Repeated daily administration of increasing doses of lipopolysaccharide provides a model of sustained inflammation-induced depressive-like behaviour in mice that is independent of the NLRP3 inflammasome

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Abstract

Mounting preclinical evidence has implicated the NLRP3 inflammasome in depression-related behaviours elicited by chronic stress or acute lipopolysaccharide (LPS) challenge. However, the relevance of acute LPS as a model of depression has been questioned and behavioural time-courses of its effects can be inconsistent. The aims of this study were (1) to develop a novel protocol for repeated daily LPS administration and (2) to use this model to assess the involvement of NLRP3 inflammasome signalling in sustained inflammation-induced depressive-like behaviour in adult C57BL/6J mice deficient in NLRP3.

Acute LPS (0.83 mg/kg; *i.p.*) induced sickness behaviour evident as hypolocomotor activity. However, there was no significant increase in depressive-like behaviour in the forced swim test 24 h post-administration. Interestingly, depressive-like behaviours were observed in the female urine sniffing test and in the sucrose preference test at 24 h, but not 48 h, post-administration of acute LPS. To mimic a period of sustained inflammation, 3-day repeated increasing LPS doses (0.1, 0.42 and 0.83 mg/kg; *i.p.*) was compared to constant LPS doses (0.83 mg/kg; i.p.). Sickness behaviour was seen in response to increasing doses, but tolerance developed to repeated constant doses of LPS. Furthermore, 3-day increasing doses of LPS resulted in a significant increase in immobility time in the forced swim test, consistent with depressive-like behaviour. When NLRP3$^{-/-}$ mice received this 3-day increasing dose regimen of LPS, sickness behaviours were attenuated compared to wild-type mice. The behaviour in the forced swim test was not significantly altered in NLRP3$^{-/-}$ mice.

We propose that this increasing repeated dosing LPS model of inflammation-induced depressive-like behaviour may better model the sustained inflammation observed in depression and may provide a more translationally relevant paradigm to study the inflammatory mechanisms that contribute to depression.

Key Words: depression; sickness; neuroinflammation; forced swim test; female urine sniffing test; C57BL/6J

Abbreviations: constant dose (CD); increasing dose (ID); lipopolysaccharide (LPS); forced swim test (FST), open field test (OFT), selective serotonin reuptake inhibitor (SSRI), sucrose preference test (SPT), female urine sniffing test (FUST), interleukin (IL), tumor necrosis factor (TNF), interferon (IFN)

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Highlights

- **A novel paradigm of sustained neuroinflammation was developed in C57BL/6 mice**
- **Repeated administration of increasing doses of lipopolysaccharide induced sickness and depressive behaviours but no tolerance over 3 days**
- **Sickness behaviours were attenuated in NLRP3$^{-/-}$ mice**
- **Studies in NLRP3$^{-/-}$ mice show this inflammasome is not required for repeated LPS-induced depressive behaviours**

1. Introduction

Neuroinflammation and the activity of cytokines have been linked with the pathology of depression (Dantzer et al., 2008; Raison et al., 2006). Based on several meta-analyses, patients with major depressive disorder exhibit elevated serum levels of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β (Dowlati et al., 2010; Hiles et al., 2012a; Howren et al., 2009; Liu et al., 2012). This elevation of pro-inflammatory cytokines has been shown to respond to antidepressant treatment, although the findings are variable. Selective serotonin reuptake inhibitors (SSRIs) have been shown to reduce serum levels of IL-1β and IL-6, with a concomitant resolution of depression symptoms (Hannestad et al., 2011; Hiles et al., 2012b). Other non-SSRI antidepressants, such as venlafaxine and duloxetine, do not appear to reduce cytokine levels although they can effectively treat depressive symptoms (Hannestad et al., 2011). This heterogeneity in response to antidepressant treatment indicates that inflammatory markers may be associated with specific subsets of depressive disorders (McNamara and Lotrich, 2012). Studies have shown that in major depressive disorder patients with attempted suicide, plasma levels of pro-inflammatory cytokines are elevated (Janelidze et al., 2011). In addition, an acute inflammatory challenge with endotoxin (derived from *E. coli*) in healthy humans can result in elevated pro-inflammatory cytokine levels and depressive symptoms (Eisenberger et al., 2010; Raison., 2006). Treatment of hepatitis C with interferon (IFN)-α induced depression in up to a third of patients where antidepressant treatments can attenuate or even prevent the development of depressive symptoms (Musselman et al., 2001). Thus a number of lines of clinical evidence implicate activation of the immune system in the pathology of depression.

The innate immune sensor nucleotide-binding domain, the leucine-rich repeat, and the pyrin domain containing protein (NLRP)3 inflammasome is a multi-protein complex that is responsible for the cleavage of proinflammatory cytokines, proIL-1β and proIL-18, in response to a range of pathogenic and endogenous danger signals (Martinon et al., 2002; Walsh et al.,

2014). The NLRP3 inflammasome has three components: the intracellular pattern recognition NLRP3 receptor, the adaptor protein ASC and the effector protein caspase-1 (Tschopp, et al., 2003). NLRP3 detection of activating stimuli results in inflammasome formation, caspase-1 activation and IL-1β cleavage and secretion, leading to the propagation of the inflammatory response (Latz et al., 2013). The NLRP3 inflammasome has been reported to be involved in the pathology of neurological disorders that are associated with neuroinflammation, such as Alzheimer's disease and depression (Heneka et al., 2014; Iwata et al., 2013). Recent studies have demonstrated a role of NLRP3 inflammasome signalling in the development of depressive-like behaviour in mice in stress- and inflammation-based models of depression (Zhang et al., 2014; Alcocer-Gómez et al., 2016; Li et al., 2016; Su et al., 2017; Zhu et al., 2017).

Animal models for studying the interactions between the immune system and depressive-like behaviours include administration of TNF-α, IFN-α or lipopolysaccharide (LPS) (Hayley et al., 2013; Kaster et al., 2012; Lawson et al., 2013). LPS is a component of the bacterial cell wall which activates microglia and induces neuroinflammation (Lawson et al., 2013; Lee et al., 2008). Acute LPS, administered systemically in mice and/or rats, induces a strong but transient sickness behaviour, evident as reduced locomotor activity and decreased body weight (Castanon et al., 2001; Kozak et al., 1994; O'Connor et al., 2009). This sickness is associated with elevated levels of pro-inflammatory cytokines within the brain, including TNF-α and IL-1β, which are thought to peak around 6 h after LPS administration, though elevated TNF-α expression in the brain has been reported to last up to 10 months (O'Connor et al., 2009; Qin et al., 2007; Quan et al., 1998). Once the overt symptoms of sickness have subsided, depressive-like behaviours can be assessed using behavioural paradigms such as the forced swim test (FST) and the sucrose preference test (SPT), typically at 24 h after acute LPS administration (Lawson et al., 2013; O'Connor et al., 2009). However, the time course of these effects varies across the literature and it is often difficult to distinguish sickness from

depressive-like behaviours. In mice, acute LPS-induced sickness has been reported to have disappeared by 24 h by some (Frenois et al., 2007; Gibb et al., 2013; Mello et al., 2013; O'Connor et al., 2009; Ohgi et al., 2013; Viana et al., 2010; Walker et al., 2013), and not by others (Biesmans et al., 2013; de Paiva et al., 2010; Godbout et al., 2005; Slalzar et al., 2012), whilst some even report sickness at 48 h (Corona et al., 2010; Godbout et al., 2008). Furthermore, the time-course of LPS-induced depressive-like behaviours is also variable, with some being reported as late as 72 h (Corona et al., 2013). Variation in housing (single or group housing) (Bogdanova et al., 2013; Karolewicz and Paul, 2001) and testing conditions (light cycle phase) (Huynh et al., 2011; Verma et al., 2010) may contribute to the inconsistency seen in the literature, although the effects of such conditions on LPS-induced behavioural changes has not been systematically assessed.

Acute administration of LPS is routinely used to study inflammation-induced depressive-like behaviours in rodents, however this may not be the best model. Depression is considered a chronic recurrent disorder, characterized by repeated or prolonged depressive episodes and sustained levels of inflammatory markers (Kling et al., 2007). In order to provide a representative inflammation-based model with more translatability, prolonged immune system activation would be required. In an attempt to mimic chronic inflammation, Kubera et al (2013) developed a repeated, intermittent schedule for LPS administration, reporting sustained anhedonia in the SPT in female mice, which was reversed with fluoxetine. Other studies have utilised repeated administration of a constant dose of LPS in mice to induce a depressive-like behaviour in the FST, including 8 injections of 0.1 mg/kg LPS (Xie et al., 2012) and 10 injections of 0.83 mg/kg LPS (Guo et al., 2014). However, repeated LPS can induce the development of tolerance resulting in a diminished behavioural response (Engeland et al., 2001).

Mounting evidence has suggested that NLRP3 signalling could be a good target for developing novel antidepressants. This is largely based on preclinical studies showing that the NLRP3 inflammasome contributes to depression-related behaviours elicited by acute LPS challenge or chronic stress (Zhang et al., 2014; Alcocer-Gómez et al., 2016; Li et al., 2016; Su et al., 2017; Zhu et al., 2017). However, the involvement of NLRP3 in the behavioural changes in response to sustained inflammatory challenge has not been reported. The aims of this study were (1) to develop and characterize a novel protocol for repeated daily LPS administration and (2) to use this model to assess the involvement of NLRP3 inflammasome signalling in sustained inflammation-induced depressive-like behaviour. In developing this model we also attempted to replicate acute LPS-induced behavioural effects under a variety of environmental conditions including group versus single housing and behavioural assessment in light or dark phases. The involvement of NLRP3 was investigated using NLRP3$^{-/-}$ mice in which the entire coding region of the NLR family, pyrin domain containing 3 (*Nlrp3*) is replaced, abolishing expression (Kovarova et al. 2012).

2. Methods

2.1. Animals

The majority of the experiments reported here were conducted at Janssen Pharmaceutica facilities, accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, and approved by the Institutional Ethical Committee on Animal Experimentation, in compliance with Belgian law (Royal Decree on the protection of laboratory animals dd. April 6, 2010). Experiments on NLRP3$^{-/-}$ mice were conducted at the University of Bath under a Home Office project license held in accordance with the Animals (Scientific Procedures) Act 1986 and European Directive 2010/63/EU.

Experiments at Janssen were performed on 10-14 week old male C57BL/6J mice obtained from Charles River (France). Mice were normally housed in groups of 2-4 except when they were singly housed as indicated in the test. Cages (L x W x H: 36 X 20 X 13 cm) contained wood shavings, nesting material and a plastic shelter (Mouse hut^{TM}, Bio-Serve; L x W x H: 9.5 x 7.6 x 4.8 cm), with access to food and water *ad libitum.* Mice were under a 12 h light cycle, with normal lighting conditions being lights on at 06:00 h and reversed lighting conditions being lights on at 18:00 h, with 30 minute dim/rise phases. Mice were acclimatised to housing conditions, including transitioning to a reversed light cycle upon arrival, for a minimum of 2 weeks prior to experimentation. Temperature was maintained at 22 ± 2 °C and humidity at 50 ± 2 %.

Experiments at the University of Bath were similarly performed on 10-14 week old male C57BL/6J or NLRP3 homozygous knockout mice on a C57BL/6J background first developed by Kovarova et al., (2012) (B6.129S6-Nlrp3$^{<tm1Bhk>}$/J - ref:021302; Jackson Laboratory, Maine, US via Charles River, Margate, UK). On arrival, both NLRP3$^{+/+}$ and NLRP3$^{-/-}$ mice were bred for at least one generation to establish the colony and were maintained in homozygous colonies. As at Janssen, mice were normally housed in groups of 3-4, in cages (L x W x H: 35 X 20 X 15 cm) containing wood shavings, nesting material and a plastic shelf, with food and

water available *ad libitum*. Mice were under a 12-hour light cycle (lights on at 07:00 h). Temperature was maintained at 21 ± 1 °C and humidity at 50 – 60 %.

All mice were handled daily for one week prior to random assignment to treatment groups and experimentation. For all behavioural studies mice were acclimatized to the behavioural room for 1 hour prior to testing. Where group or individual housing was compared, animals were individually housed for 2 weeks prior to treatment. Where normal lighting (lights on 06:00 h) was compared to reversed lighting (lights on 18:00 h) animals were maintained under these conditions for a minimum of 2 weeks prior to starting treatment. All behavioural experiments were performed between 08:00 and 17:00, under the same lighting conditions as the animals were housed under; so normal lighting meant that animals were tested in the light and reversed lighting meant that animals were tested in the dark.

2.2. Treatments

Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 055:B5, Sigma-Aldrich) was prepared fresh in sterile saline (0.9 % NaCl) prior to intraperitoneal injection. All injections were administered at a volume of 10 ml/kg. LPS doses used were within the dose range commonly used in studies of depressive-like behaviour and based on previously observed acute LPS-induced depressive-like behaviours in mice (O'Connor et al., 2009). For acute studies, mice were injected with 0.415 or 0.83 mg/kg LPS or saline. For repeated LPS studies, there were four different groups that were treated for 3 or 5 days: control (saline was injected each day); acute LPS (LPS was administered only on the final day); constant dose (CD) LPS (0.83 mg/kg LPS was injected each day) or increasing dose (ID) LPS. For ID LPS, the following doses of LPS were used: 0.052 / 0.104 / 0.208 / 0.415 / 0.83 mg/kg (5-day) or 0.208 / 0.415 / 0.83 mg/kg (3-day). Timings of injections were staggered throughout the day to enable behavioural testing after LPS administration at +6 h and at + 24h. Body weights were recorded daily prior to

injections which took place 08:00-16:00 h. Body weight is presented as % of starting weight prior to LPS administration.

2.3. Open Field Test (OFT)

The OFT protocol used was as described previously at Janssen (Biesmans et al., 2013) and the University of Bath (Almatroudi et al., 2015). At Janssen, a custom-made experimental apparatus consisted of 4 separate arenas (each 40 X 40 X 40 cm), allowing 4 mice to be tested simultaneously. An infrared camera was mounted above each arena to track the mice using Noldus EthoVision (version 6.1). At the University of Bath, the experimental apparatus consisted of a single arena (L x W x H: 41 X 41 X 38 cm) with 32 infrared photobeams (16X x 16Y) to detect locomotion (Openfield SmartFrame, Campden Instruments). The total distance travelled within the arena during a 10 min low light (<10 lux) test was recorded via beam-breaks using Motor Monitor software (Campden Instruments). Tracking began 2 seconds after placing the mouse in the centre of the arena and arenas were cleaned with 70 % ethanol in between each mouse.

2.4. Forced Swim Test (FST)

The FST protocol used was as described previously at Janssen (Biesmans et al., 2013) and the University of Bath (Almatroudi et al., 2015). At Janssen, a custom-made experimental apparatus consisted of 4 separate cylinders (11 cm diameter and 10 cm deep water), which were automatically washed and filled with water at 24-25 ˚C between each mouse. At the University of Bath, the experimental apparatus consisted of a glass cylinder (44 cm h x 22 cm diameter), which was cleaned with 70 % ethanol, rinsed and filled with water to a depth of 30 cm at 25 ± 1 ˚C between each test. At both locations, mice were placed in the cylinders for 6 minutes whilst a fixed camera was used to record the mice. Immobility was manually scored, blind to treatment, over the 6 min test period.

2.5. Rotarod

Mice were assessed in two test sessions: a 'baseline' test immediately prior to LPS or saline administration and a final test at either 6, 24 or 48 hours after treatment. Mice first underwent four training sessions in succession the day before treatment where mice were placed on the rotarod for 5 minutes each time at progressively faster speeds at 30 minute intervals: 16, 20 and 24 revolutions per minute (rpm), and finally an accelerating speed from 0-40 rpm (Med Associates, model CT-ENV-575M-X5). During training, mice that had fallen off the rotarod were placed back on. For both the baseline test and the final test, an accelerating speed of 0-40 rpm in 5 minutes was used. Latency (seconds) to fall off the rotarod was recorded automatically via infrared cameras and mice were not replaced back onto the rotarod once off.

2.6. Sucrose Preference Test (SPT)

The SPT protocol used was as described previously (Biesmans et al., 2013). Animals were housed individually in customized Plexiglas cages (35 X 31 X 16 cm; Techniplast, Italy) that fitted two water bottles. Bottles were filled with either tap water (W) or 2.5 % sucrose dissolved in tap water (S) during the habituation phase prior to a testing phase. During the habituation phase, mice were housed with a pair of water bottles (W/W) or a water bottle plus a bottle with sucrose solution (W/S) alternating for 24-hour periods over 4 days. The left versus right location of the sucrose bottle was randomised across the experimental procedure. Bottles were removed at the same time every morning (09:00-10:00 h) and fluid consumption was determined by weighing the bottles. Mice were then weighed and placed back in their cage with fresh pre-weighed bottles containing the appropriate solutions. The test phase was carried out for 2 days immediately following the administration of LPS or saline. Total consumption was calculated (water and sucrose consumption combined) as well as the preference for

sucrose (sucrose consumption as percentage of total consumption). In the event of leaking bottles, values were replaced by the mean of all bottles for the appropriate solution for that time period. This happened in less than 5 % of all bottle measurements.

2.7. Female Urine Sniffing Test (FUST)

The FUST has previously been validated using the learned helplessness (LH) model of depressive behaviour and the action of the antidepressant citalopram in mice (Malkesman et al., 2010). Prior to the experiment, female adult mice were housed in cages of 4 with grid floors for 2 hours to collect urine. 4 cotton-bud applicators were soaked in test tubes containing female urine or water for use throughout the test. Male mice were weighed and placed individually in a fresh cage (L x W x H: 36 X 20 X 13 cm) containing wood shavings and two dry cotton-bud applicators for the 30 minute habituation phase. The applicators were fixed in place by a custom-made device that clips onto one end of the cage and points the applicators down at an angle of 45 degrees and 15 cm apart, with the cotton-bud 2.5 cm above the cage floor. The protocol used here was adapted to incorporate one test period with two applicators (water and urine) as opposed to two test periods each with one applicator. For the test phase, the applicators and the holding device was removed and replaced with a new holding device with two new applicators: one soaked in water and the other soaked in female urine (left versus right was randomized). During the test phase, time spent sniffing each applicator, in which the animal was directly sniffing the bud of the applicator (biting the applicator is not counted), was manually scored with an observation timer for 3 minutes. Mice were then returned back to their home cage and the applicators placed back into the appropriate test tube for re-use. Tests were carried out in the light phase of the circadian cycle. Time spent sniffing female urine relative to water was recorded to assess anhedonic behaviour.

2.8. Statistical Analysis

Data were analysed using one-way or two-way ANOVA. When appropriate ($p < 0.05$), Dunnett posthoc tests were applied when comparing groups to only the control, or Bonferroni posthoc test when comparing between multiple treatment groups. All data are represented as mean ± SEM. Number of mice used is indicated in each figure legend. Separate groups of animals were used for the data reported in each figure where typically n=10-15 mice per treatment group.

3. Results

3.1. Effect of housing and lighting conditions on acute LPS-induced behaviours

Acute LPS has been shown to induce sickness and depressive-like behaviour in C57BL/6J adult mice (O'Connor et al., 2009). However, the influence of housing arrangements on acute LPS-induced changes has not been established. In our first experiment, separate groups of mice were housed either in groups or individually and with normal lighting (lights on 06:00 h) or reversed lighting (lights on 18:00 h) (Fig 1). A two-way ANOVA revealed a significant effect of treatment ($F_{(2,133)} = 284.7$, $p < 0.001$) and housing condition ($F_{(3,133)} = 5.56$, $p < 0.01$) on distance travelled in the OFT, but no interaction between the two ($F_{(6,133)} = 1.695$, $p = 0.127$) (Fig 1A). A significant reduction in the total distance travelled in the OFT was seen 6 h after acute administration of LPS, at both 0.415 and 0.83 mg/kg, in all housing conditions ($p < 0.001$) when compared to saline-treated controls. There were no significant differences between housing conditions in LPS-treated mice, though mice group-housed under reversed lighting and tested in the dark travelled further than mice group-housed under normal lighting and tested in the light following saline treatment ($p < 0.05$). On average, locomotion was reduced to 40 % and 32 % of control following 0.415 and 0.83 mg/kg LPS, respectively, consistent with a pronounced sickness behaviour 6 h following acute LPS administration.

Depressive-like behaviour was subsequently assessed in the FST, 24 h after acute LPS administration (Fig 1B). A two-way ANOVA revealed a significant effect of treatment ($F_{(2,131)} = 3.334$, $p < 0.05$), but no effect of housing ($F_{(3,131)} = 0.4783$, $p = 0.6979$) and no interaction ($F_{(6,131)} = 1.665$, $p = 0.1345$). Following post hoc analysis, no group showed a significant increase in FST immobility following LPS administration compared to saline-treated mice. In subsequent experiments with acute LPS all mice were group housed under normal lighting conditions (lights on 06:00 h) and tested in the light phase unless otherwise stated.

3.2. Time course of acute LPS-induced behavioural effects

We investigated whether changes in sickness behaviour or depressive-like behaviours induced by acute LPS administration were evident at later time points (Figure 2). At 24 h post-administration of LPS, there was a significant effect of LPS on distance travelled in the OFT (Fig. 2A; $F_{(2,49)} = 8.621$, $p < 0.001$). Both 0.415 and 0.83 mg/kg LPS produced a significant reduction in total distance travelled in the OFT to 81% and 79% of control, respectively ($p < 0.01$ and $p < 0.001$, respectively). There was also a significant effect of LPS on immobility in the FST 48 hours after injection (Fig. 2B; $F_{(2,25)} = 7.727$, $p < 0.01$), although no group exhibited significantly greater immobility levels compared to control. The rotarod test was also used to assess motor coordination at 6, 24 and 48 h after acute LPS administration (separate groups) and a two-way ANOVA revealed a significant effect of treatment ($F_{(1,54)} = 13.22$, $p < 0.001$; Fig. 2C; Supplementary Fig 2). Bonferroni posthoc analysis revealed a reduction in performance compared to baseline 6 h after LPS administration ($p < 0.05$), but not at 24 or 48 h after administration.

3.3. Effects of acute LPS on anhedonic behaviours

The SPT and FUST were used as additional methods of testing depressive-like behaviours, in particular, anhedonic behaviour. In the SPT, a significant effect of acute LPS administration was observed with both total volume consumed ($F_{(2,43)} = 34.31$, $p < 0.001$; Fig.. 3A) and sucrose preference ($F_{(2,43)} = 4.941$, $p < 0.001$; Fig. 3B). Both doses of LPS tested, 0.415 and 0.83 mg/kg, produced a reduction in volume consumed to 56% and 42% of control and sucrose preference to 81% and 79% of control, respectively, in the first 24 h after injection (all Ps < 0.001). Interestingly, no significant reductions in volume consumed or sucrose preference were observed from 24-48 h after injection.

The FUST was used to assess sexually motivated behaviour at 6 and 24 h (separate mice) after acute LPS administration (Fig. 4 A, B). In pilot studies, male mice showed a significant preference for sniffing at male urine over water ($p < 0.05$) and male mice also

showed a significant preference for female urine over male urine ($p < 0.001$) (Supplementary Fig. 1). These data indicate that the preference towards female urine is partially sex-based not purely an olfactory stimulus. At 6 h, a 2-way ANOVA revealed a significant interaction between treatment and odour ($F_{(2,17)} = 11.51$, $p = 0.0007$), with saline-injected control mice spending significantly more time sniffing urine when compared to water ($p < 0.001$), but not in mice treated with 0.415 or 0.83 mg/kg LPS. Furthermore, time spent sniffing urine was significantly reduced to 23% and 17% following 0.415 or 0.83 mg/kg LPS treatments, respectively, when compared to control mice ($p < 0.001$ for both). Similarly, there was a significant interaction between treatment and odour at 24 h post-administration of LPS ($F_{(2,17)} = 6.932$, p = 0.0063). Again, saline-injected control mice spent significantly more time sniffing urine compared to water ($p < 0.001$) and this effect was also evident in both 0.415 and 0.83 mg/kg LPS treated groups ($p < 0.001$ and $p < 0.05$, respectively). However, at 24 h post-LPS administration, time spent sniffing urine was significantly reduced to 67% and 48% following 0.415 or 0.83 mg/kg LPS treatments, respectively, when compared to control mice ($p < 0.01$ and $p < 0.001$, respectively). These results demonstrate that acute LPS administration produced a significant effect on behaviour in both the SPT and FUST 24 h post-administration which may reflect a decrease in hedonic drive.

3.4. Effects of repeated daily LPS administration for 3 days and 5 days

In order to develop a protocol for assessing the effects of sustained inflammatory challenge on depression-related behaviours, 3-day and 5-day repeated daily dosing with LPS was carried out. Constant dosing (CD) of 0.83 mg/kg and increasing dosing (ID) doubling up to 0.83 mg/kg on final day schedules were used to identify whether tolerance to repeated administration of LPS developed. Sickness behaviour was assessed by locomotor behaviour in the OFT 6 h after the final injections and depressive-like behaviour in the FST investigated 24 h after the final injection. In the 3-day experiment, there was a significant effect of LPS

treatment on body weight (Fig. 5A; $F_{(3,52)}$ = 160.9, p < 0.001) and distance travelled in the OFT ($F_{(3,52)}$ = 107.9, p < 0.001; Fig. 5B). Body weight at the start of the experiment was 24.5 ± 0.5 g (n=14) for saline, 24.2 ± 0.3 g (n=14) for acute LPS, 24.6 ± 0.3 g (n=14) for CD LPS and 24.9 ± 0.5 g (n=14) for ID LPS. At 24 h after the final injection, body weights were increased by 0.4% in the saline group, reduced by 8.6% for acute LPS, 12.1% for CD LPS and 11.9% for ID LPS (Figure 5A). A significant reduction in the distance travelled in the OFT was evident for all LPS-treated groups in the 3-day experiment (all p < 0.001). Taken together, the reduction in body weight and the reduction in locomotion are consistent with sickness behaviour following LPS administration. Interestingly, the CD LPS mice significantly showed less reduction in locomotion (61% of control) compared to that seen in either the ID LPS (38%) or acute LPS (37%) treated mice (p < 0.001). This observation may reflect the development of tolerance to CD LPS in sickness behaviour. Furthermore, after 3 days treatment, there was a significant effect of LPS on FST immobility (Fig. 5C; $F_{(3,52)}$ = 3.919, p < 0.05), with only ID LPS treatment causing a significant increase of 66% in immobility (p < 0.05).

In the 5-day experiment, again there was a significant effect of LPS treatment on body weight (Fig. 5D; $F_{(3,66)}$ = 191.4, p < 0.001) and distance travelled in the OFT (Fig. 5E; $F_{(3,66)}$ = 31.47, p < 0.001). Body weight at the start of the experiment was 25.1 ± 0.3 g (n=20) for saline, 24.7 ± 0.3 g (n=19) for acute LPS, 24.7 ± 0.3 g (n=20) for CD LPS and 25.1 ± 0.4 g (n=11) for ID LPS. At 24 h after the final injection, body weights were increased by 1.8% in the saline group and reduced by 7.1% for acute LPS, 6.0% for CD LPS and 4.5% for ID LPS. A significant reduction in the distance travelled in the OFT was evident for all LPS-treated groups in the 5-day experiment (all p < 0.001). The reduction in body weight and the reduction in locomotion indicate sickness behaviour was present during the 5-day LPS administration. However, the reduction in locomotion observed following acute LPS (47% of control) was significantly greater than the reductions seen in both CD LPS (75%; p < 0.001) and ID LPS (66%; p < 0.01). This finding suggests the development of tolerance to both constant and increasing doses of LPS

in sickness behaviour. There was no significant effect of LPS on FST immobility in the 5-day treatments (Fig. 5F; $F_{(3,62)} = 1.716$, $p = 0.1728$).

3.4. Effects of NLRP3 on sickness and depressive-like behaviour following acute and 3-day ID LPS

The 3-day ID LPS protocol was applied to NLRP3$^{-/-}$ and NLRP3$^{+/+}$ mice to study the influence of NLRP3 in the development of LPS-induced sickness and depressive-like behaviours (Fig. 6). A significant reduction in body weight was observed in all LPS-treated groups ($p < 0.001$ for all), with significantly lower body weights seen following 3-day ID LPS compared to acute LPS in both NLRP3$^{+/+}$ and NLRP3$^{-/-}$ mice ($p < 0.001$ for both, Fig. 6A). In addition, following 3-day ID LPS, body weight was significantly greater in NLRP3$^{-/-}$ mice when compared to NLRP3$^{+/+}$ mice ($p < 0.05$). Furthermore, at +24 h after the final injection, a two-way ANOVA of body weight revealed a significant effect of treatment ($F_{(2,138)} = 658.4$, $p < 0.001$) and genotype ($F_{(1,138)} = 8.716$, $p < 0.01$), though no significant interaction between treatment and genotype ($F_{(2,138)} = 2.872$, $p = 0.06$). In the OFT at +6 h after the final injection, a two-way ANOVA revealed a significant effect of treatment ($F_{(2,71)} = 70.14$, $p < 0.001$) and genotype ($F_{(1,71)} = 33.93$, $p < 0.001$), and a significant interaction between treatment and genotype ($F_{(2,71)} = 5.536$, $p < 0.01$), on the distance travelled (Fig. 6B). Whilst a significant reduction in distance travelled was observed in all LPS-treated groups ($p < 0.001$ for all), the reduction was attenuated in NLRP3$^{-/-}$ mice following both acute LPS ($p < 0.001$) and 3-day ID LPS ($p < 0.01$) in comparison to NLRP3$^{+/+}$ controls. As before, there was no significant difference between acute LPS and 3-day ID LPS, indicating the avoidance of LPS tolerance. At +24 h after the final injection (Fig. 6C), a two-way ANOVA revealed a significant effect of treatment ($F_{(2,61)} = 11.49$, $p < 0.001$) and genotype ($F_{(1,61)} = 6.047$, $p < 0.05$), and a significant interaction between treatment and genotype ($F_{(2,61)} = 8.113$, $p < 0.001$), on distance travelled in the OFT. Significant reductions in distance travelled were observed following both acute LPS and 3-day ID LPS in NLRP3$^{+/+}$

mice ($p < 0.001$ for both), but no significant reductions in NLRP3$^{-/-}$ mice. In addition, following 3-day ID LPS, the distance travelled was significantly lower in NLRP3$^{+/+}$ mice when compared to NLRP3$^{-/-}$ mice ($p < 0.01$). These data show that 3-day LPS-induced sickness behaviour, evident as a body weight reduction and decreased locomotion in the OFT, is attenuated in NLRP3$^{-/-}$ mice when compared to NLRP3$^{+/+}$ mice. Furthermore, the data demonstrate that NLRP3$^{+/+}$ wildtype mice still showed sickness behaviour in the OFT +24 h after treatment whereas NLRP3$^{-/-}$ mice did not.

A two-way ANOVA of time spent immobile in the FST at +24 h after the final LPS injection (Fig. 6E) revealed a significant effect of treatment ($F_{(2,71)} = 11.01$, $p < 0.001$), although there was no significant effect of genotype ($F_{(2,71)} = 1.822$, $p = 0.1813$) or interaction between these factors ($F_{(2,61)} = 0.2544$, $p = 0.7761$). Post hoc analysis revealed a significant increase in FST immobility following 3-day ID LPS in both NLRP3$^{+/+}$ mice and NLRP3$^{-/-}$ mice ($p < 0.01$ and $p < 0.05$, respectively). This finding indicates that 3-day ID LPS induced a depressive-like behaviour in the FST at +24 h after treatment in both sets of mice, independent of NLRP3 genotype.

4. Discussion

Emerging evidence suggests a role for the NLRP3 inflammasome in depression and targeting NLRP3 may be a useful therapeutic strategy (Iwata et al. 2013). Here we have directly tested the involvement of NLRP3 in depression-related behaviours using a novel model of sustained-inflammation induced depression in wildtype and NLRP3$^{-/-}$ mice. In developing the model, we first attempted to replicate the behavioural effects of acute systemic LPS administration. We found that in adult C57BL/6J mice, acute LPS induced profound sickness behaviour but, at 24 h post-LPS administration, we failed to find a significant increase in depressive-like behaviour in the forced swim test, regardless of environmental housing conditions. The impairment in locomotor activity evident at 24 h post-administration suggests that sickness behaviour persists and potentially confounds assessment of depressive-like behaviours at this time point. However, we have also assessed hedonic responses using sexually motivated behaviour in the FUST for the first time in acute LPS treated mice. Together with SPT, these data in the FUST showed a reduction in hedonic behaviour, reflecting an increase in depressive-like behaviour, 24 h post-administration of LPS. To examine the response to sustained inflammatory challenge, we have developed a repeated LPS administration paradigm, using daily increasing LPS doses to avoid the development of tolerance. This 3 day increasing dose model shows sickness behaviour at 6 h post-administration that is accompanied by a clear increase in immobility in the FST at 24 h. Intriguingly, this 3 day increasing dose LPS paradigm induced a depressive-like behaviour in NLRP3$^{-/-}$ mice, comparable to that seen in NLRP3$^{+/+}$ mice, although the sickness behaviour was somewhat attenuated. Our data indicate that while the NLRP3 inflammasome may play a role in mediating sickness behaviour, it is not required for the development of depression-related behaviours following sustained activation of the immune system.

We investigated whether the absence of depressive-like behaviour following acute LPS administration in C57BL/6J mice was influenced by social versus individual housing or the light phase in which the animals were tested. It has been shown that housing and lighting conditions can have an effect on various behaviours, including FST immobility (Bogdanova et al., 2013; Dubocovich et al., 1990; Karolewicz and Paul, 2001). In our experiments, while mice exhibited a slight increase in locomotion in the OFT when housed under reversed lighting conditions and tested in the dark, LPS-induced changes in locomotion were unaffected by group or individual housing and lighting conditions. A depressive-like behaviour was not observed in the FST. There are inconsistent reports on the effect of light phase and social conditions on FST immobility, with studies reporting positive, negative or no effects (Bogdanova et al., 2013; Huynh et al., 2011; Verma et al., 2010). For example, social isolation has been shown to both reduce immobility and increase immobility in the FST (Karolewicz and Paul, 2001; Ma et al., 2011; Yates et al., 1991). Here we report for the first time a systematic assessment of these conditions on acute LPS-induced behavioural changes. While variations in environmental conditions may have subtle effects on behaviours, we failed to observe an acute LPS-induced depressive-like behaviour in any of the tested conditions and replication of the depressive effects of acute LPS in the FST could not be achieved in the present study. This finding is supported by other work reporting no significant LPS-induced depressive-like behaviour at 24 h post-administration (Biesmans et al., 2013; Lawson et al., 2013). Inconsistencies in the behavioural effects of acute LPS and the acute inflammation associated with a single dose of LPS suggest acute LPS model of depressive-like behaviours in mice may not be a robust model of depression.

One factor contributing to apparently conflicting reports in the literature is the difficulty in separating LPS-induced sickness and depressive-like behaviours. Exploratory behaviour is often used as a measure of LPS-induced sickness and we observed a reduction in OFT locomotion at 6 and 24 h after acute LPS administration, suggesting the presence of sickness

behaviour. This finding is consistent with a number of studies which report a reduction in distance travelled at 24 h following acute LPS treatments, all using doses between 0.33-0.83 mg/kg (Biesmans et al., 2013; de Paiva et al., 2010; Godbout et al., 2005; Salazar et al., 2012) but inconsistent with others that report no effect of acute LPS on locomotion at 24 h (Frenois et al., 2007; Gibb et al., 2013; Mello et al., 2013; O'Connor et al., 2009; Ohgi et al., 2013; Viana et al., 2010; Walker et al., 2013). It has previously been reported that acute LPS caused reduced exploratory behaviour towards novel objects, even when the reduction in locomotion in the home cage was no longer seen (Haba et al., 2012). Additionally, social exploratory behaviour and novel object exploration have been shown to be reduced 24 h after LPS administration (Godbout et al., 2005; Haba et al., 2012). Assessing behaviours in the rotarod test 24 h after LPS injection revealed no deficits in motor co-ordination as others have previously shown (Custódio et al., 2013; Krzyston et al., 2008). Our data suggest that there is no locomotor deficit per se at 24 h, but rather that exploratory behaviour is reduced which could be interpreted as an indicator of depression-like behaviour.

While there was little evidence in this study that acute LPS induced consistent depressive-like behaviour in the FST at 24 hour post-administration, we did see behavioural changes in the SPT and the FUST. The SPT is a common behavioural test to assess anhedonia and has been previously shown to be effective in LPS models of depressive-like behaviour (Park et al., 2011; Zhang et al., 2014). We found that acute LPS caused a reduced preference for sucrose in the first 24 h following injection, but not at 24-48 h. In the first 24 h there was also a reduction in total consumption, an indication of sickness and a potentially confounding factor (Biesmans et al., 2013). However, during this period, a sucrose preference is still observed, indicating the mice showed attenuated hedonic behaviour for some part of the test period. If sickness behaviour was present for the entire test period, no sucrose preference would be expected (Zhang et al., 2014). The FUST is a paradigm that has not previously been used to assess behaviours following LPS treatment. The task elicits sexually motivated hedonic behaviour that

has been shown to be impaired following a chronic restraint stress model of depression in mice, as well as respond to chronic citalopram in a learned helplessness model of depression (Finger et al., 2011; Malkesman et al., 2010). The FUST has also been used to evaluate the role of proteins such as the neurokinin-1 receptor in depressive-like behaviours in mice (Berger et al., 2012). In addition, a 21-day chronic stress model of depression in rats has previously been shown to reduce sexual behaviours in male rats (Bhattacharya and Muruganandam, 2003). In the present study, a reduction in female urine sniffing was observed, suggesting a depressive effect at 24 h after LPS injection. Whilst reduced locomotion was observed in the OFT at 24 h after LPS injection, the finding that water sniffing is unaffected indicates that the reduction in urine sniffing is not a result of an overall reduction in sniffing following LPS, but an anhedonic response. Other studies have shown that while administration of LPS or IL-1 induces sickness behaviour in male rats at up to 6 h, there is no change in sexual behaviour towards female rats, such as mounting (Avitsur et al., 1997; Yirmiya et al., 1995). Such findings would indicate that these sexual behaviours are not influenced by sickness. However, our findings show a reduced sexual motivation following acute LPS, indicating a depressive-like behaviour.

In this study we have investigated whether repeated administration of LPS can produce a model of sustained inflammation-induced depressive-like behaviour. Repeated systemic administration of LPS is known to rapidly induce tolerance by the second administration in both rats and mice, whereby behavioural effects of LPS are diminished over time, even at low doses (Engeland et al., 2001; Franklin et al., 2003; Franklin et al., 2007). This is consistent with our findings when mice were administered the same LPS dose (CD) and subsequently tested in the OFT; the reduction in locomotion was attenuated when compared to acute LPS. Tolerance to LPS is a result of a refractory period in which repeated stimulation with an endotoxin results in an attenuated cytokine release, and therefore is an obstacle in developing a model of sustained inflammation (Biswas and Lopez-Collazo, 2009). However, in the 3-day increasing

dose group of mice, we found no difference in OFT locomotion when compared to acute LPS. This suggests that the mice have not developed a tolerance towards LPS regarding locomotion, and may therefore represent a useful model for studying the effect of sustained inflammation with LPS. We found that these mice exhibited a depressive-like behaviour at 24 h after final injection. However, as no further time points have been assessed in the OFT or FST, it is not yet possible to confirm the absence of any confounding sickness behaviour or the longevity of the depressive-like behaviour observed. In the 5-day experiment increasing doses of LPS still resulted in the development of tolerance, as observed in the OFT. Alternative repeated LPS protocols have been used to induce depressive-like behaviours in mice, 8 injections of 0.1 mg/kg LPS (Xie et al., 2012) and 10 injections of 0.83 mg/kg LPS (Guo et al., 2014), whereby depressive-like behaviours in the FST and SPT were observed. More long-term models include increased FST immobility following the administration of 0.25 mg/kg LPS twice a week for 12 weeks (Krishna et al., 2016), and anhedonia following a 4-month intermittent LPS administration schedule in mice, with rising and falling doses of LPS (0.75-1.25 mg/kg) being administered over 5 days each month (Kubera et al., 2013). Such models may provide greater translatability by inducing inflammation over extended periods of time. The 3-day model presented here is the first repeated LPS model to demonstrate the avoidance of LPS tolerance and induce a depressive-like behaviour. However, it remains to be established whether the depressive-like behaviour persists in the long-term which would be key to the translational relevance of this new model.

This 3-day ID LPS model was developed to enable us to investigate the involvement of the NLRP3 inflammasome in LPS-induced depressive-like behaviours. We found that sickness behaviour, as assessed by OFT locomotion and body weight reduction, was significantly attenuated in NLRP3$^{-/-}$ mice compared to NLRP3$^{+/+}$ mice. However, depressive-like behaviours were evident in both NLRP3$^{-/-}$ and NLRP3$^{+/+}$ suggesting that the depressive behaviours elicited by this 3-day ID LPS paradigm are independent of the NLRP3 inflammasome. This is surprising given a number of recent studies that have indicated the involvement of NLRP3 in

the development of depressive-like behaviour in mice in stress- and inflammation-based models of depression.

LPS is recognized by the toll-like receptor 4 (TLR4) leading to activation of the NLRP3 inflammasome *in vivo*. NLRP3 inflammsome signalling is characterized by its ability to activate pro-caspase-1 which in turn is responsible for the proteolytic cleavage of pro-IL-1β and pro-IL-18 to generate the active form of these pro-inflammatory cytokines (Agostini et al. 2004). Thus, genetic deletion of caspase 1 decreases LPS-induced IL-1β secretion and this enzyme has been reported to be required for the development of depression-like behaviour following a central, but not systemic, LPS challenge (Lawson et al. 2013). Further support for this mechanism comes from studies where pre-treatment with the caspase 1 inhibitor Ac-YVAD-CMK reduced the pro-depressive effects of acute LPS administration in BALB/c (0.8 mg/kg, ip) mice (Zhang et al. 2014) and in C57BL/6 mice (5 mg/kg ip) (Zhu et al. 2017). Furthermore, in vitro studies in LPS-primed macrophages and microglia showed that treatment with the antidepressant fluoxetine (0.1 – 10 μM) inhibited NLRP3 inflammasome activation (Du et al. 2016). These authors also showed that fluoxetine (10mg/kg/d i.p for 4 weeks) reduced the expression of NLRP3 and proIL-1β induced in a chronic mild stress model in mice (Du et al. 2016). Additionally, studies in NLRP3$^{-/-}$ mice on a C57BL/6 background have shown that depression induced by chronic restraint-stress (Alcocer-Gomez et al. 2016) or chronic unpredictable mild stress (Su et al. 2017) depends on the presence of NLRP3. Our findings are the first to show a NLRP3-independent pathway mediating depression-related behaviours following repeated sustained LPS administration. An *in vitro* study has compared acute versus chronic LPS stimulation of peripheral mouse macrophages finding that chronic LPS stimulation increases IL-10 release that dampens NLRP3 inflammasome activity (Gurung et al, 2015). Further studies are required to understand the signalling pathways underlying this NLRP3-independent depressive-like behaviour. We propose that chronic, repeated systemic LPS administration may reduce activity of the NLRP3 inflammasome pathway and shifts towards a

NLRP3-independent depressive behaviour that is not observed in acute LPS-induced and chronic stress induced models

NLRP3 could be important in the separation of sickness and depressive-like behaviours and may implicate different mechanisms in the origins of these behaviours in response to acute and sustained models of inflammation-induced depressive-like behaviour. Sickness behaviour has been suggested to be an adaptive response to infection and immune system activation that promotes recovery by conserving energy to combat acute inflammation (Maes et al. 2012). While many features of sickness behaviour overlap with depression, it is not clear that the neurobiological mechanisms underlying these behaviours are distinct. Maes et al. (2011) proposed that under inflammatory conditions, the switch from sickness behaviours to depressive behaviours may be mediated by the activation of indoleamine 2,3-dioxygenase (IDO), expressed in microglia and activated by proinflammatory cytokines, which switches tryptophan metabolism away from the synthesis of serotonin and towards the production of kynurenine. Our data show that NLRP3$^{-/-}$ mice were provided protection from LPS-induced sickness, both following acute and 3 day repeated administration, but failed to attenuate the depressive-like behaviour in 3 day repeated administration. Furthermore, the hypolocomotion seen in NLRP3$^{+/+}$ mice 24 h following acute LPS and 3-day ID LPS indicate the presence of sickness and is a confounding factor in the increased FST immobility. This confounding behaviour is not present in NLRP3$^{-/-}$ mice, indicating an absence of sickness. In a repeated stress model of depression, wildtype mice exhibited microglia activation and reduced food intake, whilst NLRP3$^{-/-}$ mice had reduced microglia activation and normal food consumption (Alcocer-Gómez et al., 2015). Locomotion 24 h after systemic acute LPS has been shown to not be affected by capase-1-knockout, though basal locomotion in those mice were significantly lower than wildtype and may therefore affect LPS-induced changes (Lawson et al., 2013). Our data suggest that NLRP3 does play a role in the sickness behaviour induced by sustained inflammatory challenge, as in the 3-day ID LPS model described here, although not in depressive-behaviours. One possible explanation for this distinction is that NLRP3 may

be able to affect immune system function independently of inflammasome activation. In T helper type 2 (TH2) cells NLRP3 has been shown to act as a transcription factor regulating TH2 differentiation (Bruchard et al. 2015). While further biochemical studies are required to support this interpretation, it may be that NLRP3 is a useful marker to distinguish between sickness and depressive behaviours.

This novel approach of using repeated increasing doses of LPS offers a potential paradigm for assessing the effects of sustained inflammation-induced depression-related behaviours and may provide a more translational model of depression than seen with acute LPS administration. Such models are also important in understanding the cellular mechanisms underlying inflammation-induced depression and defining the role of NLRP3.

FIGURE LEGENDS

Figure 1: Effects of acute LPS administration on behaviour in C57BL/6J mice. Distance travelled in the open field test assessed 6 h post-administration of LPS (A) and time spent immobile in the forced swim test assessed 24 h post-administration of LPS (B) are shown. Mice were group housed (n=3/4 per cage) or singly housed throughout the experiment and under normal lighting (lights on 06:00h) conditions where behaviours were assessed during the light or under reversed lighting (lights on 18:00 h) conditions where behaviours were assessed during the dark phase. Values shown are mean ± SEM of n=10-15 per group. ***p < 0.001 LPS treatment effect compared to saline injected controls, $^{\#}p$ < 0.05 effect of environmental factors.

Figure 2: Time course of behavioural effects of acute LPS administration in C57BL/6J mice. Distance travelled in the open field test (A) 24 h post-administration of LPS and time spent immobile in the forced swim test (B) 48 h post-administration of LPS are shown (n=9-18 per group, **p < 0.01, ***p < 0.001 compared to saline injected controls). In a separate group of animals, motor coordination in the rotarod test (C) was examined at 6, 24 and 48 h post-administration of LPS (n=10 per group, $^{\#}p$ < 0.05 compared to pre-treatment baseline performance). Mice were group housed (n=3/4 per cage) and behaviour assessed in the light cycle. All values are mean ± SEM

Figure 3. Effects of acute LPS administration in the sucrose preference test in C57BL/6J mice. Total volume of sucrose solution (2.5%) consumed (A) and sucrose preference (B) are shown at 0-24 h and 24-48 h post-administration of LPS. Mice were singly housed throughout the experiment, lights on at 06:00 h. Values shown are mean ± SEM of n=15-16 per group. ***p < 0.001 compared to saline injected controls.

Figure 4. Effects of acute LPS administration on sexual motivation in the female urine sniffing test in C57BL/6J mice. Time spent sniffing either water or female urine soaked cotton buds at 6 h (A) and 24 h (B) post-administration of LPS is shown. Mice were group housed (n=3/4 per cage) and behaviour assessed in the light cycle. Values shown are mean ± SEM of n=6-7 per group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to urine sniffing saline injected controls) and $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ within group comparison vs water.

Figure 5. Effects of repeated daily LPS administration on behaviour in C57BL/6J mice. Mice were treated for 3 days (A,B,C) or 5 days (D,E,F) with constant dose (CD) LPS (0.83 mg/kg LPS injected each day) or increasing dose (ID) LPS (doubling concentrations each day to 0.83 mg/kg LPS on final day). Saline injected controls were administered injections daily and acute LPS (0.83 mg/kg) was administered only on the last day before testing. Changes in body weight (A,D), distance travelled in the open field test (B, E) and time spent immobile in the forced swim test (C,F) are shown. Mice were group-housed (n=3/4 per cage) and behaviour assessed in the light phase. Values shown are mean ± SEM of n=11-20 per group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to saline injected controls; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ between group comparisons as indicated.

Figure 6. Effects of repeated daily LPS administration on body weight and behaviour in NLRP3$^{+/+}$ and NLRP3$^{-/-}$ mice. Mice were treated for 3 days with increasing dose (ID) LPS (0.21 / 0.42 / 0.83 mg/kg) or acute LPS (saline / saline / 0.83 mg/kg LPS). Saline injected controls were administered injections daily. Changes in body weight (% of starting weight) were monitored (A) and at 24 h post-administration of final LPS injection (D). Distance travelled in the open field test at +6 h (B) and +24 h (C) was assessed. Time spent immobile in the forced swim test at 24 h after LPS (E) is shown. Mice were group-housed (n=3/4 per cage) and behaviour assessed in the light phase. Values shown are mean ± SEM of n=12-13

per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to saline injected controls; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ between group comparisons as indicated.

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NOTE- Borders are just for size reference

Figure 1.

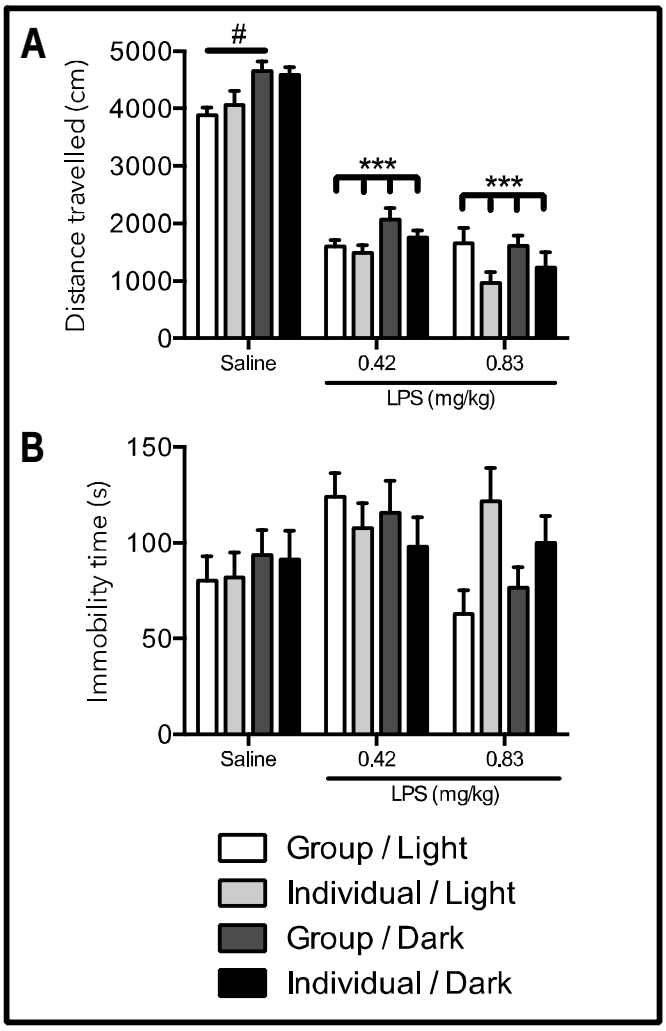


Figure 2.

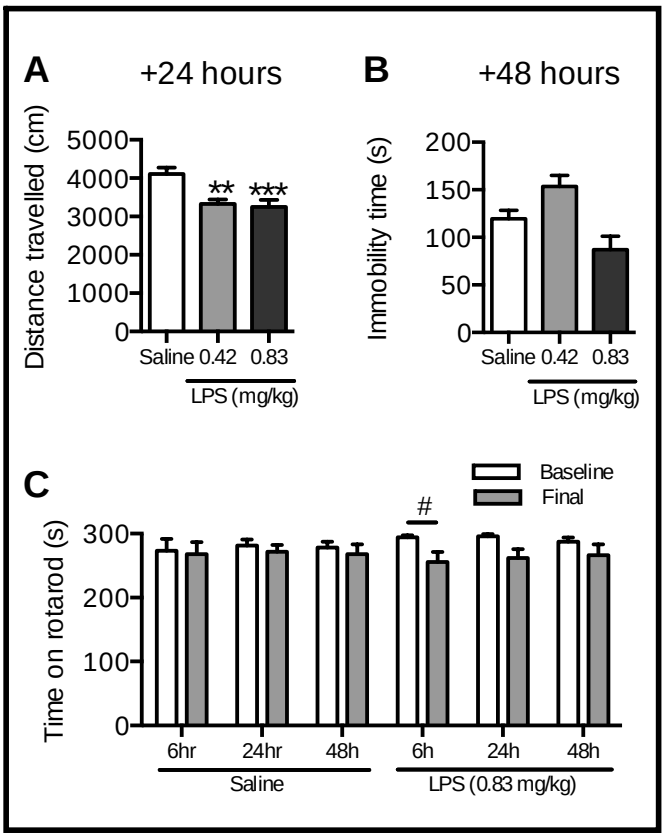

A
+24 hours
Distance travelled (cm)
5000
4000
3000
2000
1000
0
**
***
Saline 0.42 0.83
LPS (mg/kg)
B
+48 hours
Immobility time (s)
200
150
100
50
0
Saline 0.42 0.83
LPS (mg/kg)
C
Baseline
Final
#
Time on rotarod (s)
300
200
100
0
6hr 24hr 48h 6h 24h 48h
Saline
LPS (0.83 mg/kg)

Figure 3.

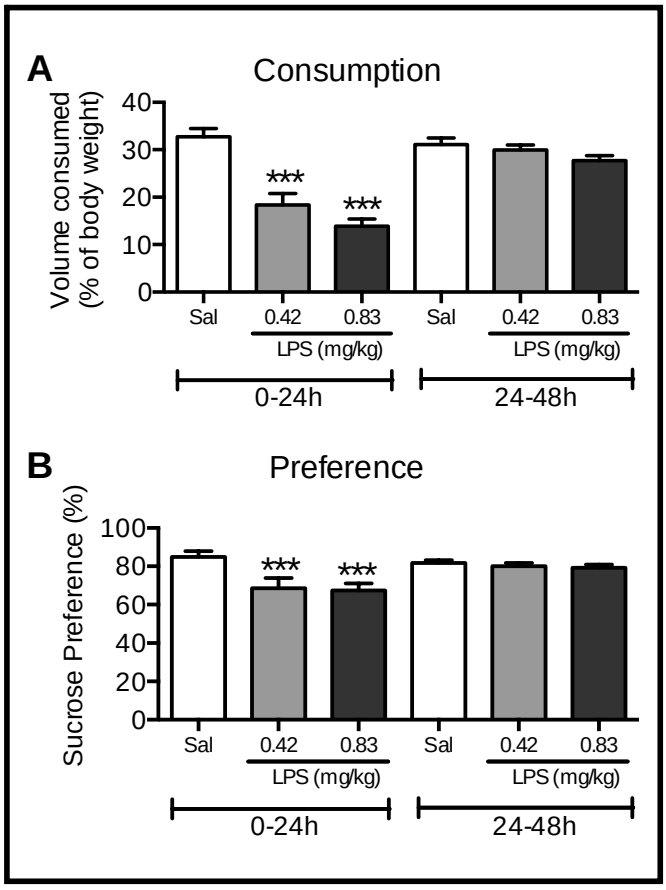

A
Consumption
Volume consumed (% of body weight)
40
30
20
10
0
***
***
Sal
0.42
0.83
Sal
0.42
0.83
LPS (mg/kg)
LPS (mg/kg)
0-24h
24-48h
B
Preference
Sucrose Preference (%)
100
80
60
40
20
0
***
***
Sal
0.42
0.83
Sal
0.42
0.83
LPS (mg/kg)
LPS (mg/kg)
0-24h
24-48h

Figure 4.

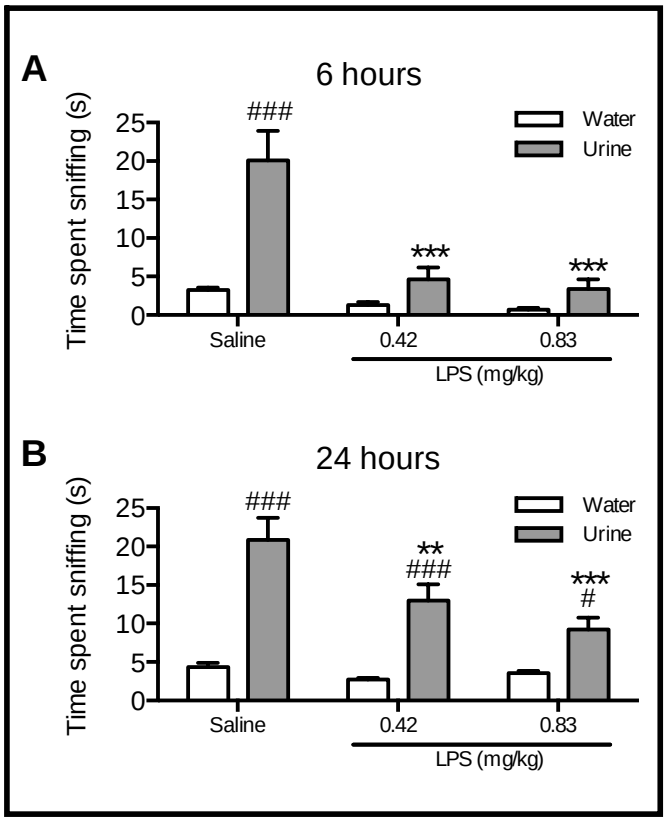

A
6 hours
Time spent sniffing (s)
25
20
15
10
5
0
###
***
***
Water
Urine
Saline
0.42
0.83
LPS (mg/kg)
B
24 hours
Time spent sniffing (s)
25
20
15
10
5
0
###
**
###
***
#
Water
Urine
Saline
0.42
0.83
LPS (mg/kg)

Figure 5.

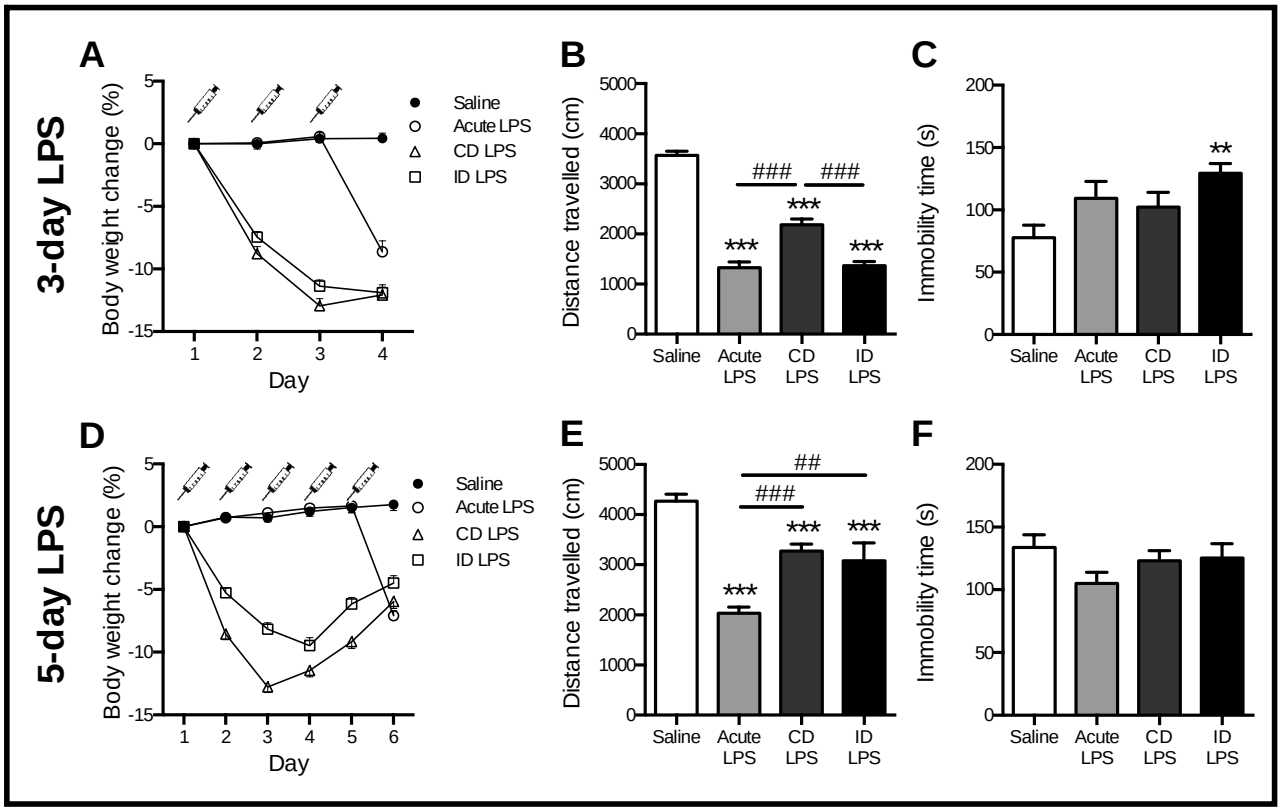
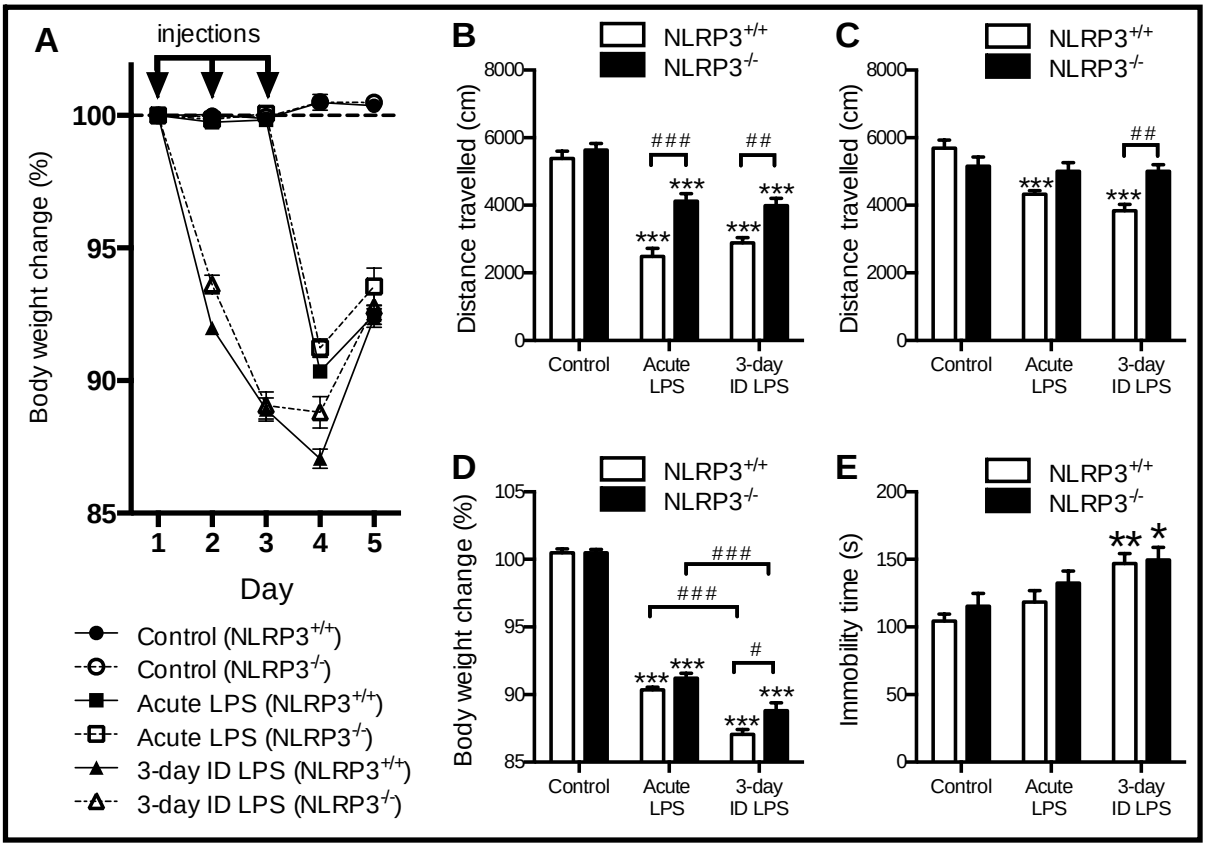
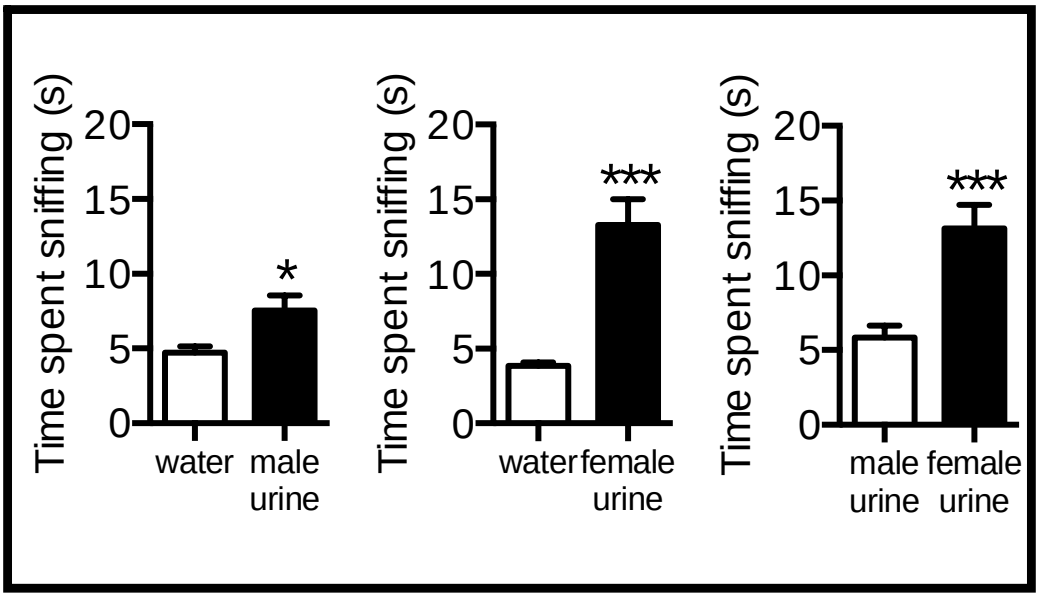
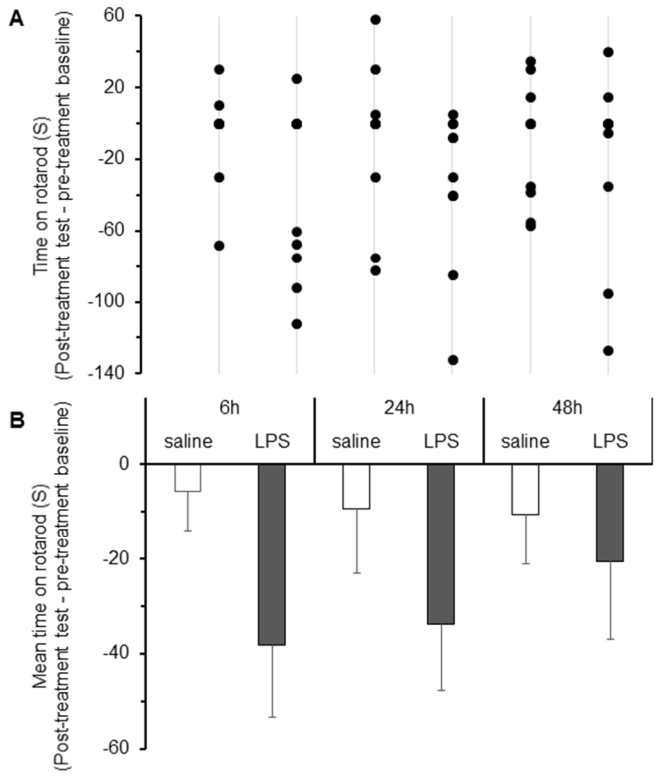

A
B
C
D
E
F
3-day LPS
5-day LPS
Body weight change (%)
Day
Saline
Acute LPS
CD LPS
ID LPS
Distance travelled (cm)
Immobility time (s)
###
***
**
##

Figure 6.


A
injections
Body weight change (%)
100
95
90
85
1
2
3
4
5
Day
Control (NLRP3+/+)
Control (NLRP3-/-)
Acute LPS (NLRP3+/+)
Acute LPS (NLRP3-/-)
3-day ID LPS (NLRP3+/+)
3-day ID LPS (NLRP3-/-)
B
NLRP3+/+
NLRP3-/-
Distance travelled (cm)
8000
6000
4000
2000
0
###
##
***
Control
Acute LPS
3-day ID LPS
C
NLRP3+/+
NLRP3-/-
Distance travelled (cm)
8000
6000
4000
2000
0
##
***
Control
Acute LPS
3-day ID LPS
D
NLRP3+/+
NLRP3-/-
Body weight change (%)
105
100
95
90
85
###
#
***
Control
Acute LPS
3-day ID LPS
E
NLRP3+/+
NLRP3-/-
Immobility time (s)
200
150
100
50
0
**
*
Control
Acute LPS
3-day ID LPS



Supplementary figure 1. Pilot study demonstrating the sniffing of female urine as a sexually motivated behaviour rather than an olfactory stimulus. The time male adult C57BL/6J mice spent sniffing cotton buds soaked in either water and male urine (A), water and female urine (B) or male urine and female urine (C) during a 3-minute test session is shown. Mice were randomly assigned to groups and exposed to two odours during the test session. Male mice showed a significant preference for male urine over water, and a significant preference for female urine over water. Panel C shows a significant preference for female urine over male urine, indicating a sexual preference, rather than urine as a novel olfactory stimulus. Values shown are mean ± SEM of n=11 per group. *$p < 0.05$, ***$p < 0.001$.



Supplementary figure 2. Analysis of time spent on rotarod by adult male C57BL/6J mice. (See also Fig 2C in manuscript). Six separate groups of mice (n=10 per group) were assessed on an accelerating rotarod test (0-40 rpm) for 5 minutes 6, 24 and 48 h after administration of either saline or acute lipopolysaccharide (LPS, 0.83 mg/kg, ip). (A) Scatter plot of time spent on rotatrod measured as the latency to drop is expressed as the difference between post-treatment test time and pre-treatment baseline for each individual mouse. (B) The mean difference ± SEM between post-treatment testing and pre-treatment baseline for each treatment group is shown. Horizontal axis label applies to both A and B. Two way ANOVA revealed a significant effect of Treatment ($F_{(1,54)}$ = 4.23 P=0.044) but not of Time nor any significant interaction between Treatment * Time. Post-hoc pairwise comparisons (1-way ANOVA) revealed an almost significant effect of LPS treatment on rotatrod performance after 6 h (p=0.07) but not after 24 h (p =0.22) or 48 h (p=0.61).In saline treated animals there appeared to be a small decrease in performance 48h post administration of saline which could be related to more time having elapsed between the training and the performance of the test session necessary in this experimental design.