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Repeated lipopolysaccharide exposure modifies immune and sickness behaviour response in an animal model of chronic inflammation

Abstract

Repeated lipopolysaccharide (LPS) exposure is often used in longitudinal preclinical models of depression. However, the potential phenotypic differences from acute depression-mimicking effects are rarely described. This study compared chronic LPS administration of doses previously used in depression research to a new mode of escalating dose injections. Adult male BALB/c mice (n=8/group) were injected intraperitoneally with either a single 0.83 mg/kg dose, a repeated 0.1 mg/kg LPS dose or a dose which escalated weekly from 0.33 to 0.83 mg/kg LPS for six weeks. The escalating LPS group demonstrated most features of sickness behaviour such as weight loss and reduction in food intake every week, whilst this effect was not sustained in other groups. Moreover, only in the escalating LPS group did most peripheral plasma cytokines levels, measured using Luminex® multiplex technology, such as interleukin 6 (IL-6), tumor necrosis factor α and IL-2 remain over 3 fold elevated on the 6th week. In addition, exposure to escalating doses led to a reduction of neuroblast maturation in the dentate

gyrus relevant for depression neurobiology. Therefore, this mode of injections might be useful in the studies attempting to replicate neurobiological aspects of the chronic inflammatory state observed in mood disorders.

Keywords

Lipopolysaccharide, chronic inflammation, sickness behaviour, adult neurogenesis, hippocampus, cytokine, microglia

Introduction

Acute administration of LPS, a proinflammatory agent derived from Gram-negative bacteria, induces a complex of behavioural reactions known as sickness behaviour (Kent et al., 1992). The parallel between sickness behaviour and symptoms of depression initially drawn by Smith (1991), as well as growing interest in inflammatory aspects of depression neurobiology encouraged the use of LPS as a model of the disorder. Yet the replication of the chronic nature of depression symptoms and neurobiology using LPS has proved to be a challenging task to date.

The analogy between sickness behaviour and depressive symptoms has its limitations, as some responses such as hyperthermia are absent in depressed patients, while other parameters could change on a much wider spectrum (Dunn et al., 2005). For example,

appetite might be increased or decreased in patients, but only its loss is linked to LPS response; sickness behaviour is associated with hypersomnia, but depressed patients can present with either insomnia or hypersomnia (Otte et al., 2016). However it has been subsequently shown that LPS exposure via i.p. injections also induces depression-like behaviour in specific behavioural tests, such as increased immobility in the forced swim test (Godbout et al., 2007; O'Connor et al., 2009) and anhedonia in the sucrose consumption test and test of sexual behaviour (Painsipp et al., 2011; Yirmiya, 1996).

While the construct validity of LPS-based models could be disputed due to pathogen-induced rather than stress-related nature of symptoms, its main advantage consists of the similarity between its biological effects and neurobiological changes seen in depression. Firstly, pro-inflammatory cytokines elevated following LPS administration have also been found to be increased in depressed patients (for meta-analysis see Liu et al., 2012). Secondly, LPS has also been shown to induce hypercortisolism observed in some patient groups (Chen et al., 2005). Next, LPS exposure has been shown to decrease the level of adult hippocampal neurogenesis (AHN) implicated in the neurobiology of mood disorders (Samuels and Hen, 2011). Lastly, systemic LPS exposure is capable of inducing microglial activation in the brain (Buttini et al., 1996; Qin et al., 2007), similar to that demonstrated by clinical neuroimaging studies detecting increased microglial

activation marker translocator protein (TSPO) in depressed patients (Husain et al., 2015; Setiawan et al., 2015).

However two major discrepancies exist between most LPS models and the course of the depressive disorder. Firstly, many studies using high doses of LPS (3-5mg/kg) have resulted in a drastic elevation in proinflammatory cytokines, as might be seen in sepsis (Anderson et al., 2016; Erickson and Banks, 2011). Such changes might not be relevant for the subtler increases seen in depressed patients. Secondly, most behavioural and neurobiological effects of LPS are short-lived (24 hours or less) and therefore do not reflect the chronic nature of the disease (Godbout et al., 2007; Graciarena et al., 2013). To prolong the effects of LPS, some studies have employed protocols involving repeated LPS injections administered at various time intervals (Elgarf et al., 2014; Kubera et al., 2013). Whilst such protocols induced more long-lasting phenotypes, they also resulted in a suppressed reaction of the immune system, known as LPS tolerance. For example, Kubera et al. (2013) showed that repeated administration of LPS (0.075-1.25mg/kg) led to a decreased sucrose preference in affected female mice, but also suppressed splenocyte proliferation and proinflammatory cytokine release in response to an acute immune challenge. In Elgarf et al.'s (2014) study, hippocampal TNF α used as a marker of brain inflammation was increased only after two but not six weeks of repeated 0.05mg/kg LPS injections, a time point when the rats displayed social interaction deficits.

Thus, the effect of repeated administration may not simply produce a longitudinal version of the phenotype as induced by acute exposure. The difference in behavioural and neurobiological responses to acute versus repeated LPS injections has not been previously described in great detail.

Therefore, the main aim of the current study was to investigate the effect of repeated administration of LPS within the dose range commonly used in depression studies on sickness behaviour response and neurobiological processes relevant for depression in comparison with the acute exposure. For this two modes of repeated LPS injections were compared to a single injection of 0.83mg/kg LPS (single LPS group), a dose which has been previously shown to induce a long-lasting phenotype (Painsipp et al., 2011). To avoid the development of LPS tolerance in repeated injections groups, several measures were taken: a weekly interval between injections was included to allow for immune system recovery; a relatively low dose (0.1 mg/kg) of LPS already utilised in depression models was used in 0.1 LPS group (Wang et al., 2011); a weekly increment from initial 0.33 mg/kg to a final 0.83 mg/kg dose was used in the escalating LPS (0.33-0.83) group as dose increase has been shown to avoid habituation of immune response (Wickens et al., 2014). Sickness behaviour, plasma cytokine profile, and the state of adult hippocampal neurogenesis and microglial activation in the hippocampus were assessed

weekly or at the end of six weekly injections in each group, and compared to a control group injected with saline.

Methods

Animals

Eight-week old male BALB/cAnNCrI mice were obtained from Charles River (Margate, Kent, U.K.). All animals were housed in the Biological Services Unit (BSU) at the Institute of Psychiatry, Psychology & Neuroscience in standard conditions (19-22°C, humidity 55%, 12h:12h light:dark cycle with lights on at 7.30am, food [Rat and Mouse No. 1 Diet; Special Diet Services, Essex, UK] and water ad libitum). All procedures described in this paper were conducted on a single set of 34 mice (n=8 per LPS treated groups, n=10 in control group). In this set, mice were pair-housed with their litter mates in large cages 45x28x13cm with environmental enrichment (small plastic and cardboard houses, cardboard tube, paper sizzle nest material; Datasand Ltd, Manchester, UK). All housing and experimental procedures were carried out in compliance with the local ethical review panel of King's College London under a U.K. Home Office project licence held in accordance with the Animals (Scientific Procedures) Act 1986 and the European Directive 2010/63/EU.

LPS injections

Sibling pairs were randomly assigned to one of four treatment groups, which received the following i.p. injections once a week (on Tuesdays at 10am) for six weeks: (i) control group (SAL; n=10) received 100 μ L of 0.9% saline each week; (ii) 0.1 LPS group (n=8) received 0.1mg/kg of LPS each week; (iii) escalating LPS (0.33-0.83) group (n=8) received 0.33 mg/kg of LPS on weeks one and two, 0.53 mg/kg of LPS on week three, 0.63 mg/kg on week four, 0.73 mg/kg on week five, 0.83 mg/kg on week six; (iv) single LPS group (n=8) received 0.83 mg/kg on week one, and 100 μ L saline on weeks 2-6. For all injections LPS from *E.Coli* Serotype O127:B8 (L3129, Sigma Aldrich, Poole, UK) was used. LPS powder was freshly dissolved prior to injections in sterile saline (Aquapharm, Argyll, UK). Body and food weight were measured immediately before and 24 hours after saline or LPS administration, and the difference between the two data points was used as weight gain or food intake measures respectively. For depiction of the experimental design see Figure 1.

Figure 1 Experimental design.

8 week old male BALB/cAnNCrl mice were injected with LPS (n=8/group) or saline (n=10/group) i.p. once a week for 6 weeks. Experimental conditions included: 0.1 LPS group injected with 0.1 mg/kg LPS once weekly; Escalating LPS group injected with 0.33mg/kg on weeks 1 and 2, 0.53mg/kg on week 3, 0.63 mg/kg on week 4, 0.73 mg/kg on week 5 and 0.83 mg/kg on week 6; Single LPS group injected with 0.83 mg/kg on week 1 and saline on weeks 2-6; SAL group was injected weekly with 0.9% saline solution. Blood was collected 2 hours after LPS injections on weeks 1 and 6; locomotion was measured 6 hours after LPS injections on weeks 1 and 6; weight change and food intake were measured during a 24-hour period after the LPS injections.

Open field test

The open field test was performed 6 hours after LPS injections on weeks one and six (between 4pm and 6pm). For this a dimly lit (20 Lux) circular arena measuring 40 cm in diameter was used. Each animal was placed in the arena facing the outer wall, and behaviour was video recorded for five minutes. The total distance travelled in the non-

threatening outer zone was measured using the automated tracking system (Ethovision, Noldus Information Technologies, Wageningen, The Netherlands) as described previously (Keers et al., 2012).

Blood collection

Blood was collected by incision method from the lateral tail vein (Sadler and Bailey, 2013) 2 hours after either LPS or saline injections on weeks one and six. Whole blood (30-50 μ L) was collected into EDTA microvette CB 300 tubes (Sarstedt, Leicester, UK) and separated by centrifugation for 10min at 3000 rpm (4°C), after which plasma was manually transferred into a new sterile tube (1.5ml 'Crystal clear' microcentrifuge tube sterile, Starlab UK Ltd) and stored at -80°C. Another blood collection by method of cardiac puncture occurred after the terminal general anaesthesia had been induced by the injection of 40mg/kg of pentobarbital sodium i.p. (Euthatal, Merial Animal Health Ltd, Harlow, UK). 100-200 μ L of whole blood was collected using syringe injected into the cardiac cavity and subsequently processed as described above to measure corticosterone levels.

Cytokine analysis

The level of cytokines in the plasma was determined using the multiplex screening assay based on magnetic Luminex® xMAP® technology as described in Hye et al. (2014). For

this assay a custom-made pre-mixed multianalyte kit was purchased from RnD systems, Minneapolis, USA (catalogue N LXSAMSM). This kit contained multi-coloured magnetic microparticles pre-coated with antibodies to 8 selected analytes (granulocyte-monocyte colony stimulating factor (GM-CSF), interleukins IL-1 β , IL-2, IL-6, IL-10, tumour necrosis factor alpha (TNF α), interferon gamma (IFN γ) and C-reactive protein (CRP)). To measure the fluorescent signal the Luminex[®] 100/200[™] system based on xMAP[®] technology was used. Plasma samples were diluted 1:2 in calibration buffer, and all assay steps were conducted according to the manufacturer's instructions. Due to small sample sizes, technical replicates for plasma samples were not included. Intraassay variability based on duplicates of the standard dilutions was estimated at 2.15 %CV. Interassay variability was not calculated as all samples were analysed on a single plate. Obtained data were analysed using five parameter logistic curve analysis using the My Assays online data tool, MyAssays Ltd., <http://www.myassays.com/five-parameter-logistic-curve.assay>.

Immunohistochemistry

For brain tissue collection, mice were anaesthetised with 40mg/kg of pentobarbital sodium i.p. (Euthatal, Merial Animal Health Ltd, Harlow, UK) seven days after the last LPS or saline injections. Animals were subsequently transcardially perfused with saline and 4% paraformaldehyde (Paraformaldehyde, prills, 95%, 441244, Sigma-Aldrich, Poole, UK) in phosphate-buffered saline (pH = 7.4 from PBS tablets, 18912-014, Gibco

by Life Technologies, Paisley, UK) through the left cardiac ventricle. Brains were post-fixed overnight in 4% PFA at 4°C, and subsequently stored at 4°C in 30% sucrose (Sigma-Aldrich, Poole, UK) in PBS.

Brain tissue was cut on a HM430 sliding microtome (Thermo Scientific) into serial 40µm thick coronal sections and stored in tris-buffered saline (TBS, pH=7.4) with 30% v/v glycerol, 15% w/v sucrose and 0.05% w/v sodium azide (all Sigma Aldrich, UK) to prevent bacterial growth and for cryoprotection at 4°C. For immunohistochemistry on free floating sections, every 6th section was washed in PBS 3x5min, incubated in 1% hydrogen peroxide (Sigma Aldrich, UK) for 30 mins with subsequent washing in PBS. Non-specific antibody binding was blocked by incubating sections in 10% NGS (Normal goat serum, S1000, Vector laboratories, UK) in PBST (PBS with 0.25% TritonX (Sigma Aldrich, UK) for 1hr. Sections were incubated in primary antibodies (anti- doublecortin (DCX) diluted 1:1000, ab18723, Abcam; Anti-ionized calcium-binding adapter molecule 1 (Iba1) diluted 1:500, 019-19741, Wako; anti CD68 diluted 1:2000, MCA 1957, ABD Serotec, Anti-Ki67 diluted 1:200, ab15580, Abcam; all made in rabbit) diluted in PBST with 10% NGS at 4°C, agitated on orbital shaker overnight. The next day sections were washed in PBS 3x5min and incubated in secondary antibody, biotinylated anti-Rabbit IgG made in goat (BA1000, Vector Laboratories, UK) for 2 hours at room temperature on an orbital shaker. Subsequently, sections were incubated in a solution containing avidin-biotin

complex (Vectastain Elite ABC Kit, PK6100, Vector Laboratories, UK) for signal amplification. The staining was visualised with diaminobenzidine (DAB, Sigma Aldrich, UK). Sections were rinsed PBS 3x5min, mounted on Superfrost Plus microscope slides (Thermo Scientific), air-dried, dehydrated in serial ethanol dilutions, cleared in xylene (Sigma Aldrich, UK) and coverslipped using Distyrene Plasticizer Xylene (DPX) (Sigma-Aldrich, UK).

Stereological analysis

Stereological method was used to quantify the density of immunopositive cells as described previously (Thuret et al., 2009). An Axioskop 2 MOT Zeiss microscope and a semiautomatic stereology system (Stereoinvestigator, Microbrightfield Inc.) were used to estimate the volume of the region of interest (ROI) and the total number of immunopositive cells in the ROI. For this estimation, the optical fractionator method in the Stereoinvestigator software was used. The optical fractionator estimates the total volume of the ROI using the sectional ROI volume manually traced at x2.5 magnification and known intersection intervals (240 μ m). To estimate the number of positive cells, the software extrapolates the number of manually counted cells in the areas of chosen size (50 μ m x 50 μ m counting frames placed over grid with chosen size of X: 94.59 μ m and Y: 182.53 μ m) of the ROI selected by systemic random sampling to the sectional volume of the region of interest. A chosen set number of average sampling sites per section was

50, while for each brain, 11 sections between stereotactic coordinates -1.06mm and -3.80 relative to bregma (Franklin and Paxinos, 2012) were included into the optical fractionator analysis. The density of Iba1 and DCX positive cells was calculated by dividing the estimated cell number by the estimated volume of the ROI.

Classification of DCX positive cells by dendritic morphology

The DCX positive cells were visually classified according to the categorization of Plumpe and co-workers (Plümpe et al., 2006). The AB group included cells with no or short plump processes, the CD group included cells with medium length processes without branching, and the EF group comprised cells with long branching dendrite(s) reaching the molecular layer. The density of each type of cells was determined using stereology as described in the previous section.

Quantification of CD68 surface area

For quantification of CD68 staining, digital images were taken using Zeiss Axioimager microscope and Zeiss AxioCam MR Rev3 camera at 40X magnification. Per each brain area (CA1, CA3, DG) identified using the Allen Mouse Brain reference coronal atlas, 1 image was taken from every 2nd section between stereotactic coordinates -1.06mm and -3.80 relative to bregma (Franklin and Paxinos, 2012) to achieve equal intersection intervals (80µm). ImageJ software (Schneider et al., 2012) and macros code were kindly

provided by Lianne Hoeijmakers and Dr Aniko Korosi (Center for Neuroscience, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, the Netherlands) were used to quantify the % surface area immunopositive for CD68.

Statistical analysis

A repeated measures two-way ANOVA was used to compare mean differences in weight gain, food intake and cytokine levels among treatment groups over the six weeks of injections. A one-way ANOVA was used to compare mean differences in DCX and Iba1 hippocampal cell density among treatment groups. The Bonferroni correction for multiple comparisons (SAL vs 0.1/Escalating/Single LPS) was applied to post-hoc analyses to determine individual differences between treatment groups and the control group.

Results

Repeated LPS injections induced sickness behaviour each week for six weeks

Body weight change and food intake were measured 24 hours after LPS injections to assess the suppressant effects of the sickness behaviour response on feeding (Biesmans et al., 2013). Both the dose and chronicity of LPS affected weight gain and food intake (see Figure 2 and Table 1). On week one, LPS treated animals showed significantly

reduced body weight gain and/or reduced food intake compared to the saline control group, with the magnitude of effect dependant on the dose of LPS injected (see Figure 2). On week two, only the 0.1 LPS group had a significant reduction in body weight gain. There were no significant differences in body weight gain in any of the LPS groups at week three, but from weeks four through six, there was a significant reduction in body weight gain in the group receiving escalating doses (0.33mg/kg-0.83mg/kg) of LPS compared to control. The effect of LPS on food intake remained significant each subsequent week for the escalating LPS (0.33-0.83) group, while the 0.1 LPS group displayed a significant reduction only on the sixth week. Locomotor activity also was assessed in the open field arena six hours after the LPS or saline injections on week one and week six. On week one, the escalating (0.33-0.83) LPS group displayed statistically significant hypolocomotion (effect of dose $F(3,28)=3.19$, $p=0.039$), but on week six the reduction in locomotor activity did not reach statistical significance in comparison with the SAL group (effect of dose $F(2,23)=1.6$, $p=0.22$, Figure 2(c)).

Table 1 Two-way repeated measures ANOVA outcomes for weight gain and food intake measures taken each week 24hrs after either LPS or saline injections for six weeks.

8 week old male BALB/cAnNCrI mice were injected with LPS or saline i.p. once a week for 6 weeks. Experimental conditions (n=8-10/group) included: 0.1 LPS group injected with 0.1 mg/kg LPS once weekly; Escalating (0.33-0.83) LPS group injected with 0.33mg/kg on weeks 1 and 2, 0.53mg/kg on week 3, 0.63 mg/kg on week 4, 0.73 mg/kg on week 5 and 0.83 mg/kg on week 6; Single LPS group injected with 0.83 mg/kg on week 1 and saline on weeks 2-6; SAL group was injected weekly with 0.9% saline solution.

Factor	Effect size (% of total variation)	F	df	df Residual	p
Weight gain					
Dose	12.33	24.66	3	30	< 0.0001
Time	18.36	11.29	5	150	< 0.0001
Interaction	16.3	3.341	15	150	< 0.0001
Food intake					
Dose	34.45	24.6	3	13	< 0.0001
Time	13.92	11.04	5	65	< 0.0001
Interaction	30.74	8.122	15	65	< 0.0001

Figure 2 Short-term sickness behaviour induced by weekly LPS injections.

8 week old male BALB/cAnNCrl mice were injected with LPS or saline i.p. once a week for 6 weeks. Experimental conditions included: 0.1 LPS group injected with 0.1 mg/kg LPS once weekly; Escalating LPS group injected with 0.33mg/kg on weeks 1 and 2, 0.53mg/kg on week 3, 0.63 mg/kg on week 4, 0.73 mg/kg on week 5 and 0.83 mg/kg on week 6; Single LPS group injected with 0.83 mg/kg on week 1 and saline on weeks 2-6; SAL group was injected weekly with 0.9% saline solution **(a)** Weight change measured during first 24hrs after injections each week expressed as % change from a baseline measured immediately before the LPS injections. **(b)** Food intake during first 24hrs after weekly injections. **(c)** locomotor activity on weeks 1 and 6 measured 6hrs after LPS or SAL injections in the open field arena; *p* values derived from Bonferroni multiple comparisons test SAL vs 0.1LPS/Escalating LPS/Single LPS, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. Data represents mean±SEM, n=8-10/group.

Repeated exposure modified cytokine response to LPS challenge

To detect systemic cytokine response to LPS challenge, selected cytokines were measured in the peripheral blood collected 2 hours after injection on weeks one and six. The time point of blood collection was chosen based on previous studies which showed that in mice exposed to LPS blood levels of most cytokines peak within the first two hours after exposure and subside at later time points (Biesmans et al., 2013; Browne et al., 2012). Animals responded to acute LPS exposure on week one with a profound elevation of pro-inflammatory cytokines IL-6 and TNF α . More specifically, IL-6 was elevated up to 700-fold compared to control levels depending on the dose of LPS injected, while TNF α showed elevations in the range of 20 to 35 fold (see Figure 3 and Table 2). Interestingly, other cytokines and inflammatory markers were not elevated at this time point, including CRP, IL-5 and IL-2, while others did not reach detection levels of the assay used (IL-4, IFN γ , IL-1 β). IL-10 showed an elevation on week one, which reached significance in the escalating LPS (0.33-0.83) and single LPS groups. GM-CSF also significantly increased in response to all doses of LPS on week one. In response to the

sixth injection the elevation of IL-6 and particularly TNF α was much reduced compared to week one. However, the levels of these pro-inflammatory cytokines were still over 570 fold (IL-6) and over nine fold (TNF α) increased in the escalating LPS (0.33-0.83) group compared to levels observed in the control group (see Figure 3). In contrast, IL-2 was over four fold elevated exclusively in response to the escalating LPS (0.33-0.83) group, accompanied by an increase in GM-CSF levels in this group, even more robust than on week one (20-fold on week one vs 30-fold on week six). Interestingly, CRP was 0.44 fold decreased compared to controls in response to the final LPS dose in the escalating LPS (0.33-0.83) group.

Figure 3 Plasma cytokine profile in response to LPS injections 2hrs after exposure on weeks one and six. 8 week old male BALB/cAnNCrI mice were injected with LPS or saline i.p. once a week for 6 weeks. Experimental conditions included: 0.1 LPS group injected with 0.1 mg/kg LPS once weekly; Escalating LPS group injected with 0.33mg/kg on weeks 1 and 2, 0.53mg/kg on week 3, 0.63 mg/kg on week 4, 0.73 mg/kg on week 5 and 0.83 mg/kg on week 6; Single LPS group injected with 0.83 mg/kg on week 1 and saline on weeks 2-6; SAL group was injected weekly with 0.9% saline solution. Data shown as percentage change from control (SAL). P values derived from Bonferroni multiple comparisons test SAL vs 0.1LPS/Escalating LPS/Single LPS, *p<0.05, **p<0.01, ***p<0.001 compared to the SAL control group. Data represents mean±SEM, n=8-10/group.

Table 2 One-way ANOVA and post-hoc Bonferroni multiple comparisons of plasma cytokine measures taken 2hrs after LPS/SAL exposure on weeks one and six.

8 week old male BALB/cAnNCrI mice were injected with LPS or saline i.p. once a week for 6 weeks. Experimental conditions (n=8-10/group) included: 0.1 LPS group injected with 0.1 mg/kg LPS once weekly; Escalating (0.33-0.83) LPS group injected with 0.33mg/kg on weeks 1 and 2, 0.53mg/kg on week 3, 0.63 mg/kg on week 4, 0.73 mg/kg on week 5 and 0.83 mg/kg on week 6; Single LPS group injected with 0.83 mg/kg on week 1 and Saline on weeks 2-6; SAL group was injected weekly with 0.9% saline solution.

Cytokine_week	One-way ANOVA				Bonferroni multiple comparisons			
		df	F	p	SAL group mean, pg/ml	LPS groups	Mean, pg/ml	p
IL-6_w1	Between Groups	3	43.40	.000	52.17	0.1LPS	13517.79	.008
	Within Groups	30				0.33-0.83 LPS	29908.75	.000
	Total	33				Single LPS	40395.00	.000
IL-6_w6	Between Groups	3	23.00	.000	65.13	0.1LPS	2749.14	.039
	Within Groups	28				0.33-0.83 LPS	5292.88	.000
	Total	31				Single LPS	58.75	1.000
TNF α _w1	Between Groups	3	14.46	.000	14.52	0.1LPS	125.13	.032
	Within Groups	30				0.33-0.83 LPS	221.65	.000
	Total	33				Single LPS	218.56	.000
TNF α _w6	Between Groups	3	6.95	.001	7.97	0.1LPS	31.23	1.000
	Within Groups	28				0.33-0.83 LPS	74.45	.002
	Total	31				Single LPS	5.45	1.000
CRP_w1	Between Groups	3	1.47	.242	87759.00	0.1LPS	112317.50	
	Within Groups	30				0.33-0.83 LPS	105770.00	
	Total	33				Single LPS	79503.75	
CRP_w6	Between Groups	3	4.86	.008	90505.00	0.1LPS	55210.00	.124
	Within Groups	28				0.33-0.83 LPS	40051.25	.006
	Total	31				Single LPS	71444.29	1.000
GM-CSF_w1	Between Groups	3	27.08	.000	1.77	0.1LPS	16.79	.024
	Within Groups	30				0.33-0.83 LPS	35.10	.000
	Total	33				Single LPS	40.45	.000
GM-CSF_w6	Between Groups	3	20.11	.000	1.71	0.1LPS	12.01	1.000
	Within Groups	28				0.33-0.83 LPS	50.60	.000
	Total	31				Single LPS	2.19	1.000
IL-2_w1	Between Groups	3	1.25	.309	15.05	0.1LPS	18.35	
	Within Groups	30				0.33-0.83 LPS	18.31	
	Total	33				Single LPS	15.66	
IL-2_w6	Between Groups	3	24.97	.000	17.94	0.1LPS	27.67	1.000
	Within Groups	28				0.33-0.83 LPS	75.31	.000
	Total	31				Single LPS	17.64	1.000
IL-10_w1	Between Groups	3	26.27	.000	8.20	0.1LPS	81.49	.151
	Within Groups	30				0.33-0.83 LPS	165.93	.000
	Total	33				Single LPS	272.39	.000
IL-5_w1	Between Groups	3	1.26	.306	27.34	0.1LPS	31.77	
	Within Groups	30				0.33-0.83 LPS	32.43	
	Total	33				Single LPS	30.00	
IL-5_w6	Between Groups	3	1.34	.283	28.08	0.1LPS	30.18	
	Within Groups	28				0.33-0.83 LPS	32.98	
	Total	31				Single LPS	29.59	

Hippocampal microglia was not affected by LPS exposure

The estimation of the number of Iba1 positive cells in neurogenic granular zone of the dentate gyrus, as well as in the whole hippocampus showed that the number of microglia was not altered in the long term (seven days later) by LPS injections (Effect of treatment $F(3,20) = 0.45$, $p = 0.72$, see Fig.4(a)). The level of microglial activation was assessed by the density of CD68-positive puncta in the tissue. According to this analysis, LPS exposure did not have a long term effect on microglial activation in DG, CA1 and CA3 areas of the hippocampus (see Figure 4(b)). Additionally, we investigated whether systemic LPS exposure affected the level of plasma corticosterone. For this purpose, blood was collected via cardiac puncture seven days after the last LPS injections. High individual variability was observed in the systemic corticosterone levels and as a consequence no statistical differences were detected amongst mean group levels (see Figure 4(c)).

Figure 4 Hippocampal microglia cell number and activation marker expression in mice exposed to chronic LPS/SAL injections.

8 week old male BALB/cAnNCrl mice were injected with LPS or saline i.p. once a week for 6 weeks. Experimental conditions (n=8-10/group) included: 0.1 LPS group injected with 0.1 mg/kg LPS once weekly; Escalating (0.33-0.83) LPS group injected with 0.33mg/kg on weeks 1 and 2, 0.53mg/kg on week 3, 0.63 mg/kg on week 4, 0.73 mg/kg on week 5 and 0.83 mg/kg on week 6; Single LPS group injected with 0.83 mg/kg on week 1 and saline on weeks 2-6; SAL group was injected weekly with 0.9% saline solution. **(a)** Number of Iba1 positive cells in the dentate gyrus of the hippocampus was unaffected by LPS exposure, ns **(b)** CD68 positive surface area, detected in images taken from different regions of the hippocampus, also was unchanged by LPS exposure (DG – dentate gyrus, CA1 and CA3 – Cornu Ammonis areas), ns **(c)** Corticosterone levels detected in the cardiac blood 7 days after last LPS injections. Data represents mean±SEM.

LPS injections affected the number of postmitotic immature neurons in the hippocampal dentate gyrus.

In the tissue collected seven days after the last injections, the level of proliferating Ki67 positive cells was not altered (see Figure 5(a)). Also, a one-way ANOVA showed that the number of DCX positive immature neurons was not affected by LPS injections ($F(3, 26) = 1.862$, $p = 0.161$). However the number of “EF” type postmitotic neuroblasts was significantly affected by treatment conditions ($F(3,26)=15.46$, $p<0.0001$). Post-hoc Bonferroni multiple comparisons showed that weekly injections of 0.1 mg/kg LPS increased the number of postmitotic DCX positive neurons and the same effect was induced by a single 0.83 mg/kg LPS injection in single LPS group. In contrast, the number of postmitotic neuroblasts in the escalating LPS (0.33-0.83) group was reduced compared to the saline-injected control (see Figure 5(b)).

Figure 5 Adult hippocampal neurogenesis and corticosterone levels in the LPS/SAL-exposed animals
 8 week old male BALB/cAnNCrl mice were injected with LPS or saline i.p. once a week for 6 weeks. Experimental conditions (n=8-10/group) included: 0.1 LPS group injected with 0.1 mg/kg LPS once weekly; Escalating (0.33-0.83) LPS group injected with 0.33mg/kg on weeks 1 and 2, 0.53mg/kg on week 3, 0.63 mg/kg on week 4, 0.73 mg/kg on week 5 and 0.83 mg/kg on week 6; Single LPS group injected with 0.83 mg/kg on week 1 and saline on weeks 2-6; SAL group was injected weekly with 0.9% saline solution. **(a)** Number of proliferating Ki67 positive cells in the dentate gyrus of the hippocampus was unchanged by LPS injections, ns **(b)** Representative micrographs of DG show DCX+ cells with their dendritic trees (in dark-brown), scale bar = 500 μ m **(c)** Total number of DCX+ cells was not significantly changed by LPS injections; DCX+ cells were then classified according to their dendritic morphology: AB – no or short dendrites, CD – medium length dendrites without branching; EF – long branching dendrites reaching the molecular layer. Data represents mean $\pm$ SEM; P values derived from Bonferroni multiple comparisons test SAL vs 0.1LPS/Escalating LPS/Single LPS, *p<0.05, **p<0.01

Discussion

In this study we investigated the change in sickness behaviour and plasma cytokine levels in response to repeated and escalating dose LPS injections. Our data showed that systemic exposure to escalating doses of LPS administered once a week for six weeks repeatedly induced core aspects of sickness behaviour, such as reduced food intake and weight loss. In contrast, the effect induced by the repeated 0.1 mg/kg LPS dose was present only intermittently. However, hypolocomotion detected in the open field test in the escalating LPS (0.33-0.83) group on week one was reduced on week six, which could be reflecting a degree of developing LPS tolerance. It is also likely that the locomotor activity data was affected by a relatively late timing of assessment (6 hours post injections) and a repeated exposure to the open field arena.

Assessment of peripheral cytokine levels in response to LPS showed that chronic escalating dose injections resulted in a cytokine profile distinct from acute response, while single and low dose repeated LPS injections did not induce any long-lasting changes in the blood cytokine levels. The escalating dose induced profile includes a lower magnitude of elevation of proinflammatory cytokines IL-6 and TNF α , reduction of CRP and contrasting elevation of IL-2 and GM-CSF. These changes partly point towards a developing tolerance to LPS, but are contradicted by an absence of the anti-inflammatory IL-10 elevation on week six typically accompanying endotoxin tolerance

(Biswas and Lopez-Collazo, 2009). The surprising absence of CRP elevation on week 1 is likely to be linked to the early timing of blood collection, as CRP levels are known to peak at 18 hours post LPS challenge (Szalai et al., 2000). Thus week 6 reduction potentially reflects chronic suppression of CRP in-between LPS stimulations. Noteworthy, TNF α levels detected on week 6 were similar to that previously reported in a chronic (2 weeks) LPS administration paradigm which included LPS injections i.p. at a dose of 0.05mg/kg (Elgarf et al., 2014).

Elevation of IL-2 and GM-CSF by escalating dose exposure is of particular interest. It has been reported that depressed patients have elevated plasma levels of soluble IL-2 receptor (Maes et al., 1995), and more recently elevation of IL-2 levels in the blood has been reported in chronic obstructive pulmonary disease patients with comorbid depression (Rybka et al., 2016). Pro-inflammatory GM-CSF (Shi et al., 2006) has been previously shown to be elevated in the blood by 3 consecutive but not single injections of 3mg/kg LPS in mice (Erickson and Banks, 2011). The increase of GM-CSF and IL-2 cytokines in response to chronic injections may reflect an alternative state of immune system activation, distinct from the conventional response to a single LPS exposure and from the previously described endotoxin tolerance. However it is likely to have some features of immunosuppression as reflected by a reduction in CRP level. Many repeated LPS studies limit their serological analysis to a single cytokine (Elgarf et al., 2014; Wang

et al., 2011), therefore it is difficult to compare current results to most of the previously published data.

Interestingly, single 0.83 mg/kg dose did not induce any long-lasting changes in the cytokine levels, albeit this dose induced long-lasting depression-like behaviour in a previous study (Painsipp et al., 2011). It is important to note that plasma cytokine levels might not be representative of the cytokine content in the brain. Indeed, several studies have shown that after repeated LPS injections levels of peripheral blood pro-inflammatory cytokines might return to control levels whilst the level of cytokine expression in the brain remains high (Chen et al., 2005; Fischer et al., 2015; Norden et al., 2016).

Due to the suggested role of adult hippocampal neurogenesis in depression neurobiology and in the mechanism of antidepressant action, we asked if systemic chronic LPS exposure would alter the rate of AHN. LPS did not affect cell proliferation in the DG in accordance with previously published studies (Belarbi et al., 2012; Ormerod et al., 2013), and did not change the level of neuronal differentiation reflected by the DCX-positive cell density. Importantly most previous studies which showed effect of LPS on AHN utilised higher doses such as 1 mg/kg or more and looked at an earlier time point of post LPS exposure assessment (Graciarena et al., 2013; Monje, 2003; Ormerod et al.,

2013). However, we found that escalating dose LPS reduced the number of postmitotic “EF”-type DCX positive neurons. It has been previously shown that LPS can disrupt morphological maturation of DCX positive cells (Valero et al., 2014). The effect of escalating dose injections on the postmitotic morphology replicates the effect which chronic mild stress, an animal model of depression, exerts on dendritic branching of DCX-positive hippocampal cells (Qiao et al., 2016; Sousa et al., 2000; Vega-Rivera et al., 2016; Vyas et al., 2002). Importantly, this effect is thought to underlie the reduction of hippocampal volume described in neuroimaging studies of depressed patients (Lorenzetti et al., 2009). As dendritic morphology is a form of neural plasticity, this effect could also be involved in a cognitive decline frequently observed in clinical depression (Femenía et al., 2012). Interestingly, a single 0.83 mg/kg LPS dose, as well as repeated 0.1 mg/kg LPS doses caused an opposite effect, an increase in the number of post-mitotic “EF” type neuroblasts, which could be part of a compensatory increase in neurogenesis previously described 7 days after LPS exposure (Valero et al., 2014).

We next speculated if activation of microglia might be affecting the adult hippocampal neurogenesis following LPS exposure. Microglia are known to have the ability to modulate neurogenesis during an inflammatory insult (Belarbi and Rosi, 2013; Ekdahl, 2012), potentially through a release of pro-inflammatory cytokines, which have been shown to exert a detrimental effect on neural stem cells proliferation and differentiation

(Koo and Duman, 2008; Zunszain et al., 2012). It has been commonly described that systemic LPS exposure induces microglial activation in the hippocampus (Ho et al., 2015; Noh et al., 2014). However, in our study LPS exposure did not have a long-lasting effect on the hippocampal microglia as reflected by Iba-1 and CD68 markers density. This negative result could indicate the insufficiency of the escalating dose LPS exposure to induce microglial activation. It is also likely that a relatively late timepoint of assessment compared to previous studies (Borges et al., 2012) and an absence of morphological assessment which could more accurately reflect activation state of microglial cells contributed to this result.

Another notable finding in the biological parameters is the absence of an increase in blood corticosterone levels previously reported in response to LPS administration (Browne et al., 2012). It has been shown that corticosterone levels return to baseline within the first 24 hours post LPS exposure (Clark et al., 2015; Silverman et al., 2013), a time period within which most studies looking at single or repeated LPS effects assess its levels (Kubera et al., 2013; Sulakhiya et al., 2016). Thus timing of blood collection (7 days post last LPS administration) could explain the lack of the effect in our study, which in turn suggests that once weekly injections might not be sufficient to maintain continuous hypercortisolism in mice. The lack of chronic corticosterone elevation could

also account for the absence of decline in the hippocampal neurogenesis, as this has been attributed to the glucocorticoid receptor activation (Anacker et al., 2011).

Conclusions

In this study we showed that weekly injections with escalating but not constant dose of LPS increasing from 0.33 to 0.83 mg/kg induce a weekly sickness behaviour response, with partial development of LPS tolerance reflected by a lack of hypolocomotion some suppression of the inflammatory pathways as reflected by blood CRP levels and a weakening of pro-inflammatory cytokine secretion. However, it also comprises an increase in factors capable of stimulating leukocyte proliferation, including an increase in plasma IL-2, previously implicated in depression, not seen in repeated or single dose exposed group. Additionally, assessment of AHN shows that only exposure to a weekly escalating doses in the range of 0.33-0.83 mg/kg of LPS decelerates dendritic maturation of new neurons in the hippocampal dentate gyrus. This effect is in line with previous observations of the effect of depression-modelling interventions on AHN in animals, and is relevant for hippocampal volume changes and cognitive decline observed in depressed patients. These findings suggest that repeated LPS injections modify sickness behaviour and immune response in mice, however weekly LPS injections with escalating

doses in the range of 0.33-0.83 mg/kg could be a suitable model to induce some neurobiological aspects of the chronic inflammatory state observed in mood disorders.

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Declaration of conflicting interests

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