in response frequency to mechanical stimulation at 2hrs, 4hrs, 8hrs and 24hrs post administration compared to saline-treated controls (Fig 2a). Although URB597-poly I:C treated animals tended to display an increase in mechanical withdrawal response frequency when compared to saline-treated counterparts, this effect was not significant at 4hrs and 8hrs post poly I:C administration. Furthermore, URB597 significantly attenuated the poly I:C-induced increase in withdrawal frequency 24hrs post administration. Further statistical analysis of the area under the curve revealed that poly I:C significantly increased nociceptive responding to a mechanical stimulus compared to saline-treated controls and this effect was attenuated by URB597 [$F_{(2,22)} = 7.259$, $p < 0.01$] (Fig 2b). In the acetone drop test, poly I:C reduced the latency of animals to respond to the cold innocuous stimulus, indicating cold allodynia (Fig 2c), an effect not observed in URB597-poly I:C treated counterparts, however analysis revealed that this effect failed to reach statistical significance ($p > 0.05$). Alternative analysis using area under the curve revealed that poly I:C elicited cold allodynia, an effect not observed in URB597-treated counterparts [$F_{(2,20)} = 4.518$, $p < 0.05$] (Fig 2d).

***3.3 FAAH inhibition attenuates TLR3-mediated anxiety-, but not depressive-, like behaviour***

Anxiety-like behaviour was assessed using the elevated plus maze and open field tests, at a time when acute sickness behaviour had resolved, 24hrs post poly I:C administration. Analysis revealed that poly I:C significantly reduced the number of transitions between inner and outer zones in the open field test (OFT) [$F_{(2,19)} = 4.347$, $p < 0.05$], and reduced the amount of time spent in the open arms of the elevated plus maze (EPM) [$F_{(2,22)} = 7.293$, $p < 0.01$] compared to saline-treated counterparts, an effect attenuated by prior administration of URB597 (Fig 3a,c). Although distance moved in the OFT did not differ significantly between the groups (Fig 3b), poly I:C-treated animals exhibited a significant decrease in total distance moved in the EPM, an effect not altered by URB597 [$F_{(2,22)} = 7.324$, $p < 0.01$] (Fig 3d). Further analysis

revealed no significant difference between the groups in relation to number of open/closed arm entries or transitions into the open arms of the EPM [data not shown]. Anhedonic and despair-like/stress-coping behaviour, both measures of depressive-like behaviour, were assessed using the sucrose preference (SPT) and forced swim tests (FST). In the SPT, all animals exhibited a baseline sucrose preference of >85% prior to experimental manipulation (data not shown). Repeated measures ANOVA revealed a significant effect of time [$F_{(1)} = 14.727$, $p < 0.01$], but not group [$F_{(1,20)} = 2.951$, $p = 0.075$] or a time × group interaction [$F_{(2)} = 2.754$, $p < 0.088$] on sucrose preference over 24hrs. Subsequent *post hoc* analysis revealed that animals treated with poly I:C displayed a significant reduction in sucrose preference compared to corresponding baseline preference, indicative of anhedonic behaviour. Although the anhedonic response appeared to be enhanced in URB597-treated rats, analysis revealed that the poly I:C-induced decrease in sucrose preference was not altered by prior URB597 administration (Fig 3e). There was no effect of poly I:C and/or URB597 on total fluid intake over this period (Fig 3f). The data revealed that animals exposed to the FST exhibited a tendency for increased immobility, indicative of despair-like/stress coping behaviour, an effect not altered by prior URB597 administration, however this failed to reach statistical significance (Fig 3g).

### *3.4 FAAH inhibition attenuates TLR3-microglia/macrophage activation*

In order to determine if URB597-mediated attenuation of the poly I:C-induced fever response, changes in nociceptive responding and anxiety-like behaviour were mediated via URB597 modulation of the neuroinflammatory response, the effects of URB597 on poly I:C-induced expression of glial activation markers were examined. ANOVA revealed a significant effect on the expression of the microglia/macrophage marker CD11b [$F_{(2,20)} = 9.282$, $p < 0.01$] and the M1 pro-inflammatory microglial/macrophage marker CD68 [$F_{(2,20)} = 4.057$, $p < 0.05$], but not the M2 anti-inflammatory/restorative microglial marker MRC2 [$F_{(2,20)} = 1.901$, $p > 0.05$]

or the astrocytic activation marker GFAP [$F_{(2,20)} = 1.432$, $p > 0.05$] (Fig 4). *Post hoc* analysis revealed that the systemic administration of poly I:C significantly increased the expression of CD11b and CD68, when compared to vehicle-saline-treated counterparts, an effect attenuated by prior URB597 administration, however this failed to reach statistical significance for CD68 expression (Fig 4a,b).

### *3.5 FAAH inhibition does not alter temperature, nociceptive responding to cold stimuli or anxiety-like behaviour in the absence of TLR3 stimulation*

The data indicated that URB597 attenuates TLR3-mediated fever, nociceptive responding and anxiety-like behaviour. As several reports suggest that enhancing FAAH substrate levels may also elicit similar effects in the absence of an immune stimulus, we examined if this was the case under the experimental conditions in the current study. Analysis revealed no effect of URB597 on temperature, nociceptive responding to a cold innocuous stimulus or time in the open arms of the elevated plus maze (Fig. 5). A significant time × group interaction [$F_{(4,36)} = 4.522$, $p < 0.01$] was found for the withdrawal response frequency in the von Frey test, with *post hoc* analysis revealing that URB597 increased the withdrawal response frequency to a mechanical stimulus 24hrs post administration when compared to vehicle-treated counterparts (Fig 5b).

## 4. **Discussion**

Aberrant TLR activation may underlie a host of psychiatric and neurodegenerative disorders and thus greater understanding of TLR-associated physiological and pathophysiological responses is of significant fundamental and therapeutic importance. The results of the present

study demonstrate that the systemic administration of the FAAH inhibitor URB597 attenuated TLR3-mediated microglia/macrophage activation and some, but not all, associated behavioural changes in female rats. Essentially, TLR3 activation was associated with enhanced microglia/macrophage activation in the hypothalamus, the induction of an acute sickness response (fever, reduced body weight gain and allodynia) and longer-term changes in mechanical allodynia, anxiety- and anhedonia/depressive-like behaviour. Systemic administration of the FAAH inhibitor URB597 attenuated TLR3-mediated fever, anxiety-like behaviour and changes in nociceptive responding, an effect associated with the attenuation of TLR3-mediated M1 pro-inflammatory microglia/macrophage activation. Such effects were not observed in the absence of TLR3 stimulation and there was no effect of URB597 on TLR3-associated body weight loss or anhedonia. Taken together, these data demonstrate for the first time that the attenuation of TLR3-mediated neuroinflammatory markers by FAAH inhibition is associated with physiological and behavioural changes including temperature, nociceptive- and anxiety-like behaviour and may have implications for the treatment of neuroinflammatory disorders associated with aberrant TLR3 activation.

The data herein confirm the well documented neuroinflammatory effect of TLR3 activation following systemic administration of the viral mimetic poly I:C and the associated acute sickness response which includes fever, reduced body weight and allodynia [8, 10, 11, 13, 14]. Sickness behaviour induced following viral infection and TLR3 activation are associated with glial activation and the release of type 1 interferons (IFN-α and IFN-β) and NFκB-inducible pro-inflammatory cytokines (e.g. IL-1β, IL-6 and TNF-α) [8-10, 13, 17]. For example, cytokines including IL-1β, IL-6, TNF-α [11, 41] and the COX-$PGE_2$ pathway have been shown to be pivotal mediators of the TLR3 febrile response [42, 43]. In addition to the acute neuroinflammatory response, TLR3 activation has been shown to result in the increased

expression of the microglia/macrophage activation marker CD11b [17, 44], reduced BDNF and increased indoleamine 2, 3, dioxygenase (IDO) and kynurenine in the CNS [17], effects which may underlie long-term neuronal and behavioural changes. Accordingly, the acute sickness response to immune stimulation in rodents has been found to resolve after 24hrs, however, lasting behavioural effects of TLR3 activation have been reported beyond this period. Anxiety-like and anhedonic behaviour [17] and enhanced mechanical nociceptive thresholds [44, 45] have been reported in rats 24hrs post poly I:C administration, effects which have been replicated and expanded upon in the current study. Accordingly, the present data demonstrated that TLR3 activation results in anxiety-like behaviour in the OFT and EPM, anhedonia in the SPT and persistent mechanical allodynia in the von Frey test at 24hrs post poly I:C administration. Thus, taken together, the current and published data highlight the short and longer-term physiological consequences of TLR3 stimulation.

Increasing evidence supports a role for FAAH, but not MAGL, substrates in the modulation of TLR3-mediated acute neuroinflammation in several brain regions [8, 9]. The current data demonstrate that the systemic administration of the FAAH inhibitor URB597 attenuates TLR3-associated increases in the microglia/macrophage activation marker CD11b and M1 pro-inflammatory microglia/macrophage marker CD68 (but not MRC2, the M2 anti-inflammatory/restorative glial activation marker) in the hypothalamus. While it remains to be determined if similar effects occur in other brain regions, URB597 has been shown to modulate the TLR3-mediated increase in expression of neuroinflammatory genes in the hypothalamus, hippocampus and frontal cortex [8, 9], although the specific genes and magnitude of the response differs between regions. Differences in the effect of FAAH inhibition on TLR3-mediated neuroinflammatory genes between brain regions may be attributed to differences in the resting state of neurons, glia or endocannabinoid activity in these regions. In accordance with the current study, FAAH inhibition also attenuates M1 microglia/macrophage activation

(CD11b and CD68) and the expression of an array of pro-inflammatory mediators following TLR4 activation [30]. Thus, taken together the data indicate that enhancing central FAAH substrate levels can attenuate both the TLR3- and TLR4-mediated activation of M1 microglia/macrophages and associated increases in inflammatory mediators in the brain. Although GFAP expression in the hypothalamus tended to be enhanced in URB597-poly I:C treated animals, analysis revealed no significant change in GFAP expression in response to poly I:C in the presence or absence of URB597. Although further histological and functional studies are required, this data suggest that the effects of URB597 on TLR3-mediated neuroinflammation and associated behavioural responding are unlikely due to the inhibition of astrocyte activity. The current data demonstrate that the physiological consequences of FAAH substrate-induced attenuation of TLR3-mediated neuroinflammation include the attenuation of the fever response, without altering other aspects of the sickness response, namely reduction in body weight. Although FAAH substrates including AEA, OEA and PEA have been shown to modulate TLR4-induced changes in temperature [27-29], this is the first study to demonstrate that enhancing FAAH substrate levels can also modulate TLR3 associated temperature changes. As the hypothalamus is a key brain region involved in TLR3-mediated fever, it is likely that FAAH substrate modulation of TLR3-mediated inflammatory processes in this region underlies the effects on temperature observed. Further studies are required in order to determine if these effects are mediated by one or a combination of FAAH substrates. However, it should be noted that $CB_1$-/- mice exhibit a robust fever response to poly I:C [46], indicating that AEA activation of $CB_1$ receptors is unlikely to be the primary mediator of the effects of URB597 on TLR3-mediated fever.

Although it is well documented that FAAH substrates can elicit analgesic effects [for review see [47, 48]] and that systemic poly I:C administration induces mechanical allodynia in rats [14], to our knowledge this is the first study to demonstrate that enhancing FAAH substrate

levels can modulate TLR3-mediated allodynia. Systemic administration of URB597 has been shown to reverse thermal hyperalgesia via $CB_1$ receptor activation [32] and mechanical hyperalgesia via $CB_1$ and $CB_2$ receptors [31] following intraplantar TLR4 activation. Thus, it is possible that enhanced FAAH substrate levels at peripheral nociceptive terminals following the systemic administration of URB597 may have modulated nociceptive input resulting in the attenuation of TLR3-induced mechanical and cold allodynia. However, previous data from our group have indicated that the systemic administration of URB597 fails to modulate TLR-induced inflammatory responses peripherally, but potently reduces neuroinflammatory tone [8, 9]. Furthermore, microinjection of IL-1β and $PGE_2$ into the preoptic area of the hypothalamus has been shown to induce thermal hyperalgesia [49, 50] and systemic LPS-induced thermal hyperalgesia was abolished by the administration of a COX-2 inhibitor into the preoptic area of the hypothalamus [51]. Thus, it is possible that the attenuation of TLR3-mediated microglial activation and neuroinflammation in key supraspinal sites such as the hypothalamus may mediate, at least in part, the anti-nociceptive effects of URB597 observed in the current study.

In addition to the attenuation of TLR3-mediated fever and allodynia, the current study demonstrates that URB597 also attenuates TLR3-associated anxiety-like behaviour, without significantly altering anhedonia or stress-coping behaviour (FST). The effects of URB597 on TLR3-induced anxiety-related behaviour were not confounded by effects on locomotor activity as URB597 did not alter the locomotor activity of poly I:C treated rats in the homecage, OFT or EPM. Recent work by our group has shown that FAAH inhibition attenuates TLR4-mediated anhedonia, but not sickness-like behaviour [30]. Thus, taken together the data indicate that enhancing FAAH substrate levels can elicit differential physiological and behavioural effects in response to TLR3 vs. TLR4 stimulation. Furthermore, individual FAAH substrates may have specific effects on the neuroinflammatory and/or behavioural effects observed following TLR stimulation. For example, OEA and PEA, have been shown to attenuate TLR4-mediated

neuroinflammatory responses, however only OEA, but not PEA, attenuated the associated anhedonia [28]. Future studies will determine if the effects of URB597 on TLR3-mediated neuroinflammation and behavioural alterations observed in the current study are mediated by one or a combination of FAAH substrates. Acute systemic administration of URB597 has been previously shown to reduce anxiety-like behaviour in the EPM [20, 21, 23-25, 52] and OFT [21]. However, the anxiolytic-like effects of FAAH inhibitors in these paradigms are primarily evaluated at a time when FAAH substrate levels would be maximally enhanced (30min - 2hrs post administration) and to our knowledge no studies have investigated the effect of a single acute administration of URB597 on anxiety-like behaviour in the EPM and OFT at time points beyond 2hrs. Accordingly, the current study demonstrated that acute systemic administration of URB597 does not alter anxiety-like behaviour in the EPM 24hrs post administration, however TLR3-mediated increases in anxiety-like behaviour were found to be attenuated at this time point. As highlighted earlier, TLR3-mediated acute neuroinflammation is followed by longer term changes in microglia/macrophage activation, activation of the kynurenic pathway, reduced BDNF and altered neuronal activity [17, 44]. Thus, it is likely FAAH substrate-induced attenuation of acute TLR3-mediated neuroinflammation results in the inhibition of these longer term changes in neuronal and glial activity in key brain areas that underlie anxiety-, but not depressive-, like behavioural responses. This hypothesis is supported by the data demonstrating that systemic administration of URB597 is associated with an attenuation of the early expression of TLR3-mediated increases in IRF3- and NFκB-related inflammatory mediators in the brain [8, 9], an effect we have now shown to be accompanied by a later suppression of CD11b and CD68 expression (markers of M1 microglia/macrophage activation) 24hrs post TLR3 activation.

It should be noted that the current studies were performed in female rats. Although the oestrous cycle has been shown to modulate inflammatory processes in the periphery, data from our group has demonstrated that the phase of the oestrus cycle does not significantly alter TLR3-mediated neuroinflammatory responses [8] or fever (unpublished data). Thus, oestrous cycle is unlikely to be a significant confounding factor in the behavioural changes observed in this study. In addition, while possible sex differences in the effects of URB597 on physiological and behavioural changes cannot be ruled out, previous data has demonstrated that the acute TLR3-mediated neuroinflammatory response in the hypothalamus and the subsequent development of fever, reduced locomotor activity and body weight do not differ between male and female rats [8]. Furthermore, URB597-induced attenuation of TLR3-mediated neuroinflammatory responses in the hypothalamus was similar in male and female rats [8]. Although further studies are required, the data suggest that FAAH-mediated modulation of TLR3-induced neuroinflammation and the resultant attenuation of fever, nociceptive responding and anxiety-like behaviour may be sex-independent.

In conclusion, the current data provide further evidence for a role of FAAH substrates in the modulation of neuroinflammatory processes in response to TLR3 activation and expands on this to include the physiological and behavioural consequences of such modulation. Overall, these findings may have implications for the development of FAAH inhibitors as a novel therapeutic strategy for pain and anxiety disorders with a neuroinflammatory component.

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**Declaration of Interest**: none

**Author's roles**

LF, MR and DF were involved in the experimental design and writing the manuscript. LF, DK and MR were involved in conducting and analysing the experiments. All authors have read and approved the manuscript

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## Figure Legends

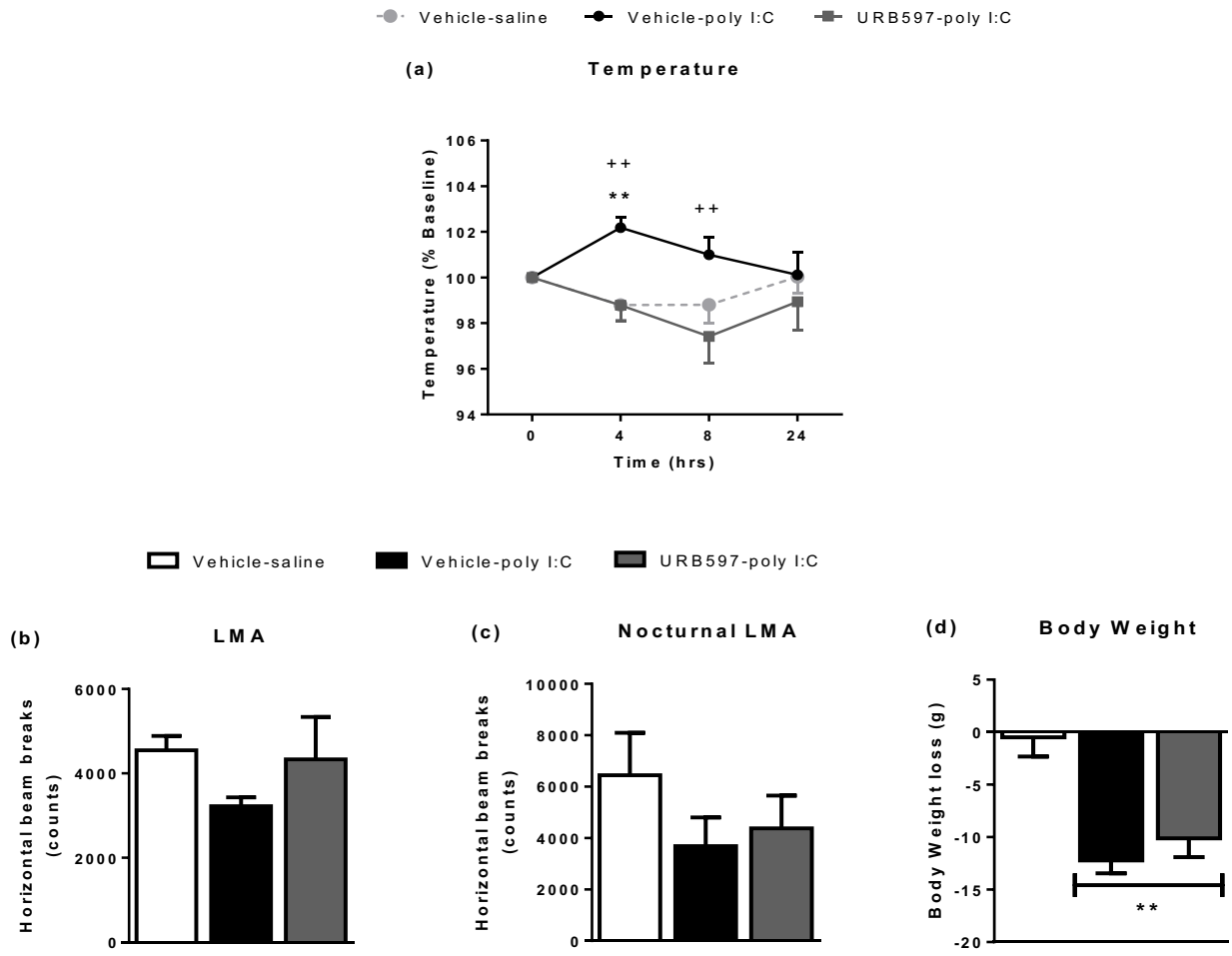


**Figure 1** The effect of systemic administration of URB597 on poly I:C-induced changes in (a) temperature, (b) homecage locomotor activity (0-8hrs), (c) nocturnal locomotor activity and (d) body weight loss over 24hrs post poly I:C administration. Data expressed as mean ± SEM (n = 4-8 per group). ** $p < 0.01$ vs vehicle-saline-treated counterparts. ++ $p < 0.01$; + $p < 0.05$ vs vehicle-poly I:C-treated counterparts.

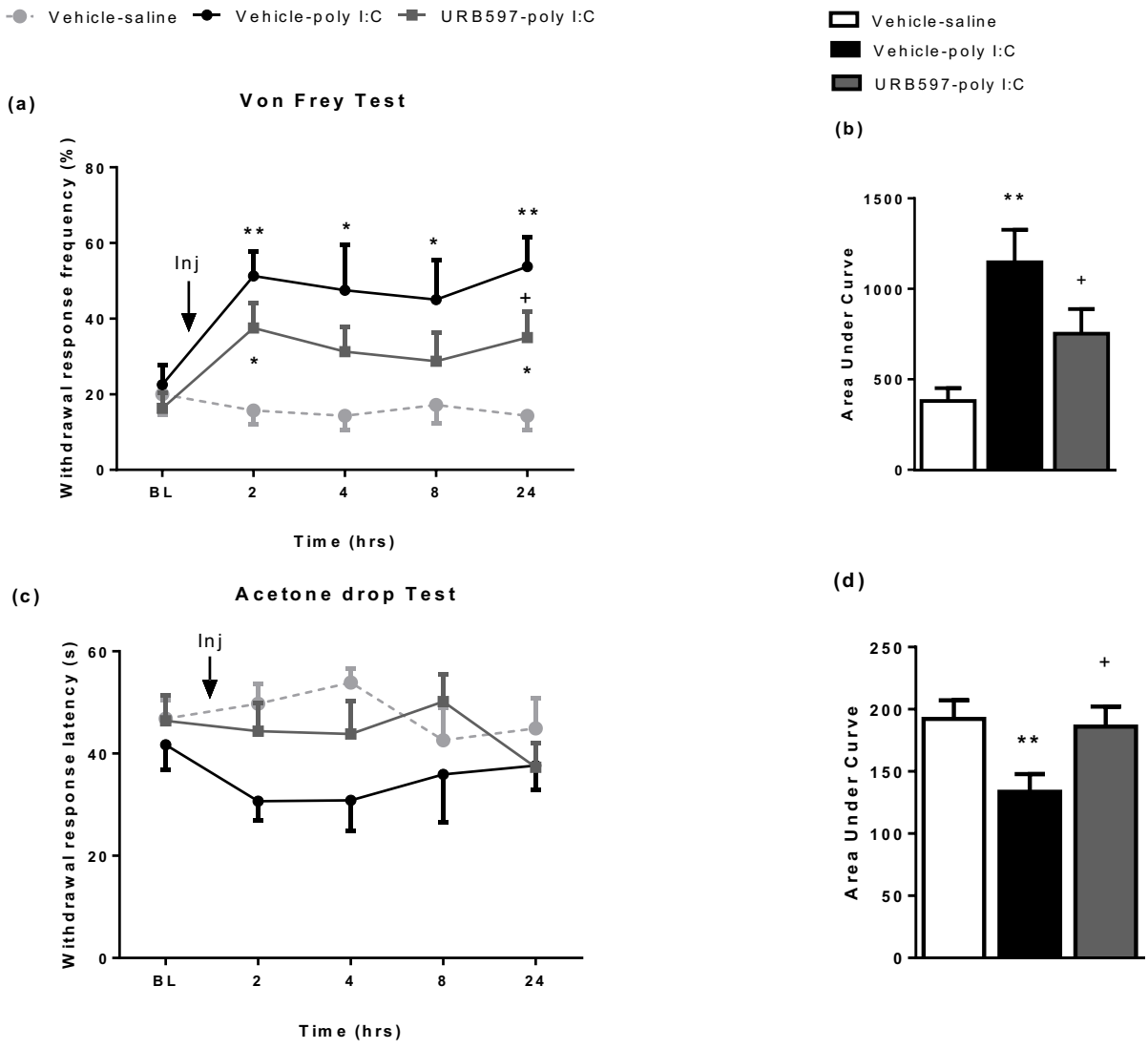


**Figure 2** The effect of systemic URB597 administration on poly I:C-induced (a, b) mechanical allodynia in the von Frey test and (c, d) cold allodynia in the acetone drop test at 2hrs, 4hrs, 8hrs and 24hrs post poly I:C administration. Data expressed as mean ± SEM (n = 8-9 per group). ** $p < 0.01$; * $p < 0.05$ vs. vehicle-saline-treated animals. + $p < 0.05$ vs. vehicle-poly I:C-treated counterparts. BL: Baseline.

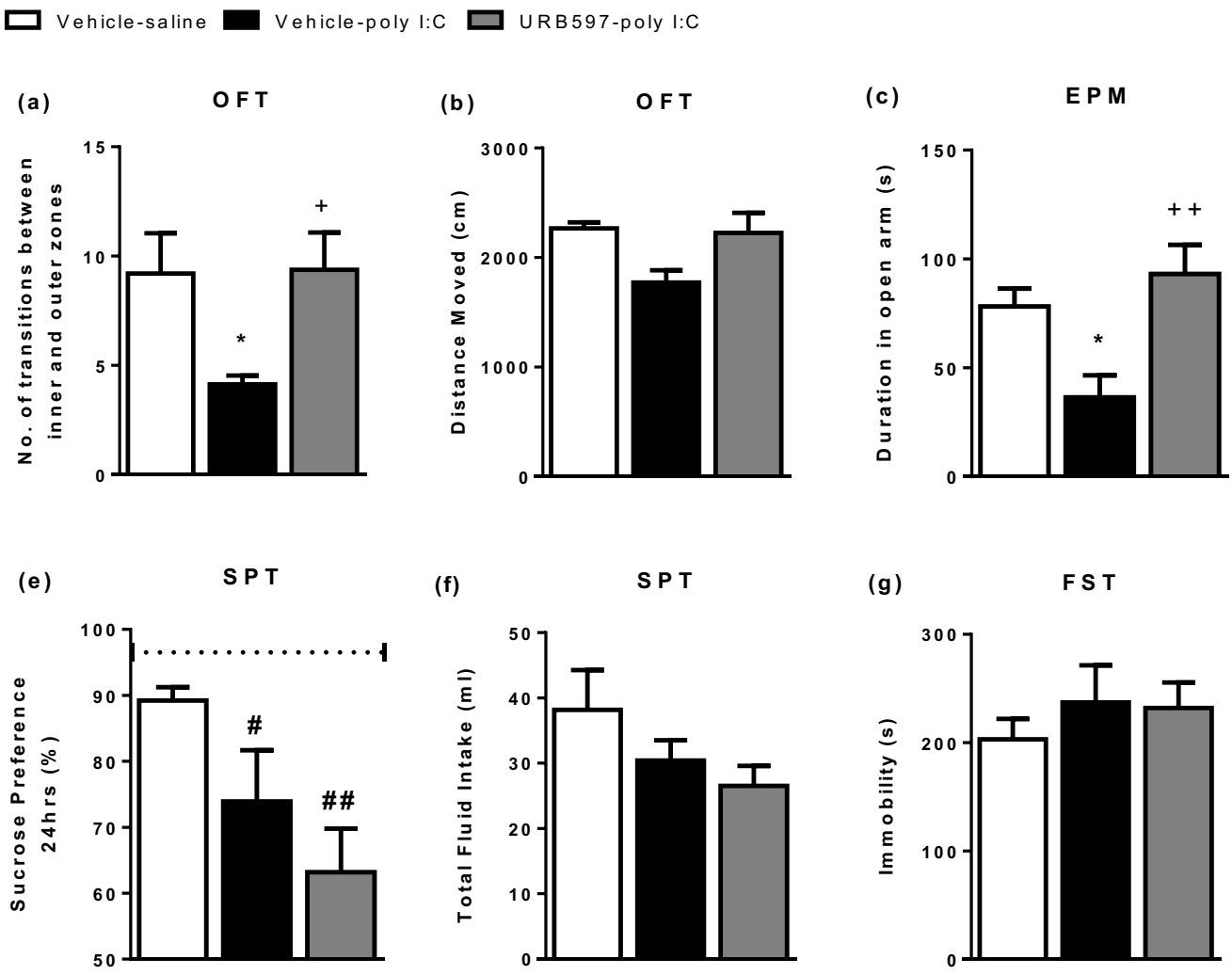


**Figure 3** The effect of URB597 and/or poly I:C on anxiety-like behaviour and locomotor activity. (a) number of transition to inner arena and (b) distanced moved in the open field test (OFT). (c) Duration of time in open arms and (d) distance moved in the elevated plus maze (EPM). Anhedonic-like behaviour was assessed by measuring (e) sucrose preference and (f) total fluid intake in the sucrose preference test (SPT). Stress coping behaviour was assessed as (g) immobility in the forced swim test (FST). Data expressed as mean ± SEM (n = 4-9 per group). ** $p < 0.01$; * $p < 0.05$ vs vehicle-saline-treated counterparts. ++ $p < 0.01$; + $p < 0.05$ vs vehicle-poly I:C-treated counterparts. ## $p < 0.01$; # $p < 0.05$ vs baseline (dotted line).

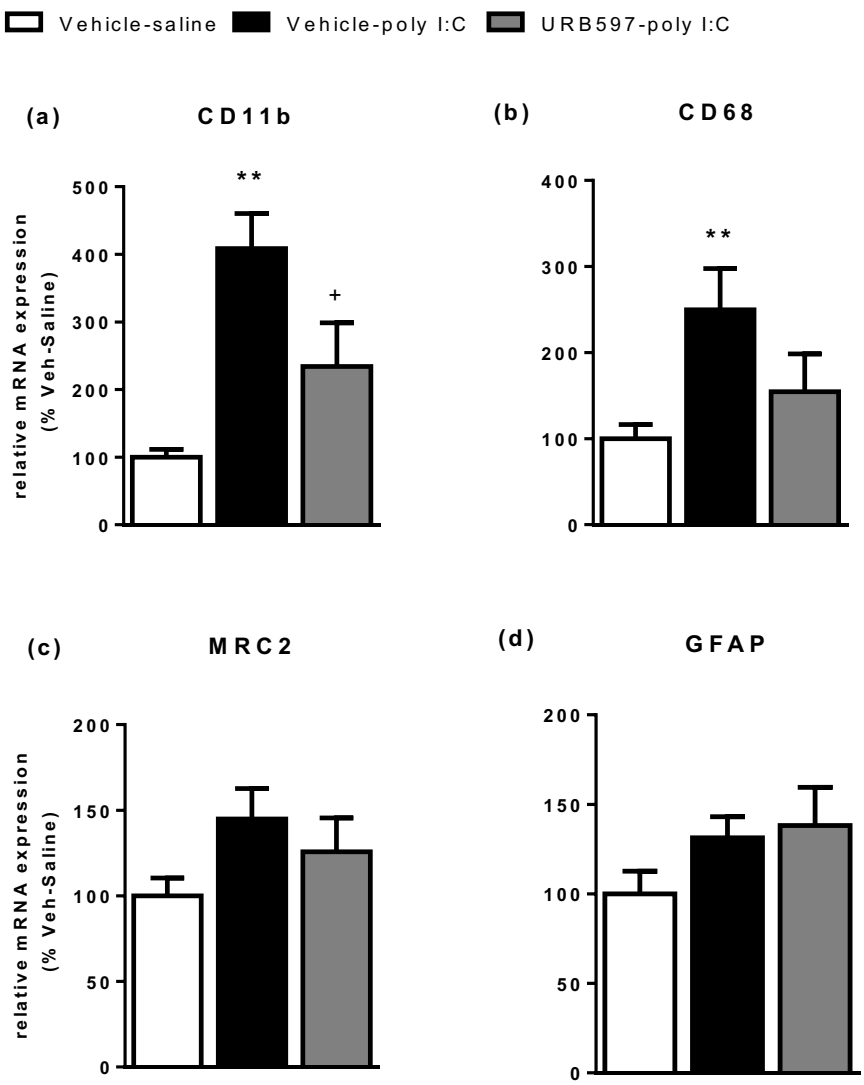


**Figure 4** The effect of URB597 and/or poly I:C on the expression (a) CD11b, (b) CD68, (c) MRC2 and (d) GFAP. Data expressed as mean ± SEM (n = 8-9 per group). ** $p < 0.01$; * $p < 0.05$ vs vehicle-saline-treated counterparts. + $p < 0.05$ vs vehicle-poly I:C-treated counterparts.

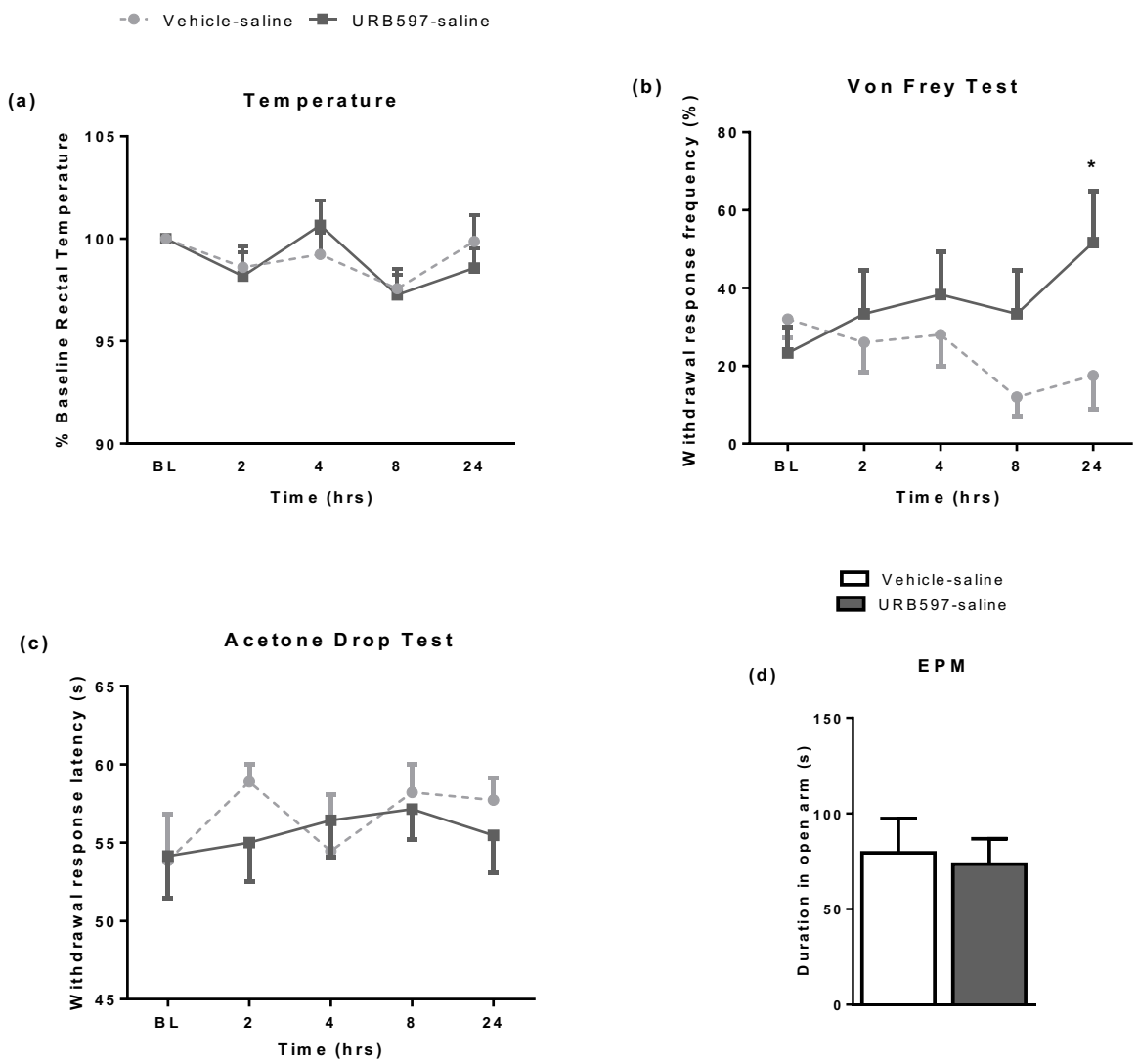


**Figure 5** The effect of URB597 on (a) temperature, (b) nociceptive responding in the von Frey test, (c) nociceptive responding in the acetone drop test and (d) anxiety-like behaviour in the elevated plus maze, in the absence of TLR3 stimulation. Data expressed as mean ± SEM (n = 5-7 per group). * $p < 0.05$ vs vehicle-saline-treated counterparts. BL: Baseline.