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Acute neuroinflammation, sickness behavior and working memory responses to acute systemic LPS challenge following noradrenergic lesion in mice

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ABSTRACT

Locus coeruleus (LC)-derived noradrenaline is important in cognition and decreases with age, but the impact of prior noradrenaline deficiency on vulnerability to inflammation-induced acute cognitive dysfunction is unclear. Here we assessed whether noradrenergic depletion, in female mice, impacted upon inflammation, locomotor activity and working memory directly after acute systemic immune challenge with bacterial lipopolysaccharide (LPS), a paradigm we have previously used to capture delirium-like acute cognitive deficits. Mice received 2 doses of the LC-selective noradrenergic toxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4; 50 mg/kg i.p.) and were challenged, 2 weeks later, with LPS (100 μg/kg i.p.). DSP-4 dramatically reduced noradrenaline concentrations and tyrosine hydroxylase-positive afferents in the frontal cortex and hippocampus. This did not significantly alter numbers of Pu.1-positive microglia, Iba1-positive microglial morphology or mRNA expression of microglia-associated gene transcripts (*Tyrobp, Sall1, Cd68, Sra2*, *Clec7a)* in the hippocampus or frontal cortex and produced modest reductions in *Cx3cr1* and *P2ry12*. LPS induced blood and brain cytokine levels, cFOS activation and locomotor responses that were highly similar in DSP-4- and vehicle-treated mice, although LPS-induced plasma TNF-α was significantly reduced in those treated with DSP-4. Importantly, prior noradrenergic depletion did not predispose to LPS-induced T-maze working memory deficits. The data demonstrate that significant depletion of noradrenaline in the hippocampus and frontal cortex does not prompt acutely exaggerated neuroinflammation or leave the brain vulnerable to acute, transient working memory deficits upon low dose LPS challenge. These findings have implications for our understanding of the impact of systemic inflammation on the aging and vulnerable brain during septic encephalopathy and delirium.

1. Introduction

The endogenous neurotransmitter noradrenaline (NA) is critical for wakefulness and arousal (Robbins, 1997), perceptual acuity (Doucette et al., 2007), attentional stability and responsiveness (Aston-Jones et al., 2000). NA also promotes memory consolidation and contextual memory retrieval via β_1-adrenergic receptor signalling in the hippocampus (Murchison et al., 2004; Sterpenich et al., 2006; Tronel et al., 2004). The selective β_2-adrenoceptor agonist clenbuterol improves cognitive performance in T-maze trials of spatial working memory and in delayed response testing in aged rats and in monkeys with existing memory impairments (Ramos et al., 2008).

Noradrenaline also exerts tonic anti-inflammatory actions in the CNS by regulating glial cell activation (O'Sullivan et al., 2009; Heneka et al., 2010). Noradrenaline reuptake inhibitors suppress increases in the chemokines IP-10 and RANTES and the cell adhesion molecules VCAM-1 and ICAM-1 in the cortex and hippocampus following systemic LPS challenge (O'Sullivan et al., 2010). Similarly, pharmacologically enhancing noradrenergic tone inhibits microglial activation, TNF-α and IL-6 gene expression and attenuates dopaminergic neurodegeneration in

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the intra-nigral LPS model of Parkinson's disease via activating β_2-adrenoceptors (O'Neill et al., 2020; Yssel et al., 2018). Moreover, noradrenergic dynamics have been shown to influence microglia process activity (Liu et al., 2019, Stowell et al., 2019). Given the key role(s) of LC-noradrenergic neurons in maintaining attention and in orchestrating memory encoding, consolidation and retrieval, as well as the potential of noradrenaline to suppress acute inflammatory responses, we hypothesised that noradrenergic depletion might lead to more severe inflammatory activation and cognitive dysfunction upon acute systemic inflammation.

Systemic inflammatory episodes are drivers of cognitive decline in the aged and vulnerable brain and can increase the risk of dementia (Cunningham and Hennessy, 2015). Systemic inflammation incites neuro-inflammation in the degenerating brain and induces acute and transient working memory deficits in mice, resembling aspects of delirium (Skelly et al., 2019). Microglia are known to be primed by prior neurodegeneration in multiple animal models (Cunningham et al., 2005; Holtman et al., 2015) and, in those conditions, systemic LPS challenge induces exaggerated microglial IL-1β responses which may contribute to more severe acute cognitive sequelae (Skelly et al., 2019). Age also leaves the brain more susceptible to acute cognitive dysfunction upon superimposed inflammatory stressors (Barrientos et al., 2012) and in neurodegenerative models we have shown that generalised hippocampal synaptic degeneration (Murray et al., 2012) and specific depletion of cholinergic projections originating in the medial septum (Field et al., 2012) both predispose animals to LPS-induced working memory dysfunction in T-maze experiments. Thus, loss of presynaptic terminals may be an important risk factor for acute cognitive deficits, such as delirium, triggered by subsequent acute systemic inflammation (Davis et al., 2015; Peters van Ton et al., 2020) and we have explicitly used the models above to begin to address neuroinflammatory and metabolic mechanisms of delirium. Given that degeneration of the LC-noradrenergic system occurs years prior to cognitive decline in Alzheimer's disease, with cortical NA-depletion correlating tightly with severity of dementia (German et al., 1992; Lyness et al., 2003; Theofilas et al., 2017) we reasoned that degeneration of the LC-noradrenergic system and loss of noradrenergic tone may constitute a specific vulnerability to secondary inflammatory insults. We hypothesised that prior depletion of hippocampal and cortical noradrenaline would exacerbate the CNS response to systemic inflammation and facilitate acute cognitive dysfunction upon exposure to such acute, immune stressors.

N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) is a neurotoxin selective for the LC-noradrenergic system (Ross and Stenfors, 2015; Jonsson et al., 1981). DSP-4-mediated loss of LC neuronal cell bodies and depletion of noradrenaline in LC-projection areas is reported to predispose to enhanced Aβ-induced cortical inflammation, and to expedite the onset of cognitive decline in APP/PS1 transgenic mice (Heneka et al., 2002; Jardanhazi-Kurutz et al., 2010) and to aggravate spatial learning and memory deficits in a transgenic mouse model of tauopathy (Chalermpalanupap et al., 2018). This suggests a role for the LC-noradrenergic system in maintaining cognitive reserve in the face of chronic neurodegenerative pathology. The impact of noradrenaline depletion on cognitive vulnerability and on the neuroinflammatory response to acute, transient systemic inflammation, is currently unknown. In the present study, we assessed the impact of prior LC-noradrenergic system dysfunction and loss of noradrenergic tone on central and peripheral inflammation and cognition in response to an acute, inflammatory stressor.

2. Methods

2.1. Animals

60 Female C57BL/6 mice aged 8–10 weeks (Harlan Olac Ltd, Bicester, United Kingdom) were housed in cages of 5 at 21 °C on a 12:12 h light–dark cycle with food and water available *ad libitum*. We used female mice for behavioral experiments since the frequency of fighting between males and the occurrence of injury and/or the need to single-house as a consequence has had, in our hands, impacts on behavior that were greater than any influence of oestrus cycle observed in our female mice. All animal experiments were in accordance with the European Commission Directive 2010/63/EU and were performed following ethical approval by the Trinity College Dublin Animal Research Ethics Committee and licensing by the Department of Health and Children and the Health Products Regulatory Authority (HPRA).

2.2. Experimental design

Mice were randomly assigned into the following four treatment groups via block (cage) randomisation to prevent accidental bias in our study design: (1) PBS + saline, (2) DSP-4 + saline, (3) PBS + LPS, (4) DSP-4 + LPS, $n = 10$ per group. Sample size estimation was conducted via power analysis (G*Power 3.1) from previous data sets, based on the prediction of exaggerated responses to LPS challenge in DSP-4 animals. DSP-4 (50 mg/kg i.p.) was injected twice with doses set one week apart (days 0 & 7). Two weeks following the second DSP-4 administration (day 21), animals were challenged systemically with 100 μg/kg i.p. bacterial lipopolysaccharide (LPS; *Salmonella equine abortus*, L5886, Sigma, Poole, UK) in a volume of 200 μl sterile saline (per 20 g body weight). We have demonstrated that this dose is sufficient to produce moderate systemic inflammation in female C57BL6 mice, and sub-threshold effects on working memory performance (Field et al., 2012), such that there was capacity for effects of NA depletion to modulate responses to LPS upward or downward. Control animals were administered 200 μl of vehicle solution (sterile saline). Behavioural and post-mortem analyses were performed by two unbiased experimenters blinded to the study design. All animals for inflammatory analysis were euthanised 3 hrs post-LPS challenge via terminal anaesthesia with sodium pentobarbital (40 mg per mouse i.p., Euthatal, Merial Animal Health, Essex, UK) since this represents an excellent compromise time point to collect information on multiple blood and brain cytokines (Murray et al., 2012) while still allowing meaningful behavioural assessment of sickness behavior. A separate cohort of mice was used for working memory experiments, as detailed below, and both cohorts are displayed on the timeline shown in Fig. 1.

2.3. T-maze alternation: Working memory

Hippocampal-dependent working memory was assessed as previously described in our "paddling" T-maze alternation task (Murray et al., 2012). Each mouse was placed in the start arm of the maze with one arm blocked by a guillotine style door so that they were forced to make a left (or right) turn to exit the maze, selected in a pseudorandom sequence (equal number of left and right turns, not >2 consecutively to the same arm). The mouse was held in a holding cage for 25 s (intra-trial interval) during which time the guillotine style door was removed, and the exit tube was switched to the alternate arm. The mouse was then replaced in the start arm and could choose either arm. Alternation from its original turn on the choice run constitutes a correct trial, meaning that 100% alternation constitutes perfect responding in this maze. On choosing incorrectly, the mice were then allowed to self-correct to find the correct exit arm. Animals were trained in blocks of 10 trials (20 min inter-trial interval) over a period of 2 weeks until reaching performance criterion: most animals reached an alternation of $\geq$ 80% but no animals were challenged with LPS or saline unless they showed consecutive days performing at 70% or above, showed no evidence of a side preference and did not score $\leq$ 80% in the last block of five trials before challenge. Animals were randomly assigned to receive LPS or saline and then tested 15 times over 6 h between 3 and 9 h post-LPS and again for 10 trials at 24 h. One week later, using a crossover design, the same animals, upon confirming stable baseline T-maze performance, were challenged with

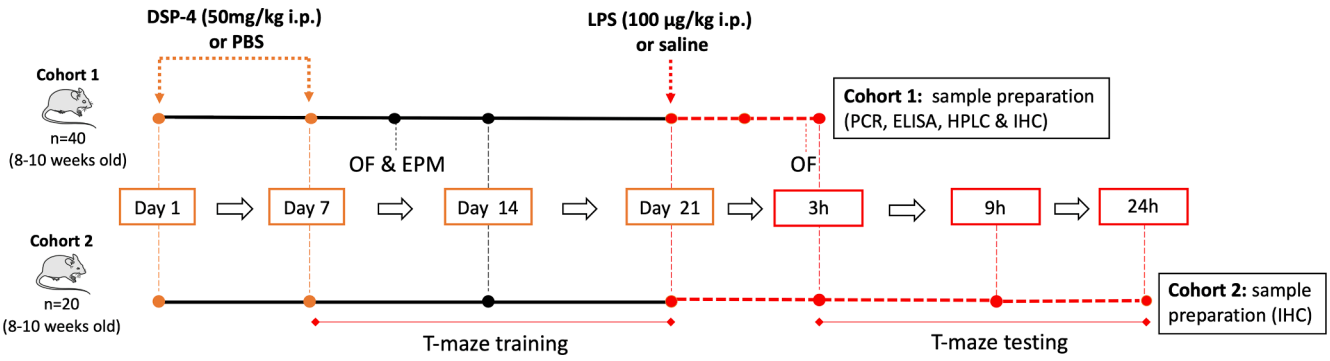

Fig. 1. Experimental timeline. Mice were administered two doses of DSP-4 (50 mg.kg i.p.) or vehicle control (sterile PBS) set one week apart (Days 0 & 7) and some animals were subsequently challenged systemically with bacterial LPS (100 µg/kg i.p.) or saline control two weeks later (Day 21). Mice were either euthanized 3 h later by transcardial perfusion-fixation in preparation for immunohistochemistry or rapidly perfused and the hippocampus and frontal cortex dissected out in preparation for HPLC analysis of monoamine concentrations (cohort 1). A separate cohort of animals (cohort 2, n = 20) were used for cognitive experiments since those experiments required performance of T-maze testing up until 9 h on the day of LPS challenge and again for 4 h on the subsequent day (i.e. at 24 h).

the opposite treatment to that received in the first challenge, and again assessed for T-maze performance 15 times over 6 h between 3 and 9 h post-LPS and again for 10 trials at 24 h.

2.4. Open field activity

Open field activity was assessed as previously described (Skelly et al., 2013). In brief, the open field consisted of an opaque grey perspex box of base (58 cm × 33 cm) with four walls of 19 cm height. The floor of the box is divided into a grid of equal sized squares. For all open field locomotor activity experiments, mice were moved from the housing room and left in the test room for 15 min prior to beginning the task to ensure an optimal state of arousal. The distance travelled (grid squares crossed) and the total number of rears performed were measured (counted manually and defined as instances where the animal stood on its hind paws only). Naïve animals had their baseline activity measured for 3 min when placed in the middle of the open field. At 2 h post-challenge, the animals' locomotor activity was measured again. This period, and this time post-LPS, are appropriate to capture the acute effect of LPS on locomotor activity. Separate cohorts of mice were tested in the open field and T maze analyses.

2.5. Elevated plus maze

The elevated plus maze task was used to test for anxiety-like behaviour in mice (Walf and Frye, 2007) and was performed after lesioning only (ie not performed post-LPS). The maze consists of four arms (two open without walls denoted North/South and two enclosed by high walls denoted East/West) 35 cm long and 5 cm wide. The maze was elevated 45 cm above the surface it was placed on. The mouse was placed in a start arm (closed arm). The groups were counterbalanced with respect to start arms. The time spent in the open and closed arms, latency to first emerge from a closed arm and the number of open and closed arm entries were recorded for five minutes. The number of entries and the time spent in the junction were also observed. When at the junction, the mouse was regarded as being neither in an open arm nor in a closed arm.

2.6. Tissue processing and immunohistochemistry

For immunohistochemistry, animals were transcardially perfused with heparinised saline followed by 10% neutral buffered formalin (Sigma, Poole, UK) for approximately 15 min/50 mls of fixative. Brains were then removed and post-fixed in the same fixative for 3 days before embedding in paraffin wax. Coronal tissue sections (10 µm) of the hippocampus and locus coeruleus were cut on a microtome (LEICA RM2235). Sections were labelled with mouse monoclonal anti-tyrosine hydroxylase [TH, (1:2000, MAB318, Millipore)], c-FOS (1:1000, sc-52, Santa Cruz), or anti-IBA1 antibody (1:2000, ab5076, Abcam) after pre-treatment with 1% H_2O_2/methanol (20 min), microwaving in citrate buffer (pH 6) for 2 × 5 min, and immune-blocking with 10% normal horse serum. IBA-1 sections were pre-treated with 0.04% pepsin for 20 min prior to blocking. Thereafter, labelling was completed using the appropriate biotinylated secondary antibody, the ABC method and 3,3′-diaminobenzidine (DAB) as a chromogenic substrate as previously described (Cunningham et al., 2005). TH-positive cells were identified and counted at specific locations through the LC. We took 10 µm coronal sections through this nucleus and, using the Watson and Paxinos mouse brain atlas and all possible orientation 'landmarks', grouped our sections according to the anterior-posterior position at which they were located (AP: −5.34 mm, −5.40 mm and −5.68 mm from bregma). TH^+ cell counting in the LC was performed by 'particle analyser' on 8 bit, binary images, thresholded manually to isolate all TH + cells from low level background labelling, followed by digital analysis (counting) of all identified 'particles', filtered to exclude particles smaller than 1000 pixels in these binary images. Isolated particles were averaged for 4 sections per animal. Analysis of TH^+ neurites and terminals was performed using a similar strategy but now quantifying much smaller objects (excluding all objects > 500 pixels) and allowing a full range of circularity to avoid exclusion of elongated structures.

$Iba1^+$ microglial morphology and $Pu1^+$ cell counts in the hippocampus were also performed using ImageJ software (Version 1.52p) followed by the 'particle analyser' function for cell counts and area and using the 'Analyze Skeleton' plugin from FIJI: to assess branch numbers in a cell-specific manner https://imagej.net/AnalyzeSkeleton .

2.7. High performance liquid chromatography with electrochemical detection (HPLC-ECD)

Mice for HPLC analysis were terminally anaesthetised with sodium pentobarbital and rapidly perfused with saline but without fixation. The hippocampus and frontal cortex were precisely scalpel-dissected on wet ice using Palkovits and Brownstein brain atlas for reference (Palkovits and Brownstein, 1988). Noradrenaline concentrations in the hippocampus and frontal cortex were measured as previously described (Yssel et al., 2018). In brief, peak heights were identified by retention times set to known biogenic amine standards and data were normalized for total wet weight of sample. Neurotransmitter concentrations were quantified by electrochemical detection and resulting chromatograms were generated using a Merck Hitachi D-2000 integrator. Inclusion of the internal standard (N-methyl serotonin) in each sample allowed for correction of processing losses. These data, together with the brain tissue

weights, were used to calculate the concentration of neurotransmitter in each sample. Results are expressed in terms of neurotransmitter (ng) per wet weight of tissue (g).

2.8. Enzyme-linked immunosorbent assay

Under terminal anaesthesia, the thoracic cavity was opened, and blood samples were collected directly into heparinized tubes from the heart. This whole blood was centrifuged to remove cells and the remaining plasma was aliquoted and stored at −20 °C until time of assay. This plasma collection was less successful in some samples, leaving slightly uneven amounts of plasma available for the 3 cytokines assessed. Quantification of serum IL-6 and IL-1β were determined using mouse IL-6 and IL-1β ELISA kits (BioLegend® ELISA MAX™ Standard Set / Cat. No. 431,301 and 432,601 respectively) and Quantification of serum TNF-α was performed using a mouse TNF-α ELISA kit (R&D Systems, Inc. DuoSet®/Cat. No. DY410) according to the manufacturer's protocol. Capture anti-IL-6 and IL-1β antibodies (diluted 1:180) and the anti-TNF-α antibody (diluted 1:200 to approximately 1 μg/ml) were used to coat 96-well maxisorb microplates (Nunc; Fisher Scientific, Leicestershire) overnight at 4 °C with 100 μl per well. Plates were then washed with PBS + 0.05% Tween and blocked with PBS + 1% BSA before addition of samples. Six 2-fold serial dilutions of the 2000 pg/ml top standard were prepared using assay diluent to produce seven standards in total; 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml (assay diluent served as the zero standard / 0 pg/ml). 100 μl/well of detection antibodies were used (1:200 in assay diluent) for 1 hr at room temperature. 100 μl of Streptavidin-poly horseradish peroxidase (diluted 1:1000 in assay diluent) was added to each well. TMB and H_2O_2 were used as substrate and the reaction was stopped with 1 M H_2SO_4 and the optical density read at 450 nm with correction at 570 nm using a plate reader (BioTek® Synergy HT, MASON Technology).

2.9. RNA extraction and quantitative PCR

Total RNA was isolated using the RNeasy Plus Mini method (Qiagen) as per the manufacturer's instructions. To ensure complete DNA elimination from the column-bound RNA, an on-column DNase step was performed. The RNA yield and quality of each sample was quantified based on optical density using the NanoDrop'ND-1000 UV–Vis spectrophotometer (Thermo Fisher Scientific) and those with insufficient yields were not analysed in PCR experiments. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). cDNA was stored at 20 °C until use in RT-PCR. All primer and probe sets were designed using Applied Biosystems Primer Express software and amplified a single sequence of the correct amplicon size, as verified by SDS-PAGE. Samples for RT-PCR were run in duplicate and contained 12.5 μl FastStart Universal Probe Master mix; 0.5 μl of each of the forward primer (10 μM), reverse primer (10 μM), and probe (10 μM); and 10 μl RNase-free water. All PCR was performed in a StepOne Real-Time PCR system (Applied Biosystems) under the cycling conditions: 95 °C for 10 min followed by 95 °C for 10 s and 60 °C for 30 s for 40 cycles (45 cycles were used for *Il1b* and *Il6*). Quantification was achieved by exploiting the relative quantitation method, using cDNA from LPS-injected mouse brain as a standard expressing all genes of interest and fourfold serial dilutions of this cDNA to construct a linear standard curve relating cycle threshold values to relative concentrations. This method has been described in detail previously (Cunningham et al., 2005). Gene expression data were normalized to the housekeeping gene GAPDH.

2.10. Statistical analysis

Data distributions were tested for normality using a Kolmogorov-Smirnov test. T-maze, behavioral and tyrosine hydroxylase labelling datasets were normally distributed while group sizes for PCR and ELISA assays were too small to assess normality. In those cases we analysed using parametric statistics due to their higher power to detect differences in groups of unequal variance and their utility is analysing main effects and interactions. One outlier was removed from the DSP-4 open field data-set, being > 3 standard deviations from the mean. Data were analysed via Student's *t*-test (HPLC and TH + immunolabelling), two-way ANOVA (open-field, ELISA, Pu.1 and Iba1-immunolabelling & PCR) or 3-way ANOVA as appropriate (T-maze working memory data, DSP-4, LPS & Time as a repeated measure) followed by a Bonferroni *post hoc* test if any significant main effect(s) were found. All data were analysed using GraphPad Prism 8 software (GraphPad Prism, San Diego, CA). Parametric data are expressed as mean ± standard error of the mean (S.E.M.).

3. Results

3.1. DSP-4 depletes noradrenaline in the hippocampus and frontal cortex

To determine whether DSP-4 (50 mg/kg i.p.; 2 doses set one week apart) had any impact on behaviour, we examined mice in tests of locomotor activity, anxiety and cognitive performance. DSP-4 had no significant effect on the number of square crossings made or rearing in the open field test of locomotor activity relative to vehicle-injected controls (Fig. 2 A,B). DSP-4 had no effect on time spent in the open arms of the elevated plus maze relative to vehicle-injected controls (Fig. 2 C).

To confirm the impact of DSP-4 on noradrenaline concentrations, we measured NA tissue content in the hippocampus and frontal cortex using HPLC-ECD. DSP-4 selectively depleted noradrenaline concentrations in the hippocampus and frontal cortex by approximately 82% ($t_{(16)} = 15.43$, $P < 0.0001$) and 58% ($t_{(17)} = 10.05$, $P < 0.0001$) respectively relative to vehicle-injected controls without affecting the tissue concentration of dopamine or serotonin (Fig. 2 D & E). These results confirm that DSP4 treatment successfully and selectively depleted noradrenaline in the hippocampus and frontal cortex.

Anti-tyrosine hydroxylase (TH) immunohistochemistry was then performed to assess the impact of DSP-4 on LC catecholaminergic neuronal cell bodies and their axonal projections. DSP-4 showed measurable effects on TH-positive neurons in the locus coeruleus, significantly reducing TH^+ cell bodies by approximately 26% relative to vehicle-injected controls ($t_{(17)} = 4.338$, $P = 0.0004$) (Fig. 2 I, J). DSP-4 reduced TH^+ synaptic terminals and fibres in the hippocampus by approximately 85% relative to vehicle-injected controls ($t_{(15)} = 12.23$, $P < 0.0001$). The few remaining immunoreactive elements showed evidence of hypertrophy. The area of TH^+ immunoreactivity in surviving hippocampal synaptic terminals/neurites was increased in DSP-4 treated mice relative to those from vehicle-injected controls ($t_{(15)} = 5.350$, $P < 0.0001$) (Fig. 2 G, H).

3.2. DSP-4 lesions do not exacerbate locomotor activity or working memory responses in response to acute, systemic LPS activation

To determine whether systemic inflammation impacted behaviour differently in normal and DSP-4 treated mice we examined mice in tests assessing locomotor activity and working memory. LPS decreased the number of square crossings and rears in the open-field but this suppression in locomotor activity was identical in normal and DSP-4 treated mice. Two-way ANOVA demonstrated an effect of systemic LPS on the number of square crossings in the open field ($F_{(1,\ 33)} = 55.63$, $P < 0.0001$) but there was no effect of DSP-4. Bonferroni *post hoc* test revealed that systemic LPS decreased the number of square crossings in the open-field in both DSP-4 treated mice and vehicle-injected mice relative to saline-injected controls ($P < 0.001$) (Fig. 3 A). Two-way ANOVA demonstrated an effect of systemic LPS on the number of rears in the open field ($F_{(1,\ 34)} = 39.88$, $P < 0.0001$) but there was no effect of DSP-4. It should be noted that there may be floor effects with

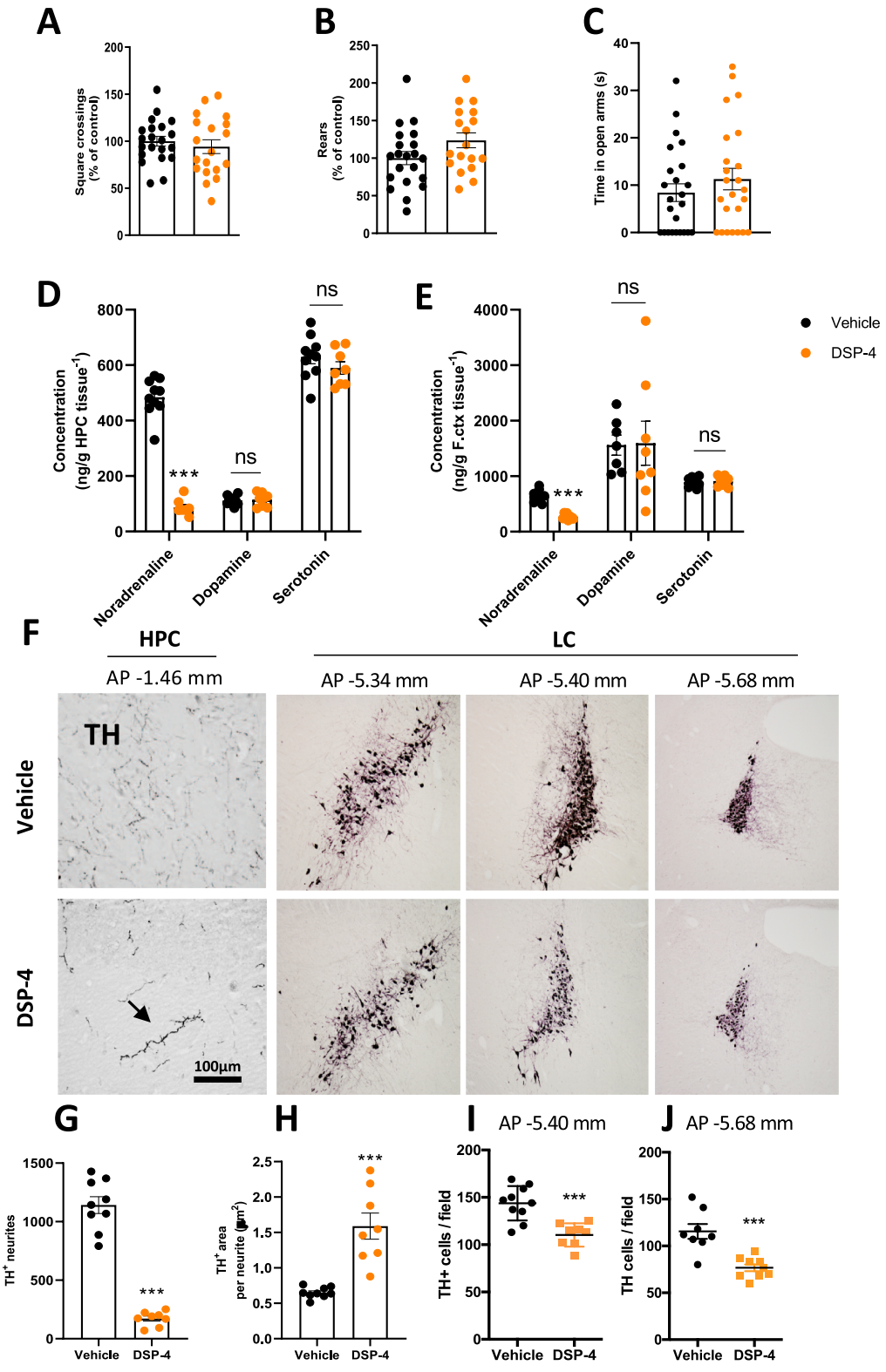

Fig. 2. DSP-4 depletes noradrenaline in the hippocampus and frontal cortex. Mice were administered two doses of DSP-4 (50 mg.kg i.p.) or vehicle control set one week apart and subsequently assessed behaviourally. DSP-4 had no effect on the number of square crossings made in the open field (A) on rearing behaviour in the open field (B) nor on the duration of time spent in open arms in the elevated plus maze (C). After transcardial perfusion LC catecholaminergic neurons were assessed via immunohistochemistry and monoamine concentrations were assessed via HPLC. DSP-4 selectively reduced noradrenaline concentrations in the hippocampus (D) and in the frontal cortex (E). (F) Representative images of TH-positive synaptic terminals / neurites in the hippocampus and TH-positive cell bodies at different anterior-posterior positions in the locus coeruleus. (G) DSP-4 induced a significant loss of TH^+ neurites in the hippocampus and (H) lead to the appearance of less, but larger, morphologically altered surviving TH^+ neurites. DSP-4 reduced TH^+ cell bodies in the locus coeruleus at AP – 5.4 mm (I) and at AP −5.68 mm (J). All values and mean ± SEM are shown for each animal. Data were analysed by students *t*-test for simple comparisons or by 2 way ANOVA where there were > 2 groups. ***P < 0.001 vs. saline-injected controls (n = 19–21, panels A-C, n = 8–10 panels D-J) and subsequently by Bonferroni *post hoc* tests upon significant main effects.

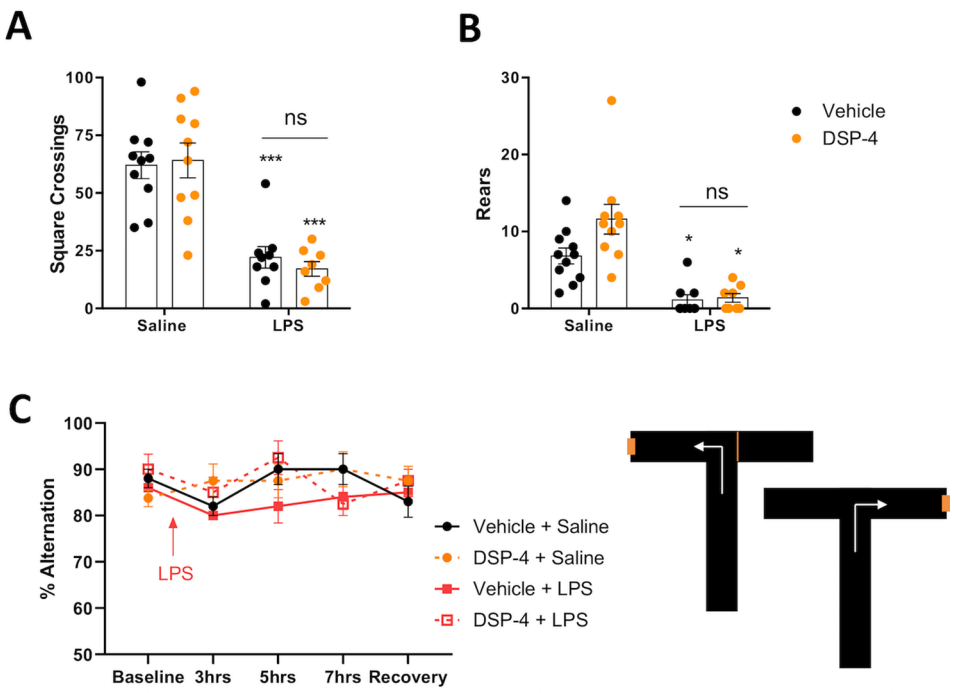

Fig. 3. DSP-4 does not alter locomotor activity or working memory responses to acute systemic inflammation. Mice were administered two doses DSP-4 (50 mg.kg i.p.) or vehicle control set one week apart and subsequently challenged systemically with bacterial LPS (100 μg/kg i.p.) or saline control two weeks later. (A) DSP-4 does not affect the decline in square crossings in response to systemic LPS in the open field when assessed two hours post-LPS challenge. (B) DSP-4 does not alter deficits in rearing behaviour in response to systemic LPS in the open field when assessed at two hours post-LPS challenge. (C) Working memory was tested in the paddling T-maze, 15 times, between 3 and 9 h post-LPS (100 μg/kg) or saline in naive and DSP-4-lesioned mice. Values are shown for activity of each animal but mean ± S.E.M is also shown. T-maze data are expressed as mean ± S.E.M. *P < 0.05, ***P < 0.001 vs. saline injected controls (n = 8–10) and analysed by three-way ANOVA with Bonferroni *post hoc* test.

respect to suppression of rears by LPS (Fig. 3 B). The data indicate that prior DSP-4 lesion did not have a significant effect on the intensity of the sickness behavior response to systemic LPS. Consistent with this, there were no obvious differences in patterns of the immediate early gene cFOS in multiple brain regions associated with cognition, arousal and systemic inflammation-induced sickness behavior (Suppl. material; S1).

To probe for acute working memory deficits, we tested mice in the escape from water T-maze, a hippocampal-dependent task. Prior NA-depletion did not prompt working memory deficits and neither were lesioned animals predisposed to show acute working memory deficits in response to systemic LPS challenge. That is, neither DSP-4 nor LPS, alone or in combination, had any statistically significant effect on percentage alternation in the T-maze trials of working memory relative to controls at baseline, 3, 5 or 7 h post-LPS challenge (Fig. 3 C). Three-way repeated measures ANOVA demonstrated that DSP-4 did not affect performance ($F_{1,80} = 1.212$, $P = 0.274$). There was a trend towards LPS-induced effects ($F_{1,80} = 3.61$, $p = 0.06$), but there was no interaction between DSP-4 and LPS ($F_{1,80} = 1.657$, $P = 0.2017$). Multiple prior studies have shown that LPS, at 100 μg/kg does not impair performance on this task in normal animals but, over the same time course, LPS does produce selective T-maze deficits in mice with prior hippocampal or cholinergic pathology (Murray et al., 2012; Field et al., 2012).

3.3. *DSP-4 lesion has limited effects on inflammatory cytokine production in response to acute, systemic LPS challenge*

To determine whether noradrenergic depletion impacted inflammatory cytokine production in response to systemic LPS challenge we performed ELISA's for plasma IL-1β, TNF- α and IL-6. Two-way ANOVA demonstrated an effect of LPS ($F_{1, 31} = 70.41$, $P < 0.0001$) on plasma IL-1β. There was no effect of DSP-4 on IL-1 levels ($F_{1,31} = 1.587$, $P = 0.2172$) and no interaction between DSP-4 and LPS ($F_{1,31} = 1.948$, $P = 0.1727$). Bonferroni *post hoc* test revealed that there was no difference between vehicle and DSP-4 treated groups in response to LPS (Fig. 4 A).

DSP-4 reduced the impact of LPS upon TNF-α levels. Two-way ANOVA demonstrated an effect of DSP-4 ($F_{1, 31} = 8.901$, $P = 0.0055$) an effect of LPS ($F_{1, 31} = 48.88$, $P < 0.0001$) and an interaction effect between DSP-4 and LPS ($F_{1, 31} = 5.666$, $P = 0.0236$) on circulating TNF-α levels. Bonferroni post-hoc test showed that circulating TNF-α levels were significantly lower in DSP-4-injected mice challenged with systemic LPS relative to vehicle-injected mice challenged with saline control ($P < 0.05$).

Two-way ANOVA demonstrated an effect of LPS ($F_{1, 33} = 64.18$, $P < 0.0001$) on blood IL-6 levels but no effect of DSP-4 ($F_{1, 33} = 0.7396$, $P = 0.3965$) and no interaction between DSP-4 and LPS ($F_{1, 33} = 0.7395$, $P = 0.3965$). Bonferroni *post hoc* test revealed that systemic LPS increased circulating IL-6 expression levels equally in both vehicle- and DSP-4-injected mice relative to saline-injected controls ($P < 0.001$).

We then assessed whether systemic inflammation differentially affected the transcription of proinflammatory cytokines in the hippocampus and frontal cortex of normal and DSP-4-lesioned mice. As expected, LPS significantly upregulated *Il1b* and *Tnfa* mRNA in the hippocampus and frontal cortex. Prior LC lesioning with DSP-4 did not significantly alter hippocampal or frontal cortex *Il1b* or *Tnfa* responses to systemic LPS (Fig. 4B). Two-way ANOVA demonstrated that there was an effect of systemic LPS on *Il1b* mRNA expression in the hippocampus ($F_{1,15} = 60.09$, $P < 0.0001$) and in the frontal cortex ($F_{1,14} = 15.82$, $p = 0.0014$). Conversely, there was no effect of DSP-4 on *Il1b* expression in the hippocampus ($F_{1,15} = 0.8306$, $P = 0.3765$) or in the frontal cortex ($F_{1,14} = 0.3976$, $P = 0.5385$) and no interactions between DSP-4 and LPS in either region ($F_{1,15} = 1.31$, $P = 0.2701$: HPC; $F_{1,14} = 0.5286$, $P = 0.4177$: F.Ctx)

Two-way ANOVA demonstrated an effect of systemic LPS on *Tnfa* mRNA expression in the hippocampus ($F_{1,14} = 25.82$, $P = 0.0002$) and in the frontal cortex ($F_{1,13} = 48.09$, $P < 0.0001$) but there was no effect of DSP-4 on TNF-α mRNA expression in the hippocampus ($F_{1,14} = 0.1240$, $P = 0.7300$) or in the frontal cortex ($F_{1,13} = 2.794$, $P = 0.1185$). (Fig. 4B, C). There were also no interactions between DSP-4 and LPS in the hippocampus ($F_{1,14} = 0.4371$, $P = 0.6823$) nor in the frontal cortex ($F_{1,13} = 2.935$, $P = 0.1104$).

COX-2 expression at the brain endothelium is a necessary step for the induction of multiple aspects of the sickness behavior response to LPS. We performed anti-cyclooxygenase-2 immunohistochemistry (COX-2) to assess, in binary terms, whether the brain endothelium was appropriately activated in noradrenaline-depleted brains. COX-2 immunolabelling showed clear endothelial induction of COX-2 in the vasculature

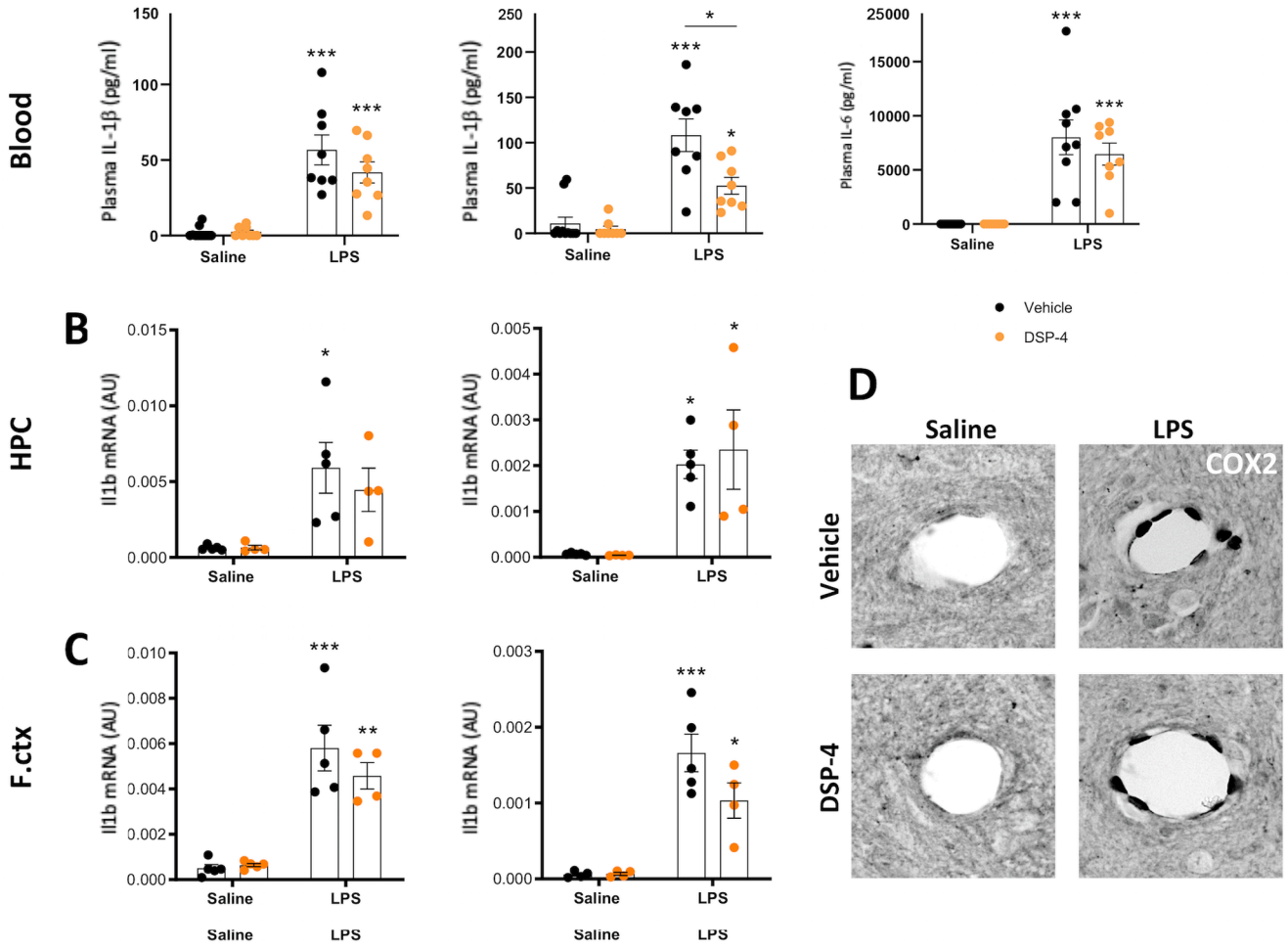

Fig. 4. Prior DSP-4-mediated noradrenergic depletion has limited effects on pro-inflammatory responses to systemic LPS. Mice were administered two doses of DSP-4 (50 mg.kg i.p.) or vehicle control set one week apart and subsequently challenged systemically with bacterial LPS (100 μg/kg i.p.). A) IL-1β, TNF-α and IL-6 concentrations in plasma were measured by ELISA at 3 h post-LPS challenge. Expression of mRNA for *Il1b* or *Tnfa* was measured by RT-PCR after isolation of RNA from hippocampus (B) or frontal cortex (C) 3 h post systemic challenge with LPS. (D) Cerebrovascular COX-2 expression was assessed by diaminobenzidine immunohistochemistry at 3 h post-LPS in DSP-4-lesioned and normal animals, showing positive cells as black cells along the circumference of blood vessels. Values are shown for each animal but mean ± S.E.M is also shown. *P < 0.05, **P < 0.01, ***P < 0.001 vs. saline-injected controls, +P < 0.05 vs. LPS-injected controls (n = 8–11, panel A, n = 4–5, panels B,C) by two-way ANOVA with *post hoc* Bonferroni.

throughout the parenchyma in response to systemic LPS challenge. Prior LC lesioning with DSP-4 did not prevent systemic LPS-induced vascular COX-2 expression (Fig. 4D).

3.4. Noradrenaline depletion does not alter microglial cell counts, morphology or functional activity in the hippocampus in response to systemic LPS.

The *Il1b* and *Tnfa* findings in Fig. 4 suggest that microglia were not 'primed' by noradrenergic depletion, thus not producing exaggerated cytokine responses to acute secondary stimulation. To determine whether DSP-4 lesion had any impact on microglial phenotype and/or influenced the impact of systemic inflammation on this cell population we assessed Pu.1$^+$ cell counts (a valid method count microglial nuclei, supplementary figure 2), Iba1$^+$ (to assess microglial morphology) and an array of microglial-associated homeostatic and activation transcripts. Neither DSP-4 nor systemic LPS alone, or in combination had any significant effect on Pu.1$^+$ cell counts nor on measures of microglial morphology as measured using Iba1-positive labelling (Fig. 5 A,B). There were no significant changes in microglial area, circularity or branching, although the latter was quite variable. Labelling with Iba1 and Pu.1 revealed equivalent density of hippocampal microglia using the 2 methods (supplementary data S2). Consistent with our Pu.1 immunohistochemistry data, *Tyrobp* mRNA levels (known to closely correlate with number of microglia) in the hippocampus and frontal cortex were statistically affected by DSP-4 or LPS when administered alone, or in combination (Fig. 5C).

We then assessed multiple transcripts associated with the homeostatic microglial phenotype and with microglial priming and phagocytic activity (Fig. 5D). Full ANOVA analysis for transcription of these genes is shown in Table 1. *Sall1* expression, a microglia-specific transcriptional regulator that defines microglia identity and function by controlling the expression of key homeostatic genes, including *P2ry12* and *Cx3cr1* (Buttgereit et al., 2016), was reduced in the hippocampus in response to systemic LPS but this suppression was equivalent in vehicle and DSP-4-lesioned mice. Two-way ANOVA showed a main effect of LPS on *Sall1* mRNA expression in the hippocampus and Bonferroni *post hoc* analysis revealed that LPS reduced *Sall1* gene expression in both vehicle-injected ($P < 0.05$) and DSP-4 lesioned mice ($P < 0.01$) relative to saline-injected controls. DSP-4 had no significant effect on *Sall1* mRNA expression in the hippocampus and there was no interaction between DSP-4 and LPS. This pattern was repeated for several genes: systemic LPS reduced the expression of *P2ry12*, *Cx3cr1* and Clec7a while increasing *Sra2* in both hippocampus and frontal cortex. LPS had no significant effect on Cd68

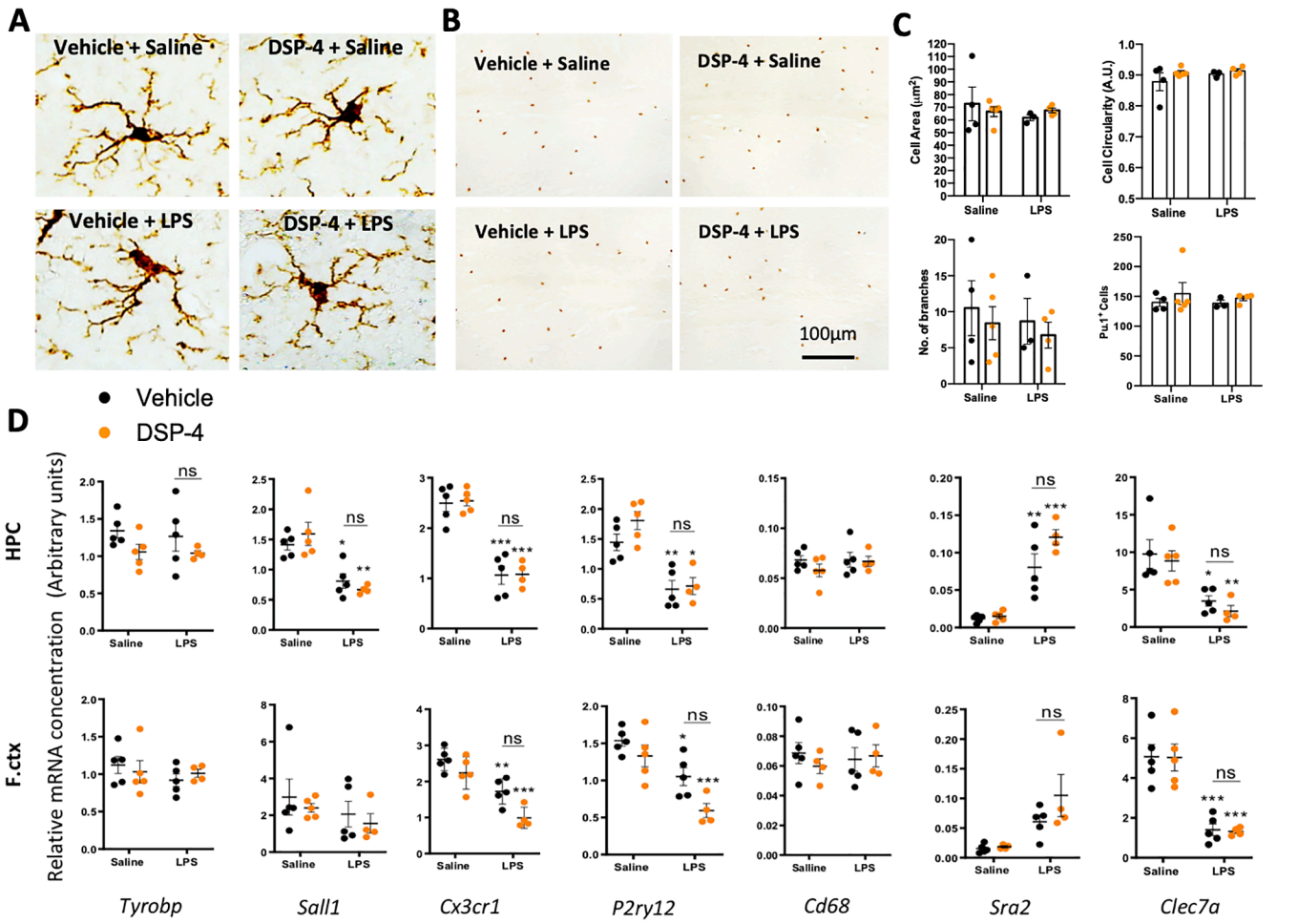

Fig. 5. Prior noradrenergic depletion has limited effects of hippocampal microglial activation and microglial response to systemic LPS challenge. Mice were administered two doses of DSP-4 (50 mg.kg i.p.) or vehicle control set one week apart and subsequently challenged systemically with bacterial LPS (100 µg/kg i.p.) or saline control. Mice were euthanised 3 h later and microglial activation was assessed via immunohistochemistry and quantitative PCR. (A) Iba1^{+} microglia in the hippocampus. (B) Pu.1^{+} microglia in the hippocampus. (C) Morphological analysis of IBA1 + microglia and quantification of Pu.1 + cells. (D) PCR analysis of microglial-associated gene transcripts in the hippocampus and frontal cortex. Values are shown for each animal but mean ± S.E.M is also shown. *P < 0.05, **P < 0.01, ***P < 0.001 vs. saline-injected controls (n = 3–5, panels A,B, n = 4–5, panel C) by two-way ANOVA with Bonferroni *post hoc tests. ns = not significant.*

Table 1
Two-way ANOVA analyses of microglial genes.

Gene	Region	Vehicle / DSP-4		Saline / LPS		Interaction	
		F	p	F	p	F	p
Tyrobp	HPC	0.1293	0.7241	3.876	0.0677	0.04981	0.8264
	F. Ctx	0.000062	0.9938	1.004	0.3322	0.6628	0.4283
Sall1	HPC	0.02	0.8884	34.44	**<0.0001*****	1.508	0.2384
	F. Ctx	0.6273	0.4407	1.658	0.2173	0.0037	0.952
Cx3cr1	HPC	0.04345	0.8377	92.5	**<0.0001*****	0.01072	0.9189
	F. Ctx	11.06	**0.0046****	41.05	**<0.0001*****	1.156	0.2993
P2yr12	HPC	2.02	0.1757	41.71	**<0.0001*****	1.15	0.3005
	F. Ctx	8.209	**0.0118***	27.76	**<0.0001*****	1.166	0.2972
Cd68	HPC	0.9929	0.3348	0.5981	0.4513	0.5136	0.4846
	F. Ctx	0.037	0.8496	0.2034	0.659	0.6013	0.45
Sra2	HPC	4.202	0.0583	67.03	**<0.0001*****	3.039	0.1017
	F. Ctx	1.951	0.1842	15.09	**0.0017****	1.465	0.2462
Clec7a	HPC	0.7157	0.4109	23.3	**0.0002*****	0.02	0.8766
	F. Ctx	0.01538	0.9029	51.59	**<0.0001*****	0.002	0.9627

Abbreviations. HPC: hippocampus; F.Ctx: frontal cortex. Significant main effects DSP-4 or LPS are represented by *, ** or *** for p < 0.05, 0.001, 0.0001 respectively. Statistically significant results are presented in bold text.

nor Tyrobp.

There were limited effects of DSP-4 on these transcripts. The only genes on which DSP-4 exerted significant effects were *P2ry12* and *Cx3cr1,* both of which were somewhat reduced in the frontal cortex of those lesioned with DSP-4, although these effects were not replicated in the hippocampus. When examining the interaction between DSP-4 and LPS (to test the hypothesis that LPS had differential effects contingent on prior lesioning of the noradrenergic system), we found no significant

effects (see Table 1). We did not find any significant interaction for any transcript in either region. There was a trend towards an interaction for *Sra2* in the hippocampus but this failed to reach significance ($P = 0.1017$). Since our hypothesis here was that microglia in DSP-4 animals would show exaggerated responses to LPS, these experiments were statistically powered to detect effect sizes proportionate with our prior experiments in microglial priming (i.e. using predicted effect sizes based on Cunningham et al., 2005; Skelly et al., 2019). Using the sum of squares method to calculate partial eta^2 (0.36) and effect size (0.75), we calculated the sample size required to give 80% power to detect a significant interaction between DSP-4 and LPS (total sample size n = 17). Given that effect sizes of that magnitude were not observed, the experiments may have been underpowered to detect the very modest effects that were observed. Thus we cannot, with confidence, rule out subtle effects, but it is clear that there are not exaggerated responses to LPS in DSP-4 treated animals.

4. Discussion

In the present study, we demonstrate that prior DSP-4 lesion leading to marked noradrenaline depletion has a limited effect on the inflammatory response, in the hippocampus and frontal cortex, to subsequent systemic immune activation. Treatment with DSP-4 dramatically depleted noradrenaline concentrations in key LC projection areas, the hippocampus and frontal cortex, and dramatically reduced catecholaminergic neurites in the hippocampus. However, the DSP-4-induced noradrenergic depletion had limited impact on the immediate neuroinflammatory response to a subsequent acute, 100 μg/kg i.p. systemic LPS challenge and did not increase the vulnerability of these animals to inflammation-induced working memory deficits. These experiments demonstrate that noradrenergic depletion does not make the brain more vulnerable to acute working memory deficits in the hours after this robust systemic inflammatory challenge, which has important implications for inflammation-induced delirium. We do not infer that all cognitive functions are unaffected by lesions of the locus ceruleus and the generalisability of our observations to the anti-inflammatory hypothesis of noradrenergic function more broadly is also limited by the age of the animals, by the LPS dose used and by the short temporal window examined after LPS challenges.

4.1. Noradrenergic depletion and preserved cognitive function (section shortened)

As had previously been reported (Li et al., 2018), DSP-4 depleted noradrenaline concentrations in the hippocampus and frontal cortex by 82% and 60% respectively whilst leaving other monoamine concentrations intact. These data confirm that noradrenergic depletion is robust, and selective for the LC-noradrenergic system. Consistent with previous reports (Bharani et al., 2017; Hassani et al., 2020) DSP-4 diminished the area of TH$^+$ neurites in the hippocampus by 86% and reduced the number of TH$^+$ cell bodies in the middle and late LC by approximately 25%. The remaining LC-derived TH$^+$ terminals/neurites in the CA3 layer of the hippocampus of DSP-4-treated mice were also enlarged and dysmorphic in appearance, a previously reported feature following DSP-4 administration that occurs secondary to the depletion of noradrenaline (Kalinin et al., 2006). Given the magnitude of DSP-4-induced catecholaminergic neurite loss (86%) and noradrenaline depletion (82%) in the hippocampus and the relatively modest reduction of TH$^+$ cells in the LC, it seems likely that DSP-4 induces degeneration of terminals but has less marked impact on the survival of the ascending noradrenergic neurons from which these terminals arise, consistent with the idea that axon terminals are the principal site of action for DSP-4 due to the higher expression of noradrenaline transporters (Fritschy and Grzanna, 1989).

Noradrenaline has a well established role in working memory and LC noradrenergic input to hippocampal CA3 has been shown to facilitate episodic memory formation (Wagatsuma et al., 2018). However, despite the extensive LC-noradrenergic system deficiency and diminished noradrenaline tissue concentrations achieved in the hippocampus and frontal cortex in the current study, DSP-4 did not impair cognitive performance in the T-maze alternation test of working memory. We had no aspiration to interrogate the role of noradrenaline in cognitive function *per se*. Specifically we wished to interrogate vulnerability to acute dysfunction upon subsequent exposure to systemic inflammation. However, LPS did not precipitate working memory impairments in noradrenaline-depleted mice. We have previously shown that p75^{NTR}-saporin lesions of the medial septum, producing hippocampal hypocholinergia, predispose to working memory deficits in response to systemic LPS challenges that were identical to those used here (Field et al., 2012). The latter studies support an existing hypocholinergic hypothesis of delirium and given the important neuromodulatory roles of noradrenaline in promoting arousal and attention, which underpin cognitive function in dynamic tasks requiring sustained attention to trial-specific information (Arnsten, 2009) we had predicted that LPS might produce similar impairments in noradrenaline-depleted mice. Contrary to the findings with hypocholinergia, depletion of noradrenaline concentrations in the hippocampus and frontal cortex did not confer a heightened susceptibility to working memory deficits in response to systemic LPS.

The hippocampus and frontal cortex can begin to become reinnervated by 4 weeks post DSP-4 treatment (Fritschy & Grzanna, 1992). Moreover an increased rate of NA turnover, mediated by presynaptic α2-adrenoceptors, has been reported in noradrenergic neurons that have survived the lesion (Logue et al., 1985), perhaps partially preserving noradrenergic transmission. The observation of enlarged/swollen residual TH$^+$ neurites in the hippocampus of DSP-4 lesioned mice may be consistent with a compensatory adaptation to LC-noradrenergic system degeneration. However tissue levels of NA remained very much decreased at 4 weeks in the current study and given the low level of tissue noradrenaline, functional reinnervation had clearly not occurred within the 4 weeks of this study. The failure of this lesion to increase vulnerability to acute cognitive dysfunction indicates that noradrenergic loss does not increase predisposition to acute cognitive dysfunction and may suggest that loss of noradrenergic tone is not a major contributor to delirium risk in older patients or those with neurodegenerative disease. This somewhat surprising result may suggest that functionality to which noradrenaline normally contributes may be compensated for by other neurotransmitters under the circumstances of noradrenergic denervation, at least in the current model system. However, these interpretations come with the caveat that our experiments have been performed in a reductionist paradigm inducing noradrenergic lesions in young healthy animals.

4.2. Microglia were not primed by loss of noradrenergic tone

Selectively depleting noradrenaline concentrations in the hippocampus and frontal cortex with DSP-4 did not amplify the magnitude of acute neuroinflammation in response to the subsequent acute LPS challenge. We previously demonstrated that microglia in the degenerating brain are primed to produce exaggerated IL-1β responses to subsequent systemic inflammation (Cunningham et al., 2005) and this has been replicated in multiple degenerative states (Holtman et al., 2015). In the current study, selectively depleting noradrenaline concentrations with DSP-4 did not prime hippocampal or cortical microglia to produce exaggerated *Il1b* and *Tnfa* responses to acute, systemic inflammation.

That these microglia are not significantly 'primed' is emphasised by our analysis of microglial cell counts, phenotype, and activation state. *Tyrobp* codes for DAP12, an adaptor proteins for TREM2, and is a target gene for the microglial-specific transcription factor Pu.1, a master regulator of myeloid cell development and microglial gene expression (Rustenhoven et al., 2018). DSP-4 did not affect Pu.1$^+$ cell counts, *Tyrobp* gene expression or Iba1$^+$ morphology, nor did it affect changes in these markers in response to systemic LPS. Therefore, at least with

respect to the current DSP-4 treatment, the 100 μg/kg LPS dose and the short temporal window of the current experiments, noradrenaline depletion does not alter the acute microglial response to acute, systemic LPS challenge.

It has recently been shown that network activity-associated increases in noradrenaline significantly reduce microglial process motility (Liu et al., 2019). Anaesthesia and the noradrenaline α_1 agonist, dexmedetomidine, which suppresses LC noradrenaline release, both increase microglia surveillance and response to injury (Stowell et al., 2019) while other studies show that bath application of noradrenaline to microglia *ex vivo* triggers process retraction (Gyoneva & Traynelis, 2013). While the concordance of those findings is unclear, they are all descriptive of dynamic effects of noradrenaline on microglial activities. There were limited indications that noradrenergic depletion affected microglial phenotype in the current study. The homeostatic microglial transcript *Sall1* (Buttgereit et al., 2016) was not significantly affected by > 80% noradrenergic depletion. With respect to the homeostatic genes *Cx3cr1* and *P2yr12*, there were lesion-associated decreases in their expression in the frontal cortex, but not in the hippocampus, which might indicate some deviation from a basal homeostatic state selectively in the cortex. It is worth recalling that the noradrenaline depletion was less marked in the frontal cortex than in the hippocampus, but also that basal noradrenaline concentrations were initially higher in the frontal cortex. One obvious difference between the current study and those examining dynamic effects of noradrenaline levels on microglial activity is that in the current study noradrenergic changes are severe but relatively stable rather than in continuous dynamic flux.

Consistent with the interpretation that microglial activation status is minimally altered by noradrenegic depletion, there were limited DSP-4-induced increases in scavenger receptor class A, type II (*Sra2)*, macrosialin (*Cd68*) or *Clec7a*, which are descriptive of endocytic/phagocytic activation and microglial priming or hyper-reactivity (Holtman et al., 2015; Krasemann et al., 2017; Li et al., 2019) and are strongly upregulated in microglia proportionate to the extent of immune activation (Hendrickx et al., 2017). There was a modest but statistically significant increase in LPS-induced *Sra2* expression in the hippocampus of DSP-4-lesioned animals when compared to naive mice treated with LPS.

Broadly speaking, responses to LPS were normal: homeostatic transcripts were significantly reduced by LPS, but roughly equally in DSP-4 treated and in naive animals and the LPS-induced increases in *Il1b* and *Tnfa* transcription were equivalent in normal and DSP-4 treated animals, confirming that DSP-4 and subsequent noradrenaline depletion does not prime microglia to produce exaggerated responses to acute, systemic immune activation. Previous studies suggest that synaptic loss, rather than overt neuronal death, is sufficient to prime microglia (Cunningham et al., 2005) but it is not clear whether the withdrawal of noradrenergic terminals here constitutes remodelling of TH + synaptic terminals or synaptic degeneration *per se*. Therefore hippocampal and cortical microglia may not have been exposed to key ''priming'' signals. However a lack of significant synaptic *loss* in the projection areas would only simplify the interpretation of events in those projection areas: it is difficult to escape the conclusion that depletion of local synaptic noradrenaline simply does not prime microglia, at least in young healthy animals.

4.3. Temporal and spatial determinants of microglial consequences of NE depletion

It was not an aim of this study, and the experimental design also would not permit us, to fully interrogate the widely accepted hypothesis that noradrenaline has anti-inflammatory effects. Timing, LPS dose and presence or absence of other pathological features (such as those that would have been induced by aging) may be important factors in determining the impact of diminishing noradrenergic tone on neuroinflammation and cognitive function. Prior studies (Song et al., 2019a) demonstrated that a single dose of DSP-4 (50 mg/kg i.p.) depletes noradrenaline concentrations in the hippocampus by 78% in as little as 24 h, and increases $CD11b^+$ microgliosis in the hippocampus by 14 days. This indicates that DSP-4 depletes intra-neuronal noradrenaline concentrations rapidly but apparently activates microglia in the LC-projection areas much more slowly. It is possible therefore that slow development of a noradrenaline depletion-mediated microgliosis could facilitate exaggerated responses to secondary inflammation. Nonetheless, given that we examined microglial activation and cytokine responses to secondary challenge with LPS at a time when noradrenaline levels were demonstrably reduced by 80%, our data nonetheless demonstrate that this depletion of noradrenaline does not, of itself, lead to an increased reactivity of microglia that is manifest by 3 h post-LPS. This choice of temporal window was dictated by our prior descriptions of peak hippocampal cytokine induction at 3–4 h post-LPS (at 100 μg/kg) and, most importantly, by our demonstration that neurodegenerative features leave animals vulnerable to acute working memory dysfunction starting as early as 3 h post-LPS (Murray et al., 2012, Field et al., 2012).

Our findings in this short temporal window should not detract from findings that lesioning of the LC noradrenergic neurons in models of chronic neurodegeneration does exacerbate neuroinflammation and worsen features of neurodegeneration in other experimental designs. Monthly injections of DSP-4 significantly exacerbate neuroinflammation and neurodegeneration in the P301S model of Tauopathy (Chalermpalanupap et al., 2018). A similar regime of DSP-4 treatment promotes β-amyloidosis and increases the expression of neuroinflammatory mediators in the APP/PS1 double transgenic model of AD (Jardanhazi--Kurutz et al., 2010). Administration of a single dose of DSP-4 (50 mg/kg i.p.) also potentiates microglial activation in the hippocampus arising from systemic LPS, given 1 week later, at neurotoxic doses ($1.5x10^7$ EU/Kg or approx 7.5 mg/Kg i.p.; 75-fold higher than those used in the current study). This potentiation of microglia is most obvious at 3 and 6 months. However, DSP-4 has no additive effect with respect to cognitive impairment: hippocampal-dependent spatial memory deficits in the Morris water maze probe trial were caused by this severe dose of LPS but this was not exacerbated by the DSP-4 lesion (Song et al., 2019b). In each of these studies, noradrenaline depletion is being executed simultaneously with additional neurodegenerative pathologies, unlike the moderate, non-neurotoxic LPS dose used in the current study. Therefore, one might speculate that danger-associated molecular patterns (DAMPs) released from damaged LC-noradrenergic terminals coincident with loss of noradrenaline (rather than either alone), are influencing microglial activation in the hippocampus. In the studies of Song et al., DSP-4-induced hippocampal microglial activation persists at 10 months post-DSP-4 treatment, even though the hippocampus is once again replete again with NA by that time (Song et al., 2019a). Therefore while it is possible that there is some reinnervation of the hippocampus due to axonal sprouting of surviving noradrenergic neurons, which may supply extra-synaptic noradrenaline in the hippocampus (Fritschy & Grzanna, 1992; Song et al., 2019a) and bind microglial adrenergic receptors (Jiang et al., 2015), this does not appear to be sufficient to restore microglia to a homeostatic state.

Reducing noradrenergic tone with DSP-4 deprives the hippocampus of an innate immunomodulator that might help to maintain microglial homeostasis in the face of evolving neurodegenerative pathology. It's loss may eventually contribute to chronic, deleterious levels of inflammation and progressive neuronal loss over an extended time period, but in the current study, without an additional neurodegenerative pathological process, the depletion of noradrenaline does not, of itself, lead to significant microglial activation or hyper-responsiveness. It would be of significant interest to repeat these experiments in aged animals since aging would lead to an altered baseline pathological and neuroinflammatory state, at which point depletion of noradrenaline may then have more marked effects on microglial activation. Nonetheless, the current experimental design allowed us to address a more reductionist hypothesis, not confounded by additional pathological stimuli: does the

loss of noradrenaline, *per se*, increase risk for inflammation-induced working memory dysfunction? The data indicate that it does not.

4.4. Impact of noradrenergic depletion on circulating cytokine response to LPS

The acute LPS challenges made in this study were intraperitoneal and systemic cytokine levels are the standard metric of the severity of the response to this pro-inflammatory challenge. Systemic LPS increased IL-1β, TNF-α and IL-6 cytokine secretion in the blood and DSP-4 did not influence LPS-induced IL-1β or IL-6 expression. Interestingly, DSP-4 did significantly reduce the magnitude of LPS-induced TNF-α levels, indicating that noradrenaline-depletion suppresses the peripheral TNF-α response to systemic LPS. This finding was surprising considering that endogenous noradrenaline is thought to exert regulatory control over TNF-α secretion by elevating intracellular cAMP via β-adrenergic receptor signalling in macrophages (Magistretti and Schorderet, 1984; Spengler et al., 1994). One possible explanation, however, is that DSP-4 is reducing sympathetic nervous system-derived noradrenaline from acting on α_2-adrenoceptors, which could decrease intracellular cAMP (Umemura et al., 1986), leading to suppressed TNF-α secretion from peripheral blood macrophages (Straub et al., 2000). The LC influences NA levels in the periphery by acting on sympathetic dorsal horn spinal nerves and can by acting on circulating macrophages and on the spleen to enhance peripheral inflammatory tone in response to stress. Recent studies have demonstrated that prior treatment with DSP-4 (400 μg/rat i.c.v) reduces plasma noradrenaline concentrations and inhibits social defeat stress-induced enhancement of TNF-α expression (Finnell et al., 2019). The spleen, which is rich in macrophages and T-cells, receives strong sympathetic noradrenergic innervation from cell groups A5 and A6 (Felten et al., 1987; Cano et al., 2001). DSP-4 (50 mg/kg i.p.) induces the loss of LC and brainstem (A5) noradrenergic neurons, which reduces splenic noradrenaline concentrations, by 20–40%, 8 weeks later and diminishes the production of IL-1β, TNF-α and IL-6 by LPS-stimulated splenocytes *ex vivo* (Engler et al., 2010). Therefore, DSP-4 suppression of LPS-induced increases in circulating TNF-α may be explained by diminished regulatory effects of noradrenaline on splenocytes, arising from the loss of ennervation that is normally supplied by descending A5 and LC-noradrenergic projections.

5. Conclusion

Our data demonstrate that significant noradrenergic depletion in young animals does not leave the brain more vulnerable to acute working memory deficits or exaggerated neuroinflammatory changes in the hours after secondary challenge with bacterial LPS. That is, LPS challenge and acute, transient systemic inflammation did not produce acute deficits in working memory, even in noradrenaline-depleted animals. The findings suggest significant resilience of hippocampal and frontal cortical regions to maintain function under acute inflammatory stressors despite depletion of noradrenaline. Noradrenaline is likely to regulate brain inflammation and cognitive performance differentially dependent on timing, nature, severity and locality of CNS pathology. Further studies are clearly necessary to understand the interplay between neurodegenerative changes and loss of noradrenergic tone per se and the vulnerability of aging and degenerating brain structures to secondary inflammation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.12.002.

References

Arnsten, A.F., 2009. Stress signalling pathways that impair prefrontal cortex structure and function. Nat. Rev. Neurosci. 10 (6), 410–422.
Aston-Jones, G., Rajkowski, J., Cohen, J., 2000. Locus coeruleus and regulation of behavioral flexibility and attention. Progress in Brain Research. Elsevier 165–182.
Barrientos, R.M., Hein, A.M., Frank, M.G., Watkins, L.R., Maier, S.F., 2012. Intracisternal interleukin-1 receptor antagonist prevents postoperative cognitive decline and neuroinflammatory response in aged rats. J. Neurosci. 32 (42), 14641–14648.
Bharani, K.L., Derex, R., Granholm, A.-C., Ledreux, A., 2017. A noradrenergic lesion aggravates the effects of systemic inflammation on the hippocampus of aged rats. PLoS ONE 12, e0189821.
Buttgereit, A., Lelios, I., Yu, X., Vrohlings, M., Krakoski, N.R., Gautier, E.L., Nishinakamura, R., Becher, B., Greter, M., 2016. Sall1 is a transcriptional regulator defining microglia identity and function. Nat. Immunol. 17, 1397–1406.
Cano, G., Sved, A.F., Rinaman, L., Rabin, B.S., Card, J.P., 2001. Characterization of the central nervous system innervation of the rat spleen using viral transneuronal tracing. Journal of Comparative Neurology 439 (1), 1–18.
Chalermpalanupap, T., Schroeder, J.P., Rorabaugh, J.M., Liles, L.C., Lah, J.J., Levey, A.I., Weinshenker, D., 2018. Locus coeruleus ablation exacerbates cognitive deficits, neuropathology, and lethality in P301S tau transgenic mice. J. Neurosci. 38 (1), 74–92.
Cunningham, C., Hennessy, E., 2015. Co-morbidity and systemic inflammation as drivers of cognitive decline: new experimental models adopting a broader paradigm in dementia research. Alzheimer's Research & Therapy 7, 33.
Cunningham, C., Wilcockson, D.C., Campion, S., Lunnon, K., Perry, V.H., 2005. Central and Systemic Endotoxin Challenges Exacerbate the Local Inflammatory Response and Increase Neuronal Death during Chronic Neurodegeneration. The Journal of Neuroscience 25, 9275–9284.
Davis, D.H.J., Skelly, D.T., Murray, C., Hennessy, E., Bowen, J., Norton, S., Brayne, C., Rahkonen, T., Sulkava, R., Sanderson, D.J., Rawlins, J.N., Bannerman, D.M., MacLullich, A.M.J., Cunningham, C., 2015. Worsening cognitive impairment and neurodegenerative pathology progressively increase risk for delirium. American Journal of Geriatric Psychiatry 23 (4), 403–415. https://doi.org/10.1016/j.jagp.2014.08.005.
Doucette, W., Milder, J., Restrepo, D., 2007. Adrenergic modulation of olfactory bulb circuitry affects odor discrimination. Learning & memory 14 (8), 539–547.
Engler, H., Doenlen, R., Riether, C., Engler, A., Besedovsky, H.O., Del Rey, A., Pacheco-López, G., Schedlowski, M., 2010. Chemical destruction of brain noradrenergic neurons affects splenic cytokine production. J. Neuroimmunol. 219 (1–2), 75–80.
Felten, D.L., Felten, S.Y., Bellinger, D.L., Carlson, S.L., Ackerman, K.D., Madden, K.S., Olschowki, J.A., Livnat, S., 1987. Noradrenergic sympathetic neural interactions with the immune system: structure and function. Immunol. Rev. 100 (1), 225–260.
Field, R.H., Gossen, A., Cunningham, C., 2012. Prior Pathology in the Basal Forebrain Cholinergic System Predisposes to Inflammation-Induced Working Memory Deficits: Reconciling Inflammatory and Cholinergic Hypotheses of Delirium. The Journal of Neuroscience 32, 6288–6294.
Finnell, J.E., Moffitt, C.M., Hesser, L.A., Harrington, E., Melson, M.N., Wood, C.S., Wood, S.K., 2019. The contribution of the locus coeruleus-norepinephrine system in the emergence of defeat-induced inflammatory priming. Brain Behav. Immun. 79, 102–113.
Fritschy, J.M., Grzanna, R., 1989. Immunohistochemical analysis of the neurotoxic effects of DSP-4 identifies two populations of noradrenergic axon terminals. Neuroscience 30 (1), 181–197.
Fritschy, J.M., Grzanna, R., 1992. Restoration of ascending noradrenergic projections by residual locus coeruleus neurons: Compensatory response to neurotoxin-induced cell death in the adult rat brain. Journal of Comparative Neurology 321 (3), 421–441.
German, D.C., Manaye, K.F., White Iii, C.L., Woodward, D.J., McIntire, D.D., Smith, W.K., Kalaria, R.N., Mann, D.M.A., 1992. Disease-specific patterns of locus coeruleus cell loss. Ann. Neurol. 32, 667–676.
Gyoneva, S., Traynelis, S.F., 2013. Norepinephrine modulates the motility of resting and activated microglia via different adrenergic receptors. J. Biol. Chem. 288 (21), 15291–15302.
Hassani, O.K., Rymar, V.V., Nguyen, K.Q., Huo, L., Cloutier, J.-F., Miller, F.D., Sadikot, A. F., 2020. The noradrenergic system is necessary for survival of vulnerable midbrain dopaminergic neurons: implications for development and Parkinson's disease. Neurobiol. Aging 85, 22–37.
Hendrickx, D.A.E., van Eden, C.G., Schuurman, K.G., Hamann, J., Huitinga, I., 2017. Staining of HLA-DR, Iba1 and CD68 in human microglia reveals partially overlapping expression depending on cellular morphology and pathology. J. Neuroimmunol. 309, 12–22.

Heneka, M.T., Galea, E., Gavriluyk, V., Dumitrescu-Ozimek, L., Daeschner, J., O'Banion, M.K., Weinberg, G., Klockgether, T., Feinstein, D.L., 2002. Noradrenergic Depletion Potentiates β-Amyloid-Induced Cortical Inflammation: Implications for Alzheimer's Disease 22, 2434–2442.

Heneka, M.T., Nadrigny, F., Regen, T., Martinez-Hernandez, A., Dumitrescu-Ozimek, L., Terwel, D., Jardanhazi-Kurutz, D., Walter, J., Kirchhoff, F., Hanisch, U.-K., Kummer, M.P., 2010. Locus ceruleus controls Alzheimer's disease pathology by modulating microglial functions through norepinephrine. Proc. Natl. Acad. Sci. 107, 6058–6063.

Holtman, I.R., Raj, D.D., Miller, J.A., Schaafsma, W., Yin, Z., Brouwer, N., Wes, P.D., Möller, T., Orre, M., Kamphuis, W., Hol, E.M., 2015. Induction of a common microglia gene expression signature by aging and neurodegenerative conditions: a co-expression meta-analysis. Acta neuropathologica communications 3 (1), 31.

Jardanhazi-Kurutz, D., Kummer, M.P., Terwel, D., Vogel, K., Dyrks, T., Thiele, A., Heneka, M.T., 2010. Induced LC degeneration in APP/PS1 transgenic mice accelerates early cerebral amyloidosis and cognitive deficits. Neurochem Int. 57 (4), 375–382. https://doi.org/10.1016/j.neuint.2010.02.001.

Jiang, L., Chen, S.H., Chu, C.H., Wang, S.J., Oyarzabal, E., Wilson, B., Sanders, V., Xie, K., Wang, Q., Hong, J.S., 2015. A novel role of microglial NADPH oxidase in mediating extra-synaptic function of norepinephrine in regulating brain immune homeostasis. Glia 63 (6), 1057–1072.

Jonsson, G., Hallman, H., Ponzio, F., Ross, S., 1981. DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine)—a useful denervation tool for central and peripheral noradrenaline neurons. Eur. J. Pharmacol. 72 (2–3), 173–188.

Kalinin, S., Feinstein, D.L., Xu, H.-L., Huesa, G., Pelligrino, D.A., Galea, E., 2006. Degeneration of noradrenergic fibres from the locus coeruleus causes tight-junction disorganisation in the rat brain. Eur. J. Neurosci. 24, 3393–3400.

Krasemann, S., Madore, C., Cialic, R., Baufeld, C., Calcagno, N., El Fatimy, R., Beckers, L., O'Loughlin, E., Xu, Y., Fanek, Z., Greco, D.J., Smith, S.T., Tweet, G., Humulock, Z., Zrzavy, T., Conde-Sanroman, P., Gacias, M., Weng, Z., Chen, H., Tjon, E., Mazaheri, F., Hartmann, K., Madi, A., Ulrich, J.D., Glatzel, M., Worthmann, A., Heeren, J., Budnik, B., Lemere, C., Ikezu, T., Heppner, F.L., Litvak, V., Holtzman, D. M., Lassmann, H., Weiner, H.L., Ochando, J., Haass, C., Butovsky, O., 2017. The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. Immunity 47, 566–581.e569.

Li, Q., Cheng, Z., Zhou, L., Darmanis, S., Neff, N.F., Okamoto, J., Gulati, G., Bennett, M. L., Sun, L.O., Clarke, L.E., Marschallinger, J., Yu, G., Quake, S.R., Wyss-Coray, T., Barres, B.A., 2019. Developmental Heterogeneity of Microglia and Brain Myeloid Cells Revealed by Deep Single-Cell RNA Sequencing. Neuron 101, 207–223.e210.

Li, Y., Jiao, Q., Du, X., Bi, M., Han, S., Jiao, L., Jiang, H., 2018. Investigation of behavioral dysfunctions induced by monoamine depletions in a mouse model of Parkinson's disease. Front. Cell. Neurosci. 12, 241.

Liu, Y.U., Ying, Y., Li, Y., Eyo, U.B., Chen, T., Zheng, J., Umpierre, A.D., Zhu, J., Bosco, D. B., Dong, H., Wu, L.J., 2019. Neuronal network activity controls microglial process surveillance in awake mice via norepinephrine signaling. Nat. Neurosci. 1–11.

Logue, M.P., Growdon, J.H., Coviella, I.L.G., Wurtman, R.J., 1985. Differential effects of DSP-4 administration on regional brain norepinephrine turnover in rats. Life Sci. 37 (5), 403–409.

Lyness, S.A., Zarow, C., Chui, H.C., 2003. Neuron loss in key cholinergic and aminergic nuclei in Alzheimer disease: a meta-analysis. Neurobiol. Aging 24, 1–23.

Magistretti, P.J., Schorderet, M., 1984. VIP and noradrenaline act synergistically to increase cyclic AMP in cerebral cortex. Nature 308, 280–282.

Murchison, C.F., Zhang, X.-Y., Zhang, W.-P., Ouyang, M., Lee, A., Thomas, S.A., 2004. A Distinct Role for Norepinephrine in Memory Retrieval. Cell 117, 131–143.

Murray, C., Sanderson, D.J., Barkus, C., Deacon, R.M.J., Rawlins, J.N.P., Bannerman, D. M., Cunningham, C., 2012. Systemic inflammation induces acute working memory deficits in the primed brain: relevance for delirium. Neurobiol. Aging 33, 603–616. e603.

O'Neill, E., Yssel, J.D., McNamara, C., Harkin, A., 2020. Pharmacological targeting of β2-adrenoceptors is neuroprotective in the LPS inflammatory rat model of Parkinson's disease. Br. J. Pharmacol. 177 (2), 282–297.

O'Sullivan, J.B., Ryan, K.M., Curtin, N.M., Harkin, A., Connor, T.J., 2009. Noradrenaline reuptake inhibitors limit neuroinflammation in rat cortex following a systemic inflammatory challenge: implications for depression and neurodegeneration. Int. J. Neuropsychopharmacol. 12, 687–699.

O'Sullivan, J.B., Ryan, K.M., Harkin, A., Connor, T.J., 2010. Noradrenaline reuptake inhibitors inhibit expression of chemokines IP-10 and RANTES and cell adhesion molecules VCAM-1 and ICAM-1 in the CNS following a systemic inflammatory challenge. J. Neuroimmunol. 220, 34–42.

Palkovits, M., Brownstein, M.J., 1988. Maps and guide to microdissection of the rat brain. Elsevier.

Peters van Ton, P.A.M., Verbeek, M.M., Alkema, W., Pickkers, P., Abdo, W.F., 2020. Downregulation of synapse-associated protein expression and loss of homeostatic microglial control in cerebrospinal fluid of infectious patients with delirium and patients with Alzheimer's disease. Brain Behav. Immun. 89, 656–667. https://doi.org/10.1016/j.bbi.2020.06.027.

Ramos, B.P., Colgan, L.A., Nou, E., Arnsten, A.F.T., 2008. β2 adrenergic agonist, clenbuterol, enhances working memory performance in aging animals. Neurobiol. Aging 29, 1060–1069.

Robbins, T.W.J.B.p., 1997. Arousal systems and attentional processes 45, 57-71.

Ross, S.B., Stenfors, C., 2015. DSP4, a Selective Neurotoxin for the Locus Coeruleus Noradrenergic System. A Review of Its Mode of Action. Neurotox. Res. 27, 15–30.

Rustenhoven, J., Smith, A.M., Smyth, L.C., Jansson, D., Scotter, E.L., Swanson, M.E.V., Aalderink, M., Coppieters, N., Narayan, P., Handley, R., Overall, C., Park, T.I.H., Schweder, P., Heppner, P., Curtis, M.A., Faull, R.L.M., Dragunow, M., 2018. PU.1 regulates Alzheimer's disease-associated genes in primary human microglia. Mol. Neurodegener. 13, 44.

Skelly, D.T., Griffin, É.W., Murray, C.L., Harney, S., O'Boyle, C., Hennessy, E., Dansereau, M.-A., Nazmi, A., Tortorelli, L., Rawlins, J.N., Bannerman, D.M., Cunningham, C., 2019. Acute transient cognitive dysfunction and acute brain injury induced by systemic inflammation occur by dissociable IL-1-dependent mechanisms. Mol. Psychiatry 24, 1533–1548.

Skelly, D.T., Hennessy, E., Dansereau, M.-A., Cunningham, C., 2013. A systematic analysis of the peripheral and CNS effects of systemic LPS, IL-1β, [corrected] TNF-α and IL-6 challenges in C57BL/6 mice. PloS one 8, e69123-e69123.

Song, S., Jiang, L., Oyarzabal, E.A., Wilson, B., Li, Z., Shih, Y.-Y.I., Wang, Q., Hong, J.-S., 2019a. Loss of Brain Norepinephrine Elicits Neuroinflammation-Mediated Oxidative Injury and Selective Caudo-Rostral Neurodegeneration. Mol. Neurobiol. 56, 2653–2669.

Song, S., Wang, Q., Jiang, L., Oyarzabal, E., Riddick, N.V., Wilson, B., Moy, S.S., Shih, Y.-Y.I., Hong, J.-S., 2019b. Noradrenergic dysfunction accelerates LPS-elicited inflammation-related ascending sequential neurodegeneration and deficits in non-motor/motor functions. Brain Behav. Immun. 81, 374–387.

Spengler, R.N., Chensue, S.W., Giacherio, D.A., Blenk, N., Kunkel, S.L., 1994. Endogenous norepinephrine regulates tumor necrosis factor-alpha production from macrophages in vitro. J. Immunol. 152, 3024–3031.

Sterpenich, V., D'Argembeau, A., Desseilles, M., Balteau, E., Albouy, G., Vandewalle, G., Degueldre, C., Luxen, A., Collette, F., Maquet, P., 2006. The Locus Ceruleus Is Involved in the Successful Retrieval of Emotional Memories in Humans 26, 7416–7423.

Stowell, R.D., Sipe, G.O., Dawes, R.P., Batchelor, H.N., Lordy, K.A., Whitelaw, B.S., Stoessel, M.B., Bidlack, J.M., Brown, E., Sur, M., Majewska, A.K., 2019. Noradrenergic signaling in the wakeful state inhibits microglial surveillance and synaptic plasticity in the mouse visual cortex. Nat. Neurosci. 22, 1782–1792.

Straub, R.H., Linde, H.-J., Männel, D.N., Schölmerich, J., Falk, W., 2000. A bacteria-induced switch of sympathetic effector mechanisms augments local inhibition of TNF-α and IL-6 secretion in the spleen. FASEB J. 14, 1380–1388.

Theofilas, P., Ehrenberg, A.J., Dunlop, S., Di Lorenzo Alho, A.T., Nguy, A., Leite, R.E.P., Rodriguez, R.D., Mejia, M.B., Suemoto, C.K., Ferretti-Rebustini, R.E.D.L., Polichiso, L., Nascimento, C.F., Seeley, W.W., Nitrini, R., Pasqualucci, C.A., Jacob Filho, W., Rueb, U., Neuhaus, J., Heinsen, H., Grinberg, L.T., 2017. Locus coeruleus volume and cell population changes during Alzheimer's disease progression: A stereological study in human postmortem brains with potential implication for early-stage biomarker discovery. Alzheimer's & Dementia 13, 236–246.

Tronel, S., Feenstra, M.G., Sara, S.J., 2004. Noradrenergic action in prefrontal cortex in the late stage of memory consolidation. Learning & memory 11 (4), 453–458.

Umemura, S., Smyth, D.D., Pettinger, W.A., 1986. Alpha 2-adrenoceptor stimulation and cellular cAMP levels in microdissected rat glomeruli. American Journal of Physiology-Renal Physiology 250, F103–F108.

Wagatsuma, A., Okuyama, T., Sun, C., Smith, L.M., Abe, K., Tonegawa, S., 2018. Locus coeruleus input to hippocampal CA3 drives single-trial learning of a novel context 115, E310–E316.

Walf, A.A., Frye, C.A., 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protoc. 2, 322.

Yssel, J.D., O'Neill, E., Nolan, Y.M., Connor, T.J., Harkin, A., 2018. Treatment with the noradrenaline re-uptake inhibitor atomoxetine alone and in combination with the α2-adrenoceptor antagonist idazoxan attenuates loss of dopamine and associated motor deficits in the LPS inflammatory rat model of Parkinson's disease. Brain Behav. Immun. 69, 456–469.