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Neonatal overfeeding by small-litter rearing sensitizes hippocampal microglial responses to immune challenge: reversal with neonatal repeated injections of saline as well as minocycline.

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Abstract

The early life period is extremely vulnerable to programming effects from the environment, many of which persist into adulthood. We have previously demonstrated that adult rats that were overfed as neonates have hypothalamic microglia that are hyper-responsive to an immune challenge and hippocampal microglia that respond less efficiently to learning. We therefore hypothesized that neonatal overfeeding would alter the ability of hippocampal microglia to respond to an immune challenge with lipopolysaccharide (LPS) and that concomitant minocycline, a tetracycline antibiotic that suppresses microglial activity, could restore these responses. We induced neonatal overfeeding by manipulating the litter sizes in which Wistar rat pups were raised, so the pups were suckled in litters of 4 (neonatally overfed) or 12 (control-fed). We then examined the hippocampal microglial profiles 24 hr after an immune challenge with LPS, and found that the neonatally overfed rats had dramatically increased microglial numbers in the hippocampus after immune challenge compared with control-fed. Attempts to reverse these effects with minocycline revealed repeated neonatal injections, whether with minocycline or with saline, markedly suppressed microglial number and density throughout the hippocampus and abolished the difference between the groups in their responses to LPS. These data suggest neonatal overfeeding can have lasting effects on hippocampal immune responses, but also that neonatal exposure to a protocol of repeated injections, irrespective of treatment, has pronounced long-term impact, highlighting the importance of considering these effects when interpreting experimental data.

Introduction

Early life diet in children and animals can strongly influence body weight long-term¹⁻⁵. As such, children and rodents that overeat or have poor diet during vulnerable windows of development are at significant risk of long-term obesity and the myriad associated comorbidities that ensue⁶. For instance, rodents suckled in small litters, where they have greater access to their mother's milk compared with controls, have accelerated growth and weight gain early on; this excess weight and

body fat persists at least to young adulthood^{5,7-9}. In addition to metabolic dysfunction^{7,10,11}, hypothalamic-pituitary-adrenal (HPA) axis dysfunction^{12,13}, reproductive problems¹⁴, and other complications, neonatal overfeeding leads to a potentiated febrile response¹⁵ as well as hypersensitivity or “priming” of hypothalamic microglia, one of the major immune cell populations in the brain⁵. Hypothalamic microglia in the neonatally overfed are increased in number and density under basal conditions, reflective of a pro-inflammatory, hyper-activated profile. They also hyper-respond to stimulation with lipopolysaccharide (LPS) and this is associated with elevated circulating cytokines⁵, suggesting neonatal overfeeding leads to an increased response to immune challenge.

This programming effect of early life diet on microglia extends beyond the hypothalamus; microglia are also increased in density in the hippocampus in adults that have been overfed as neonates¹⁶. Similarly, recent studies have shown a primed hippocampal microglial profile in adult offspring from high-saturated-fat diet-fed dams^{4,17}. Performance in hippocampally-mediated learning tasks is less efficient in these rats^{4,17} and it appears microglia are less responsive to a learning task in neonatally overfed rats than in control-fed¹⁶. Microglia have region-specific heterogeneity with different responses to stimuli in different brain regions¹⁸⁻²¹. However, given that neonatally overfed rats have a primed microglial profile under basal conditions in both the hypothalamus and hippocampus, and noting the integral role of the hippocampus in responding to immune challenge^{22,23}, we hypothesized that hippocampal microglia from neonatally overfed rats would be hyper-activated in response to LPS, leading to an altered ability to respond to the immune challenge.

We therefore tested hippocampal microglial responses to LPS *in vitro* and *in vivo* in rats that had been suckled in small litters (neonatally overfed; SL) compared to those that had been suckled in normalized litters (control-fed; CL). To restore normal hippocampal microglial responses, we then attempted to suppress microglial activation during the neonatal period with the second generation antibiotic, minocycline²⁴. Minocycline easily crosses the blood-brain after systemic administration

^{25,26} and is a known inhibitor of microglial activation ^{27,28}. Minocycline has been shown to successfully ameliorate neuroinflammation at the site of hypoxic-ischemic insult in neonatal rodent studies ²⁸⁻³². Unexpectedly, we found that the injection protocol, even in the saline-treated rats, markedly suppressed microglial number and density throughout the hippocampus. It also reversed the effects of neonatal overfeeding on microglia. There was no additional effect of minocycline. Our outcomes have significant implications for future study design and also suggest that the negative effects of neonatal overfeeding, on at least microglial responsiveness, may be mitigated by neonatal experience.

Materials and Methods

Animals

We obtained timed pregnant Wistar rats from the Animal Resources Centre, Murdoch, WA Australia. On arrival at the RMIT University Animal Facility at gestational day 16, we singly-housed the dams at 22 °C on a 12 hr light/dark cycle (light: 0700 – 1900 hr) and provided them with *ad libitum* pelleted rat chow and water. All procedures were conducted in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals, and experiments were approved by the RMIT University Animal Ethics Committee.

Litter manipulation and minocycline treatment

On the day of birth (postnatal day 0; P0) we removed all pups from their dams and randomly reallocated them to new dams in litters of 12 (CL) or 4 (SL) as we have previously described ^{2,5,11,15}. Dams did not receive any of their own pups and each new litter was made up of 50% males and 50% females. Excess pups were culled by decapitation. We have previously shown that this manipulation results in the neonatally overfed pups being significantly heavier by P7 and throughout life ^{2,5,11,15}. We

have previously reported weight data from this cohort of rats (those shown here in Fig. 2 and Fig. 3) and have analysed their HPA axis responses to an immune challenge³³.

At P0, control-fed and neonatally overfed pups were allocated to either a three-week treatment regimen or were untreated, except for the litter size manipulation, until experimentation in adulthood; approximately P70 (Fig. 1). For the neonatal treatment, we administered either minocycline (22.5 mg/kg i.p. in saline, 100 µL) or vehicle (saline, 100 µL) once every second day during the three-week suckling period starting on P1 to control-fed and neonatally overfed pups²⁴ (Fig. 1B). Each litter had equal numbers of minocycline and saline-treated pups. To administer the minocycline (or vehicle), we removed the entire litter of pups from the dam, completed the injections, then returned the whole litter unit to the dam. Each pup was handled for approximately 10 s on each injection day and was separated from the dam for a maximum of 10 min. We refer to this treatment throughout the manuscript as the injection protocol.

The pups were separated into same-sex littermate pairs upon weaning at P21 and left undisturbed until experimentation, except for the usual animal husbandry. We used only males in these experiments; females were kept for use in other studies. For these experiments we used 136 rats. No more than two rats per litter were used per experimental group³⁴. N are as indicated in the results. We performed all adult experiments between 0900 and 1300 hr to avoid the potential influence of circadian rhythms.

Hippocampal microglial responses to immune challenge

To determine if hippocampal microglia are more susceptible to an immune challenge in neonatally overfed animals we gave adult (P70) control-fed and neonatally overfed rats an i.p. injection of LPS (100 µg/kg/mL; *E. coli*, serotype 0.127:B8; L-3129; Sigma-Aldrich, St Louis, MO, USA) or pyrogen-free saline, 24 hr prior to cull (Fig. 1A).

At 24 hr after LPS injection, we deeply anaesthetized the rats with sodium pentobarbital (approximately 150 mg/kg/mL, i.p.). We then transcardially perfused the rats with phosphate buffered saline (PBS; 4 °C, pH 7.4), followed by 4% paraformaldehyde in PBS (4°C, pH 7.4). We then removed the brains and post-fixed them for 24 hr in the same fixative before placing them in 20% sucrose in PBS (4 °C). Forebrains were cut into 30 µm-thick coronal sections using a cryostat. Sections were serially cut into a one in five series and were stored at 4 °C until use for immunohistochemistry.

For immunohistochemistry, sections through the hippocampus were immunolabelled for ionized calcium-binding adapter molecule-1 (Iba-1; expressed on microglia). Iba1 is a commonly used marker for identification of microglia³⁵⁻³⁷. It is clearly constitutively expressed in microglia and is not expressed in neurons, astrocytes, or oligodendroglia^{38,39}. However, we should note it is expressed on cells of the monocyte / macrophage lineage, including non-microglial CNS macrophages. Sections from each treatment group were randomly selected and processed at the same time to reduce variability. Briefly we incubated sections in primary Iba-1 antibody (overnight; 4 °C; 1:1000; rabbit; Wako Chemicals USA Inc., Richmond, VA, USA), followed by secondary antibody (1.5 hr; 1:200; biotinylated anti-rabbit; Vector Laboratories, Burlingame, CA, USA) and avidin-biotin horseradish peroxidase (HRP) complex (ABC; 45 min; Vector Elite Kit; Vector). To visualize the HRP activity, the sections were incubated in diaminobenzidine (DAB), seen as amber staining. The reactions were stopped when the contrast between specific cellular and non-specific background labelling was optimal. Sections were mounted and air-dried, dehydrated in a series of alcohols, cleared in histolene and coverslipped.

Hippocampal sections were assessed by an experimenter blinded to treatment conditions for differences in numbers of cells with Iba-1 labelling and in density of Iba-1 labelling, as previously described^{40,41}. Briefly, we used the thresholding method on photomicrograph images imported into

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image analysis software ImageJ (National Institutes of Health, Bethesda, MD, USA). For the hippocampus we analysed four sections 120 μm apart between 2.52 and 4.56 mm caudal to bregma per animal. We saw no differences between the rostrocaudal levels for any of the regions, so we took the sum counts and mean density of the four images as our sampled result. We present analysis from several subfields of the hippocampus since there are regional differences in microglial distribution between these regions, with microglial density in the CA3 region being lower than in the CA1 region and the dentate gyrus (DG), and this heterogeneous distribution may participate in the modulation of hippocampal activity⁴².

Microglial cell isolation and in vitro immune stimulation

To assess microglial responsiveness *in vitro*, we euthanized a cohort of otherwise untreated control-fed and neonatally overfed rats in early adulthood (P70) with a lethal dose of sodium pentobarbital (Fig. 1A). We rapidly extracted the brains, and dissected the two hippocampi on ice. Whole hippocampi were placed in ice cold Dulbecco's PBS supplemented with 0.2% glucose (sDPBS). These experiments were conducted in accordance with previous literature that states isolated microglial cultures do not change their phenotype after 4 hrs post-isolation⁴³⁻⁴⁵. Moreover, we directly analysed freshly isolated microglia, which have been shown to be more representative for the *in vivo* status of microglial cells at the time of isolation^{45,46}.

Tissue was homogenized according to the methods previously described by Frank et al^{45,47,48}. Briefly, we finely minced the whole hippocampi with a razor blade in DPBS and transferred the minced tissue to an iced tissue homogenizer. The homogenate was filtered through a 40 μm cell strainer (BD Biosciences Discovery Labware, Australia) and was pelleted at 1500 rpm for 10 min. A Percoll® density gradient was created by resuspending the pellet in 70% isotonic Percoll® (Sigma-Aldrich), followed by 37% and 30% Percoll® layers and topped with DPBS. The gradient was spun for 45 min

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at 3400 x rpm at 20 °C with minimum acceleration and brake. Microglia were extracted from the 37/70% interface. Following isolation, hippocampal microglia from cage-mate pairs were combined and stained with Trypan blue and the numbers of viable cells were counted under the microscope based on dye exclusion. We obtained a density of $>5 \times 10^5$ cells. Cells were pelleted at 1500 x rpm for 5 min at room temperature (RT) and then separated for either flow cytometric assessment or *in vitro* immune stimulation.

For flow cytometric assessment of microglial purity and immunophenotype, microglia were stained with both CD11b-FITC (a microglial marker to assess microglial purity) and CD45-PE (a non-microglial macrophage marker to assess contamination with other brain macrophages)⁴⁵. Briefly, microglia ($>3 \times 10^4$ cells) were suspended in incubation buffer (1 x DPBS + 0.2% glucose) and kept on ice for 30 min prior to staining. Antibodies and the isotype controls (1:100; Anti-Rat CD45-PE, Anti-Rat CD11b-FITC, Mouse IgG2a K Isotype Control FITC, Mouse IgG2a K Isotype Control PE; EBiosciences, Waltham, MA, USA) were diluted in the incubation buffer and incubated on ice for 1 hr. Cells were washed and resuspended in DPBS and immediately analysed by flow cytometry.

Isolated microglia were analysed on a FACSCanto II cytometer (BDBiosciences, North Ryde, NSW, AUS). For each staining condition (isotype control and antibody of interest), 1×10^4 events were collected. Quantitation of positively labelled cells was determined by setting threshold on background staining of isotype control. The mean fluorescence intensity of each sample was measure and expressed as a fold increase. To ensure that the population of cells analysed were a pure population of microglia (CD11b^{high}/CD45^{low}), we performed gating on size and granularity to exclude the debris.

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For *in vitro* immune stimulation, microglia (3×10^4 cells per well) were resuspended in 100 μL /well of media (RMPI + 10% Fetal Bovine Serum (FBS)). To assess microglial cytokine responsiveness, cells were challenged with LPS (*E. coli*, serotype 0.127:B8; L-3129; Sigma) at a concentration of either 10 or 100 ng/mL, or media alone⁴⁵ for 4 hr at 37 °C, 5% CO₂. At the end of incubation, 100 μL of media was collected, centrifuged at 1500 x rpm for 1 min at RT, and kept frozen for cytokine analysis.

To assess changes in LPS-stimulated hippocampal microglia, cultured supernatant fluids were analysed for two pro-inflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in a simultaneous, multiplexed format using a Bio-Plex Pro rat cytokine assay. All procedures were carried out according to the recommended procedure (Bio-Plex Pro Array System, Bio-Rad Laboratories Inc., Hercules, CA, USA). The data were analysed using Bio-Plex Manager Software 6.1 (Bio-Rad).

Assessment of body fat composition via EchoMRI in adult neonatally injected rats

One week prior to cull, a cohort of adult (P63) control-fed and neonatally overfed rats that had been treated as neonates with minocycline or saline underwent a magnetic resonance imaging (MRI) scan to assess fat and lean mass (Fig. 1B). Rats were briefly restrained for the duration of the 90 s scan.

Effects of minocycline on adult microglia and central susceptibility to LPS

To determine if the increased microglial activation after LPS in adult rats that had been overfed as neonates could be reversed by neonatal microglial inactivation with minocycline, we also examined immunohistochemical responses to LPS (as described above; Fig. 1B). We assessed this in a cohort of

adult control-fed and neonatally overfed rats that had been treated as neonates with minocycline or saline (as described above).

Assessment of plasma corticosterone

To assess basal HPA axis function in adults that had been overfed as neonates, versus control-fed, and either not injected (Fig. 1A) or given the injection protocol (saline; Fig. 1B), we measured plasma corticosterone concentrations. We used a standard rat corticosterone ELISA (Abnova Corp., Taipei, Taiwan). The inter-assay variability for this assay was 7.2% coefficient of variation (CV), intra-assay variability 4.8% CV, and lower limit of detection 40 pg/ mL. We assayed samples from all treatment groups together in duplicate.

Data Analysis

Data were analysed by SPSS using multi-factorial analyses of variance (ANOVAs) with Tukey's *post hoc* tests where significant interactions were found. We analysed weight data using repeated measures ANOVA. Data are mean + SEM and statistical significance was assumed when $p < 0.05$.

Results

Neonatal overfeeding increases the microglial response to LPS in adults

Neonatal overfeeding increased susceptibility to the central effects of an immune challenge in the CA3 and CA1 regions of the hippocampus at 24 hr after i.p. LPS (Fig. 2A-E), however, there were no differences in density. Thus, after LPS injection, the numbers of microglia were significantly higher in the CA3 region in neonatally overfed than control-fed rats, and were also significantly elevated after LPS compared with saline-treated animals (significant effect of litter size: $F_{(1,36)} = 12.28$, $p = 0.001$ and LPS: $F_{(1,36)} = 8.61$, $p = 0.006$; $n = 6-16$). The neonatally overfed rats also showed increased

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numbers of microglia in the CA1 region compared to control-fed rats (significant effect of litter size: $F_{(1,39)} = 13.61, p = 0.001$ and LPS: $F_{(1,39)} = 5.88, p = 0.02$). Numbers of microglial cells were also increased in the hilus with neonatal overfeeding (significant effect of litter size: $F_{(1,35)} = 14.68, p = 0.001$; Fig. 2 G) and subgranular/granular (significant effect of litter size: ($F_{(1,35)} = 13.17, p = 0.001$; Fig. 2 I) regions of the dentate gyrus (DG) but not in the molecular region of the DG (Fig. 2K).

Additionally, in the DG, the previously reported finding that density was increased in neonatally overfed rats with respect to control-fed rats under basal and LPS-stimulated conditions¹⁶ was replicated here (Fig. 2H, J, L). Thus, there was a significant effect of litter size on density in the hilus, subgranular/granular, and molecular regions, with neonatally overfed rats having higher density than control-fed (hilus: $F_{(1,36)} = 30.44, p < 0.001$; Fig. 2H, subgranular/ granular: $F_{(1,36)} = 27.87, p < 0.001$; Fig. 2J and molecular: $F_{(1,35)} = 39.40, p < 0.001$; Fig. 2L).

Neonatal overfeeding does not affect microglial sensitivity to an LPS challenge in vitro

Since our *in vivo* studies had revealed that the hippocampus, at least the CA3 and CA1 regions, was more sensitive to the effects of LPS on microglia in neonatally overfed than control-fed rats, we next aimed to determine if this was an effect specific to microglia. We thus examined specific microglial responses to LPS by stimulating them in isolation.

Quantitation of cells positively labelled for CD11b and CD45 verified there were no differences in the expression of both either marker between the groups, indicating our isolation protocol was equally effective in both the neonatally overfed and control-fed rats (Fig. 3A). For both TNF α and IL-6 there was a significant effect of treatment with LPS. 100 ng/mL LPS significantly increased TNF α ($F_{(2,39)} = 13.32, p < 0.001$; n = 8; Fig. 3B) in neonatally overfed rats only compared with no LPS and significantly increased IL-6 ($F_{(2,39)} = 44.78, p < 0.001$; Fig. 3C) in control-fed and neonatally overfed

rats compared with no LPS. There were no significant differences between the control-fed and neonatally overfed groups, indicating microglial sensitivity to LPS *in vitro* was not affected by neonatal overfeeding, and suggesting the response is potentially modulated by other cell types.

Neonatal overfeeding and effects of minocycline

Although we did not see *in vitro* differences between the microglial responses of the neonatally overfed control-fed rats in adulthood, our *in vivo* results showed microglial responses to an immune challenge in adults are increased in these animals relative to control-fed responses. We thus aimed to reverse or mitigate the long-term effects of neonatal overfeeding on microglial responses by inhibiting microglial activation concomitant with overfeeding.

The course of minocycline injections had no immediate effect on neonatal weight (significant time by litter size effect: $F_{(3, 108)} = 107.54, p < 0.001$; Fig. 4A; $n = 16-18$) with both groups of neonatally overfed rats weighing more than control-fed rats at P14 and P21. The weight difference in adulthood between control-fed and neonatally overfed rats was also maintained, even slightly increased, after minocycline injections (significant effect of litter size: $F_{(1, 60)} = 25.41, p < 0.001$ and treatment: $F_{(1, 60)} = 9.33, p = 0.003$; Fig. 4B).

The differences in body mass in control-fed and neonatally overfed rats were also maintained despite neonatal minocycline. Lean mass, total fat mass and percentage fat mass were significantly increased in the neonatally overfed compared with the control-fed rats (lean mass: significant effect of litter size: $F_{(1, 60)} = 17.44, p < 0.001$ and treatment: $F_{(1, 60)} = 5.41, p = 0.023$; Fig. 4C, total fat mass: significant effect of litter size: $F_{(1, 60)} = 45.18, p < 0.001$ and treatment: $F_{(1, 60)} = 5.08, p = 0.028$; Fig. 4D and percentage fat mass: significant effect of litter size: $F_{(1, 60)} = 29.28, p < 0.001$; Fig. 4E).

Neonatal injection protocol markedly reduces microglial numbers and density; no additional effect of minocycline.

We hypothesized that the altered microglial profile that we have previously seen in the neonatally overfed rats¹⁶ would be suppressed to that of control-fed levels with minocycline administered during the suckling period. We thus analysed hippocampal microglia from these rats that had been injected as neonates with either saline or minocycline. We unexpectedly found not only no beneficial effect of minocycline, but that our finding (previously observed in two separate experiments¹⁶ and here) of neonatal overfeeding-induced increases in hippocampal microglia number and density was no longer evident in this cohort (Fig. 5; n = 7-9). In the CA3 region, there was a main effect of immune challenge on the number of microglia (significant effect of immune challenge: $F_{(1,54)} = 12.312$, $p = 0.01$; Fig 5A) but no effect on density (Fig. 5B). In the CA1 region, there was a significant interaction between litter size and immune challenge on density of microglia: $F_{(1,54)} = 4.576$, $p = 0.037$; Fig. 5F), but no specific effects of neonatal overfeeding with *post hoc* comparisons. Neonatal overfeeding did not affect the number of microglia in the hilus region of the DG (Fig. 5G), however, it suppressed the density of microglia overall (significant interaction between litter size and minocycline injection: $F_{(1,55)} = 4.518$, $p = 0.038$ and an interaction between litter size and immune challenge: $F_{(1,55)} = 4.042$, $p = 0.005$; Fig. 5H). Specifically, there was reduced microglial density in the neonatally overfed vehicle-treated rats that were given LPS as adults (SL nSal/ aLPS) compared to control-fed (CL nSal/ aLPS), and reduced microglial density in the control-fed minocycline-treated rats that were given LPS as adults (CL nMino/ aLPS) compared to control-fed vehicle-treated that were given adult LPS rats (CL nSal/ aLPS). In the sub-granular/granular zone, there was an interaction between litter size, minocycline-injection and immune challenge ($F_{(1,57)} = 4.049$, $p = 0.049$; Fig. 5J) on microglia density, however, there were no effects with *post hoc* analysis. We also found no of any of the treatments the number of microglia between the control-fed and neonatally overfed animals regardless of injection in the sub-granular/ granular region (Fig. 5I). Neonatal overfeeding suppressed the number and density of microglia in the molecular region of the DG (number: significant interaction between litter size and immune challenge: $F_{(1,55)} = 4.869$, $p = 0.032$; Fig. 5K, and, density: significant interaction between

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litter size and immune challenge: $F_{(1,54)} = 4.244, p = 0.044$; Fig. 5L) with specifically fewer microglia in the neonatally overfed vehicle-treated rats that were given LPS (SL nSal/ aLPS) compared to control-fed vehicle-treated (CL nSal/ aLPS).

Interestingly, the neonatal injection protocol, whether with minocycline or with saline, appeared to significantly suppress microglial numbers and density in all groups. Figure 6 illustrates the data shown in Figure 5 superimposed on those from the experiment in Figure 2. Data from Figure 2 show high numerical similarity with data we have previously published¹⁶. Given the apparent effect of the neonatal treatment regimen, we re-processed the hippocampal tissue from our previously published animals⁴⁹ to verify this result was not due to tissue processing or age of the tissue/duration of storage. Microglial numbers and density were not different from the published data and Figure 2 (data not shown).

To statistically compare the effects of the neonatal injection protocol and neonatal overfeeding alone, we examined an additional cohort of control-fed and neonatally injected rats that had been processed at the same time as our minocycline cohort, but for a different study (Fig. 1C). These were not otherwise handled except for the usual husbandry and weekly weighing during the neonatal period. The non-injected animals (CL and SL non-injected) showed the same microglial profiles as previously reported. The neonatally overfed animals that were not injected (SL non-injected) during the suckling period showed an increased microglial density in the hilus region of the DG, compared to the control-fed (CL non-injected) and neonatally overfed saline-injected animals (SL nSal/ aSal) (number: significant effect of litter: $F_{(1,21)} = 5.326, p = 0.031$; Fig. 7A and injection $F_{(1,25)} = 23.793, p < 0.001$; Fig. 7B and density: significant interaction between litter and injection: $F_{(1,23)} = 32.797, p < 0.001$; Fig. 7B). In the sub-granular/granular zone, the neonatally injected rats (both CL nSal/ aSal and SL nSal/ aSal) had significantly fewer microglia and reduced microglial density compared to non-injected rats (CL and SL non-injected; number: significant effect of injection: $F_{(1,23)} = 15.747, p =$

0.01; Fig. 7C and density: significant interaction between litter and injection: $F_{(1,23)} = 125.472, p < 0.01$; Fig. 7D). Neonatal injections (both CL nSal/ aSal and SL nSal/ aSal) also suppressed the number and density of microglia in the molecular region compared to non-injected animals (CL and SL non-injected; number: significant effect of injection: $F_{(1,22)} = 28.466, p < 0.01$; Fig. 7E and density: significant interaction between litter and injection: $F_{(1,23)} = 55.876, p < 0.01$; Fig. 7F).

To assess if the neonatal injection protocol caused a generalized suppression of microglia throughout the brain, we also assessed two regions we had investigated in previous studies, the arcuate nucleus of the hypothalamus (ARC) and paraventricular nucleus of the hypothalamus (PVN). Similar to previously published data ⁵, there was no change in either the microglial number or density in the ARC under basal conditions (Fig. 8A, B), but neonatal overfeeding (SL non-injected) led to a significant increase in microglial numbers in the PVN (Fig. 8C), with no change in density (Fig. 8D), compared with CL non-injected. Neonatally overfed animals that were saline-injected (SL nSal/ aSal) had a reduced number and density of microglia in the ARC compared to the non-injected animals (CL and SL non-injected; number: significant effect of treatment: $F_{(1,20)} = 54.312, p < 0.001$; Fig. 8A and density: significant effect of treatment: $F_{(1,20)} = 38.591, p < 0.001$; Fig. 8B). The previously reported effect of neonatal overfeeding to increase PVN microglial number and density was also abolished in the neonatally injected group (both CL nSal/ aSal and SL nSal/ aSal; number: significant litter size by treatment interaction: $F_{(1,19)} = 7.668, p = 0.012$; Fig. 8C and density: significant effect of treatment: $F_{(1,19)} = 63.696, p < 0.001$; Fig. 8D). Together these data suggest that our neonatal injection protocol caused a generalized suppression of microglial activity that was able to over-ride any effects of neonatal overfeeding to increase numbers of these cells, with no additional effect of minocycline.

Neonatal injection protocol increases basal corticosterone concentrations in neonatally overfed offspring

To determine if the generalized suppression of microglial activity is due to the neonatal injection protocol causing a long-term upregulation in HPA axis activity or basal glucocorticoid secretion, we assessed basal corticosterone concentrations. We saw no basal corticosterone differences between the non-injected neonatally overfed and control-fed adult rats 24 hr after saline injection (Fig. 9) and saline-injected control-fed (CL nSal/ aSal) corticosterone concentrations were not significantly different from those of non-injected animals. Basal corticosterone levels were elevated in neonatally overfed rats that were saline injected (SL nSal/ aSal) during the suckling period compared with both the neonatally overfed non-injected (SL non-injected) and the control-fed injected rats (CL nSal/ aSal; significant litter size by treatment interaction: $F_{(1,16)}=4.769$, $p = 0.044$; $n=5$; Fig. 9). However, this is unlikely to account for our microglial findings since the control-fed rats were not similarly affected.

Discussion

This study is the first to show that neonatal overfeeding increases hippocampal microglial responses to an immune challenge with LPS and that neonatal injections (in this case with either saline or minocycline) can have pronounced long-term effects on microglia sufficient to over-ride the impact of the neonatal overfeeding intervention. Notably, saline injections were just as effective as minocycline at suppressing microglial activity long-term.

The neonatal nutritional environment, as induced by altering the litter size in which the animals were raised, can have significant long-term programming effects. We have previously demonstrated that changes to the neonatal nutritional environment can alter body weight into adulthood as well as altering microglial profiles within developing brains and it predisposes the animal to a sensitized basal microglial profile in the hypothalamus during adulthood^{5,49}. We have also previously shown that

adult neonatally overfed animals have dramatically increased microglial numbers in the PVN 24 hr after an LPS challenge⁵. This microglial programming in adults that have been overfed as neonates extends beyond the hypothalamus into the hippocampus¹⁶. Here we demonstrate that adult neonatally overfed rats have increased microglial numbers in the CA3 in response to an LPS challenge compared with control-fed rats. Other early life manipulations can lead to similar hyper-responsive microglia. A peripheral immune challenge with *E.coli* or LPS in early life results in increased microglial numbers and an activated phenotype in the adult brain^{4,50}. Similarly, maternal high fat diet predisposes the offspring to an increase in hippocampal expression of microglial activation markers at birth, as well as increased microglial activation both basally and in response to an LPS challenge in adulthood⁴. It should be noted that previous groups have used numbers and density of Iba-1 staining to provide an index of microglial activation states^{5,40,41}. We acknowledge that this method of microglial characterisation does have its limitations. However, other more refined analysis techniques such as Sholl analysis also have restrictions; despite the Sholl analysis being able to quantitatively analyse processes and branching complexity of microglia⁵¹⁻⁵³, this technique is unable to determine microglial function⁵⁴. For instance, a ramified microglial cells may be de-ramifying in response to a stimulus such as LPS or hyper-ramifying⁵⁴. Additionally, functional changes can be seen in microglial cells in the absence of morphological change⁵⁵. Nonetheless, our findings that neonatally overfed microglial cell numbers, particularly in the CA3, are increased after an LPS challenge support our findings that neonatally overfed rats have hyper-responsive microglia.

This increase in microglial numbers in the CA3 region in our neonatally overfed animals led us to investigate whether these findings are likely to be due to an exclusive effect on microglia. It is noteworthy that culturing isolated microglia for an extended period can result in loss of the microglial phenotype⁴³⁻⁴⁵. However, we only stimulated the microglia culture for 4 hours therefore the microglial phenotype should be similar to *in vivo* microglial cells. We found that microglial sensitivity to LPS *in vitro* was not affected by neonatal overfeeding suggesting that other cell types may be interacting with microglia to produce the response. Astrocytes are known to form an

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interaction with microglia and this is an important mechanism allowing the inhibition of excessive microglial activation⁵⁶. Therefore, it is possible that our neonatally overfed animals have reduced astrocyte numbers or complexity enabling them to hyper-respond to an immune challenge *in vivo* compared to control-fed rats. The absence of this and other cell types in our *in vitro* isolated microglia experiments could explain why the differences induced by neonatal overfeeding are not seen in this preparation. Furthermore, the neonatal injection protocol caused generalized microglial suppression in both adult neonatally overfed and control-fed rats, however it failed to alter the neonatal overfeeding-induced weight gain, further suggesting that other cells types are involved alongside microglia to maintain long-term changes in response to early life diet.

To our knowledge this is the first study to demonstrate that repeated injections during the suckling period suppress the number and density of microglia in the hippocampus in adulthood. However, other early life treatments can have similar effects on microglia^{57,58}. Handling refers to neonatal rats being exposed to short periods of maternal absence in a novel environment (approximately 15 mins) daily for the first 2-3 weeks of life. Adult rats that are infected with *E. coli* at P4 coupled with neonatal handling (P1-10, 15 mins daily) exhibit decreased gene expression of cluster of differentiation molecule 11b (CD11b), glial fibrillary acidic protein (GFAP), and IL-1 β in the hippocampus under basal conditions compared with non-handled control-fed rats⁵⁷. These adult rats also display a suppression of CD11b gene expression 2 hr after an immune challenge with LPS and have dramatically improved spatial learning and memory in the Morris water maze compared with those that are not handled as neonates⁵⁷. It has been speculated that microglia of neonatally handled rats shift to take on an anti-inflammatory profile via epigenetic modification of the IL-10 gene specifically within the microglia⁵⁸, but this remains to be tested with either neonatal handling or multiple injections. We have demonstrated here that non-injected neonatally overfed animals have an increased microglial response to LPS in the hippocampus relative to control-fed, particularly in the CA3 region, and that this response is suppressed in adult neonatally injected rats. Thus, our data

support the hypothesis that neonatal handling coupled with an injection can produce an anti-inflammatory microglial profile.

Obesity and high-fat diets have been associated with systemic inflammation and more recently central inflammation⁵⁹. It has become increasingly apparent that a high-fat diet can cause hypothalamic inflammation, which can disrupt normal feeding- and metabolism-related signalling via the activation of microglia⁵⁹⁻⁶¹. This central inflammation can contribute to leptin and insulin resistance therefore causing weight gain and maintaining an elevated body weight^{59,60}. We have demonstrated that neonatal overfeeding contributes to a primed basal pro-inflammatory profile with increases in number and density of Iba-1 positive microglia in the PVN during early life, which persists into adulthood⁵ and this co-occurs with the maintenance of accelerated weight gain compared to control-fed rats. Interestingly, in the present study we found the drastic suppression of microglial number and density in adult rats that underwent repeated injections was not localized to the hippocampus but this trend was also seen in the hypothalamus. Furthermore, this microglial suppression did not affect weight gain during the suckling period or in adulthood. Maternal separation does not affect body weight or ingestion of standard lab chow⁶², therefore it is possible that an additional increase in microglia is occurring in these neonatally overfed pups during the suckling period, that dissipates after weaning, which is affecting normal feeding- and metabolism- related signalling compared to control-fed rats.

Under normal physiological conditions, corticosterone is considered to be anti-inflammatory. However, stressful stimuli can sensitize central pro-inflammatory responses and activation of microglia. To investigate if the repeated neonatal injections effects on microglia were associated with long-term changes to the HPA axis, we measured basal circulating corticosterone in neonatally non-injected and injected (saline) adult rats. Our current findings are consistent with previous studies from our group, showing that corticosterone levels in non-injected neonatally overfed rats are not significantly different from non-injected control-fed rats^{2,15,33}. Surprisingly, we found that the

neonataly overfed animals that underwent repeated neonatal injections had significantly increased basal corticosterone levels compared to the non-injected equivalent and this difference was not seen in the neonataly injected control-fed animals. Repeated neonatal injections induced a potentiated corticosterone response in animals that were overfed during development which could suggest that the HPA axis in these animals is sensitized.

Previous literature shows confounding evidence on the effects of maternal separation of pups during development on basal corticosterone; however, this is likely due to the different separation protocols. In adulthood, animals that were handled during the suckling period display a reduction in plasma corticosterone concentrations^{57,63,64}. However, a longer period of maternal separation during this developmental period (180 min/day) increases basal corticosterone concentrations⁶⁵⁻⁷¹. Interestingly, Llorente and colleagues have also shown similar findings to our study, whereby a neonatal injection period between P7-P12 increases basal corticosterone compared to the non-injected rats⁷².

The neonatal litter size manipulation model undoubtedly has elements incidental to feeding that may influence physiology long-term^{5,73}. However, these do not provide a simple explanation for our findings. For example, Meaney and colleagues have shown maternal attention suppresses HPA axis responses to stress throughout life via maternal contact-mediated changes in histone acetylation and NGF1-A binding to the glucocorticoid receptor promoter to increase expression of this receptor and enhance the efficiency of glucocorticoid negative feedback⁷⁴. Dams will spend approximately the same time in high intensity nursing and grooming behaviours irrespective of the size of the litter, therefore more attention would be imparted to each of the neonataly overfed pups than to the control-fed pups⁷⁵⁻⁷⁷. Thus, one would anticipate those from the neonataly overfed group to have suppressed HPA axis responses to stress and perhaps reduced basal circulating corticosterone; the opposite profile to the one we see here. Likewise, neonatal overfeeding can induce changes in circulating satiety hormones such as ghrelin that have a role in stress regulation^{78,79}. However, this effect on

ghrelin's role in stress regulation appears to be exclusive to females despite persistent changes in other function of this hormone in males^{79,80}. We thus currently do not have an explanation for why basal corticosterone is elevated in the neonatally overfed that have had the neonatal injection protocol and not in the other groups.

In the current study, we aimed to test the effects of neonatal minocycline; a known microglial inhibitor²⁸, on the hippocampal microglial profile both under basal conditions and after an LPS challenge. We hypothesized that a minocycline regimen during the suckling period would attenuate the increased microglial response to an LPS challenge in our adult neonatally overfed rats. Neonatal minocycline had no effect on the number or density of microglia in adulthood regardless of whether the rats were exposed to LPS or not. The results from the minocycline aspect of this experiment were inconclusive due to the over-riding effect of the repeated injection regimen itself. Ideally, an alternative form of drug administration would need to be adopted to gain a conclusive result as to whether minocycline administration could reduce microglial activation after neonatal overfeeding. Our treatment regimen was based on studies of hypoxia-ischemia showing this dose, administered via i.p. injection, could successfully ameliorate neuroinflammation at the site of hypoxic-ischemic insult²⁴. A common alternative form of minocycline administration is via oral administration from drinking water^{81,82}. However, delivery of drugs via voluntary consumption of water does not take into consideration the individual variability in the amount of water consumed. Additionally, the drug must be able to accumulate in the dam's milk to enable delivery to the suckling pups and may therefore not be reliably delivered to each pup at the same dose⁸³. Importantly, the nature of our model where the neonatally overfed pups consume more milk from the dams means the neonatally overfed pups would unavoidably receive more minocycline. Alternatively, drug delivery could be achieved via oral gavage of the drug into either the pharynx or the esophagus, which would give exact concentrations of minocycline^{83,84}. Previous literature recommends oral gavage to commence after P4 to reduce damage to the tissue⁸⁴. We needed to begin our regimen following litter manipulation to try and prevent the early effects on microglia, therefore this technique would not be suitable. This process is

also stressful to the pups and involves removing the litter and handling them, therefore, likely, providing the same result as we have shown here with a reduction in microglia despite early life overfeeding. Thus, the effect of reversing microglial priming during neonatal overfeeding remains untested but our experiments reveal important effects of repeated injections in the neonatal period.

In summary, we have shown that neonatal overfeeding leads to a hyper-responsive basal microglial profile in the hippocampus and this is associated with an increased response to LPS in this region relative to control-fed responses, and that cellular populations additional to microglia may be important for these responses. In our attempts to reverse the neonatal overfeeding-induced microglial effects, we also revealed that our neonatal injection protocol, whether with minocycline or with saline, strongly suppressed microglial numbers and density without reversing any of the metabolic effects of neonatal overfeeding. Although the mechanism behind the impact of neonatal injection on microglia remains to be determined, it does not appear to be explained by an effect on HPA axis function. These findings have significant implications for all studies employing injection protocols in neonatal experimental animals and raise interesting questions about the impact of various stimuli on neonates in general.

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Figure Legends

Fig. 1. Timeline of the experimental design. Litter size manipulation is performed on the day of birth (postnatal (P)0) into litters of 12 (control-fed; CL) or 4 (neonatally overfed; SL). Litters are assigned to either A) non-injected or B) the neonatal injection protocol during the suckling period until weaning (P21). Pups are weaned into pairs and left undisturbed until experiment during adulthood (P70). C) To further examine the impact of the injection protocol on microglia, we performed another experiment on a different cohort of non-injected pups.

Fig. 2. Numbers and density of ionized calcium-binding adapter molecule-1 (Iba-1)-immunolabelled cells 24 hours after an i.p. saline or lipopolysaccharide (LPS) injection at postnatal day (P)70 in rats raised in control-fed litters (CL) and small litters (neonatally overfed; SL). A, B) CA3 region. C, D)

CA1 region. G, H) Dentate gyrus (DG) hilus. I, J) DG subgranular/granular region. K, L) DG molecular region. Data are mean + SEM. N = 6-16 per group. Stripes represent animals that are injected with LPS. E, F) Representative photomicrographs of the CA3 (E) or the dentate gyrus (F) region from control-fed and neonatally overfed rats at P70 injected with saline or LPS i.p. illustrating differences in numbers of Iba-1-positive microglia. Scale bar = 50 μ m. * main effect of litter size. # main effect of LPS. $p < 0.05$.

Fig. 3. A) Characterisation of hippocampal microglial culture from rats raised in control-fed (CL) and small (neonatally overfed; SL) litters. Hippocampal cell expression of B) tumour necrosis factor α (TNF α) and C) interleukin-6 (IL-6) in response to either 10 ng/mL or 100 ng/mL lipopolysaccharide (LPS) stimulation *in vitro* from control-fed and neonatally overfed rats. Data are mean + SEM. N = 9 per group. ^ $p < 0.05$ Tukey *post hoc* after main effect of dose.

Fig. 4. Effects of injections on A) pre-weaning weight (P7-P21) * saline and # minocycline $p < 0.05$ Tukey *post hoc* after litter size by day interaction. B) adult weight in rats raised in control-fed (CL) and small (neonatally overfed; SL) litters. C) lean mass, D) fat mass and E) percentage of fat mass at P70 in rats raised in control-fed and small (neonatally overfed) litters. Data are mean + SEM. N = 14-16 per group. * main effect of litter size. \$ main effect of minocycline treatment. $p < 0.05$.

Fig. 5. Numbers and density of ionized calcium-binding adapter molecule-1 (Iba-1)-labelled cells at postnatal day (P)70 in rats raised in control-fed (CL) and small (neonatally overfed; SL) litters that were saline or minocycline injected during the suckling period (P1-21) as well as given an adult immune challenge with lipopolysaccharide (LPS) (or saline). A, B) CA3 region. E, F) CA1 region. G, H) Dentate gyrus (DG) hilus. I, J) DG subgranular/ granular region. K, L) DG molecular region. Data are mean + SEM. N = 7-9 per group. C, D) Representative photomicrographs of the CA3 region from

P70 injected neonatally overfed rats (SL) that were injected with saline (C) or LPS (D) during adulthood illustrating no differences in numbers and density of Iba-1-labelled cells. Scale bar = 50 μm . # main effect of LPS. * x # litter size by minocycline treatment interaction. ^ Tukey *post hoc* after litter size by minocycline treatment interaction. $p < 0.05$.

Fig. 6. Comparison between Fig. 2 and Fig. 5 of numbers and density of ionized calcium-binding adapter molecule-1 (Iba-1)-labelled cells at postnatal day (P)70 in rats raised in control-fed (CL) and small (neonatally overfed; SL) litters. These animals were injected with saline or minocycline during the suckling period (P1-21) as well as given an adult immune challenge with lipopolysaccharide (LPS). Blue shaded bars on the graphs represent previously seen non-injected microglial profile from Fig. 2. A, B) CA3 region. C, D) CA1 region. E, F) Dentate gyrus (DG) Hilus. G, H) DG subgranular/ granular region. I, J) DG molecular region. Data are mean + SEM. N = 7-9 per group.

Fig. 7. Comparison of numbers and density of ionized calcium-binding adapter molecule-1 (Iba-1)-stained cells at postnatal day (P)70 in injected (saline) rats and a separate cohort of non-injected rats raised in control-fed (CL) and small (neonatally overfed; SL) litters. A, B) Dentate gyrus (DG) hilus. C, D) DG subgranular/ granular region. E, F) DG molecular region. Data are mean + SEM. N = 7-9 per group. * main effect of litter size. & main effect of injection. ^ Tukey *post hoc* after litter size by injection interaction. $p < 0.05$.

Fig. 8. Numbers and density of ionized calcium-binding adapter molecule-1 (Iba-1)-stained cells at postnatal day (P)70 in rats raised in control-fed (CL) and small (neonatally overfed; SL) litters that were non-injected or injected with saline. A, B) Arcuate nucleus of the hypothalamus (ARC). C, D) Paraventricular nucleus of the hypothalamus (PVN). Data are mean +SEM. N = 5-8. * main effect of

litter size. & main effect of injection. ^ Tukey *post hoc* after litter size by injection interaction. $p < 0.05$.

Fig. 9. Basal plasma corticosterone concentrations of non-injected and injected rats raised in control-fed (CL) and small (neonatally overfed; SL) litters. Data are mean + SEM. N = 5. ^ Tukey *post hoc* after litter size by injection interaction. $p < 0.05$.

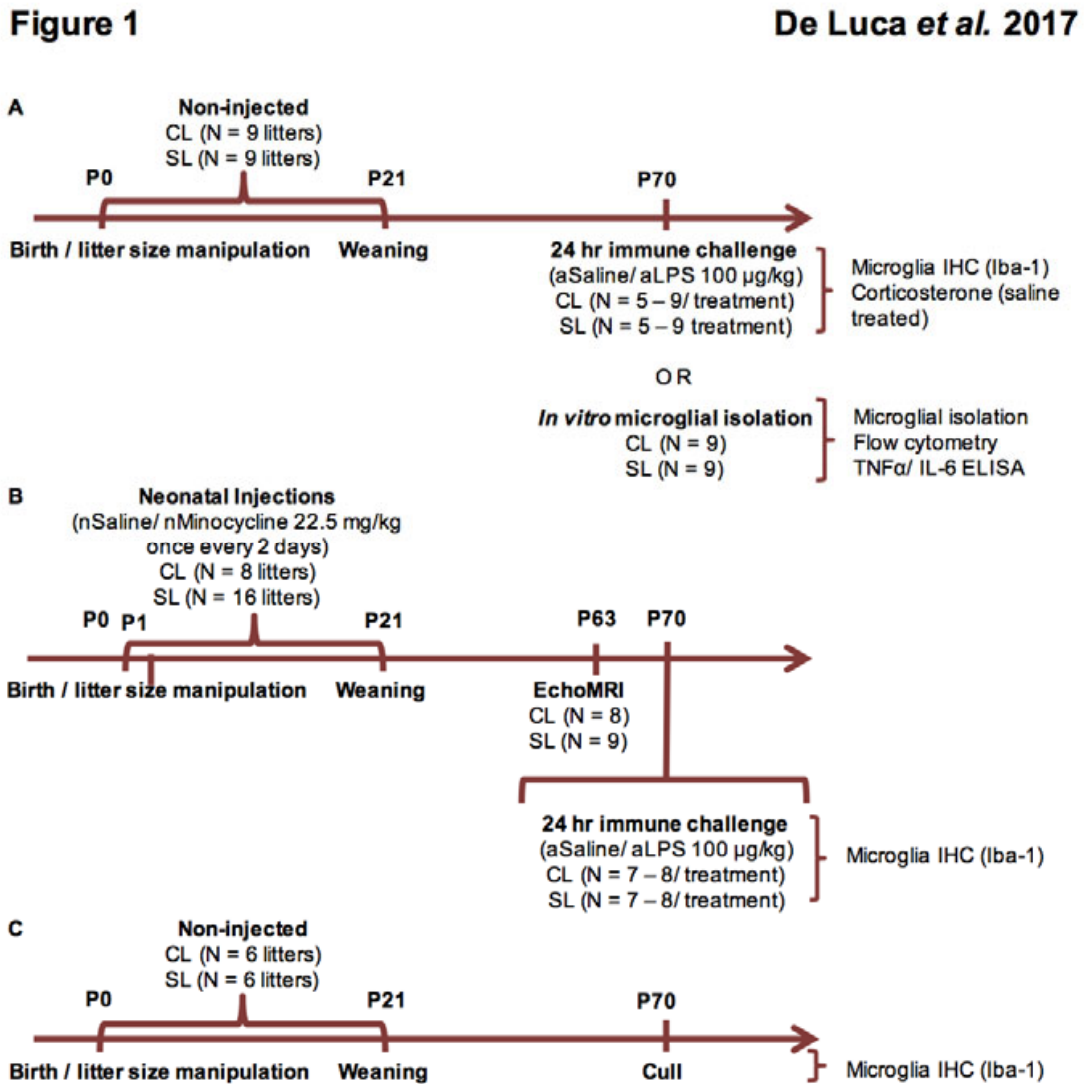


Figure 2

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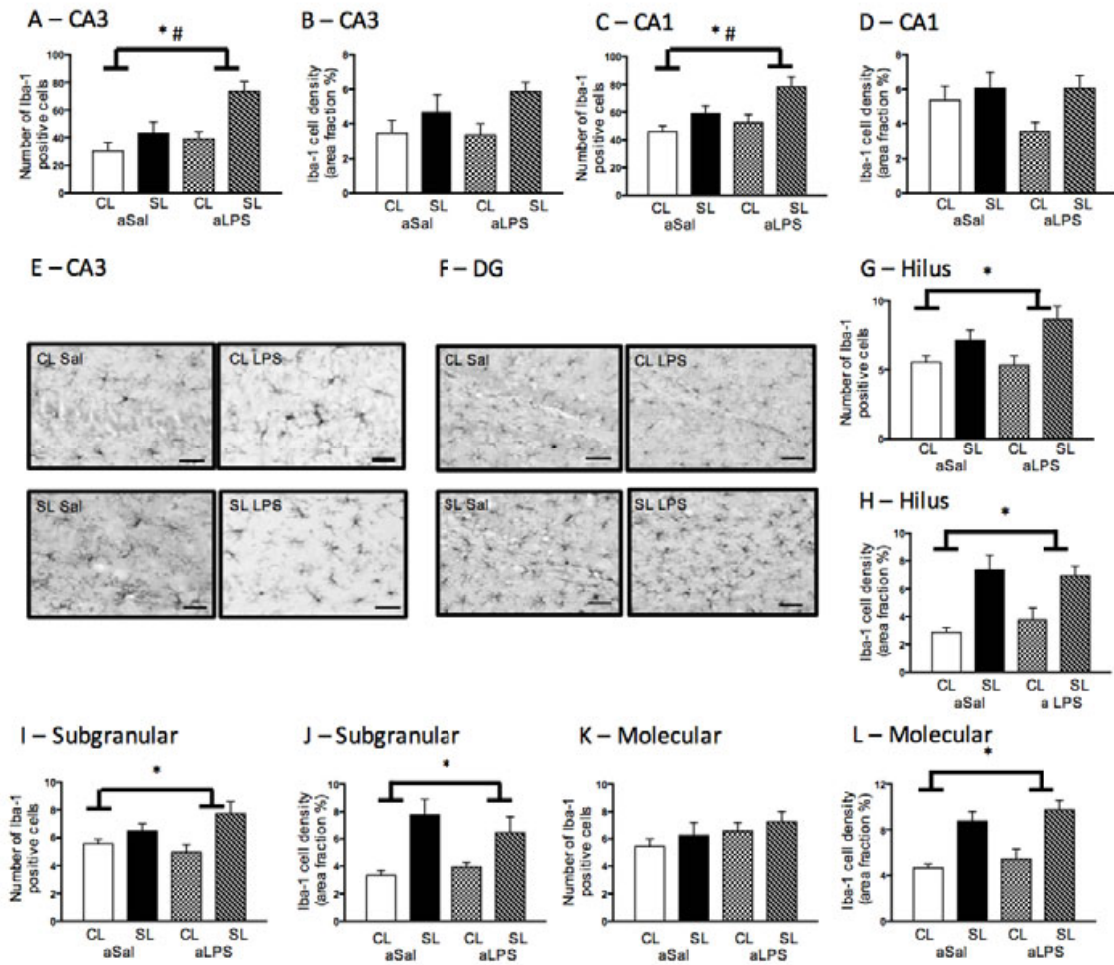


Figure 3

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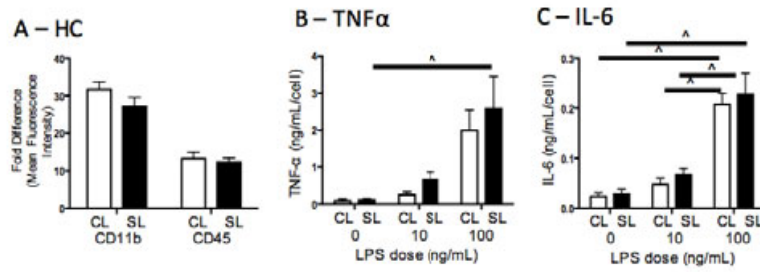


Figure 4

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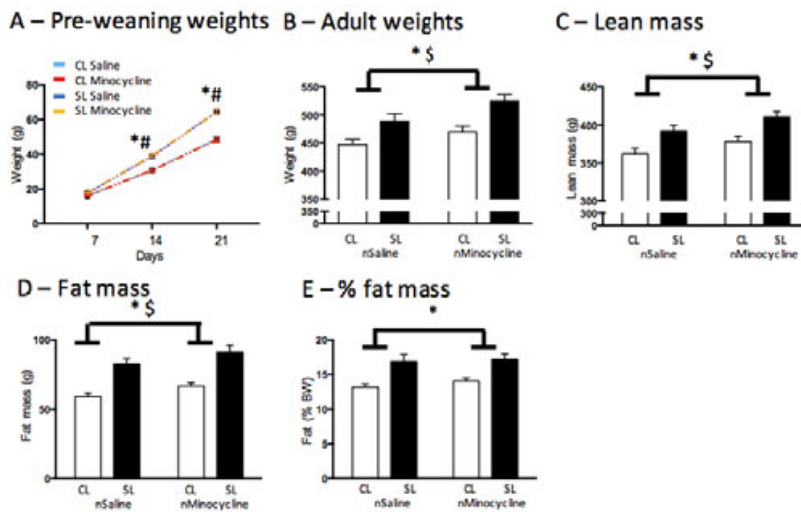


Figure 5

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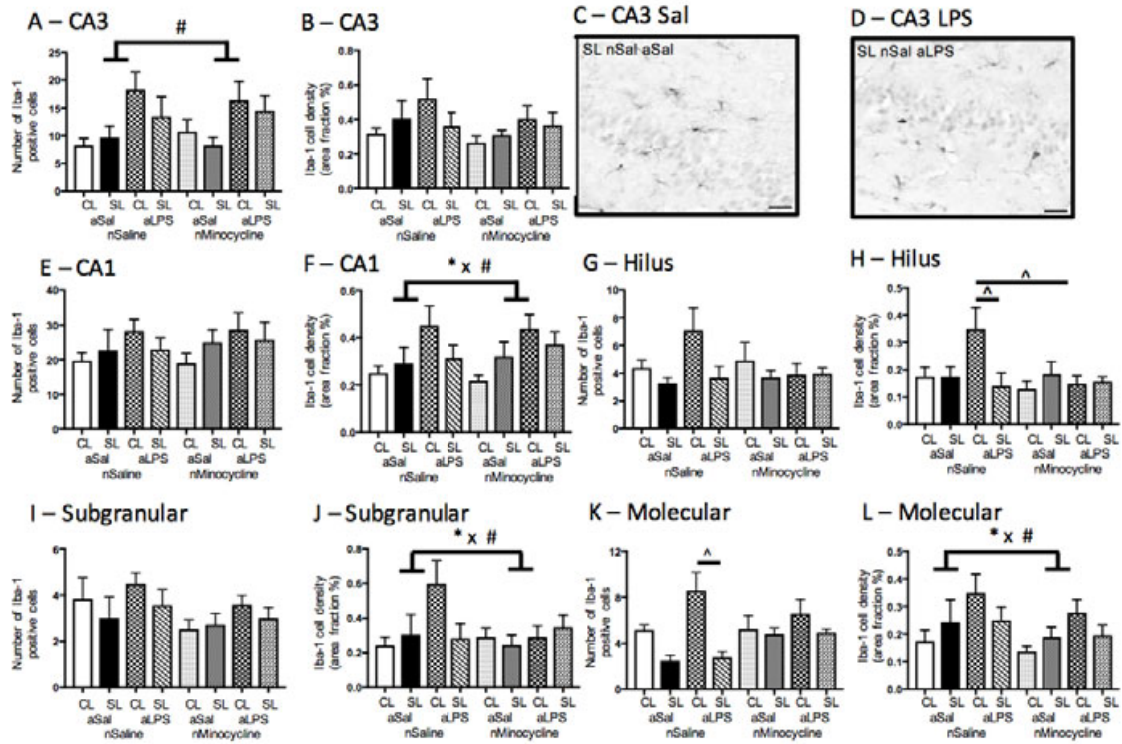


Figure 6

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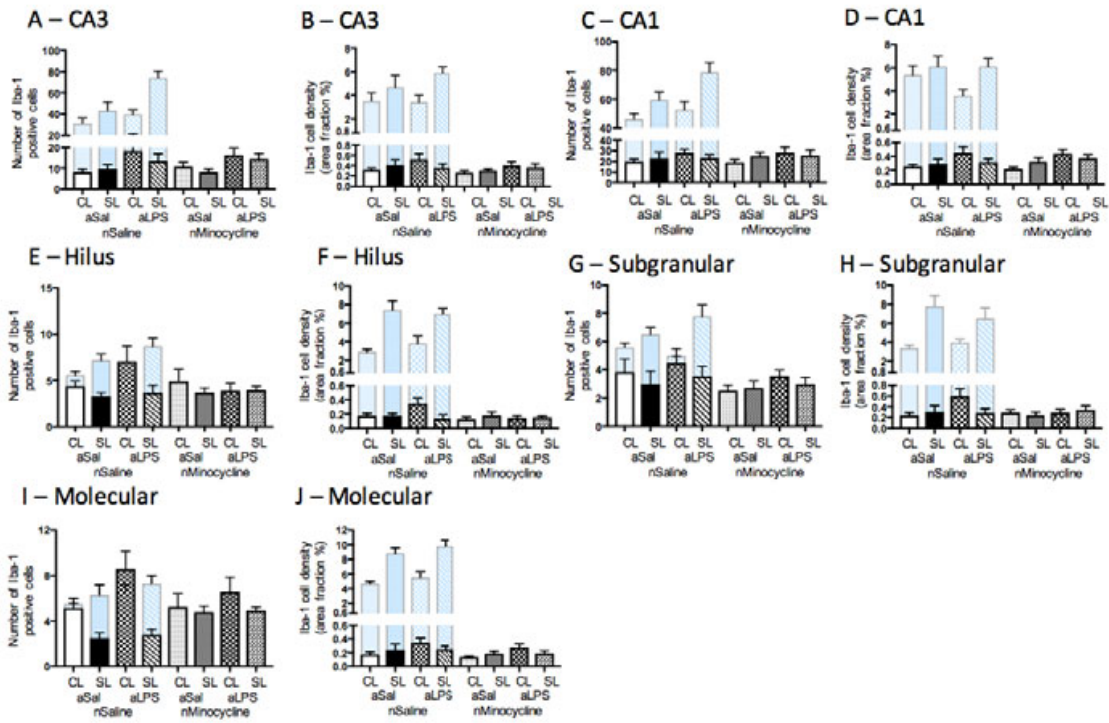


Figure 7

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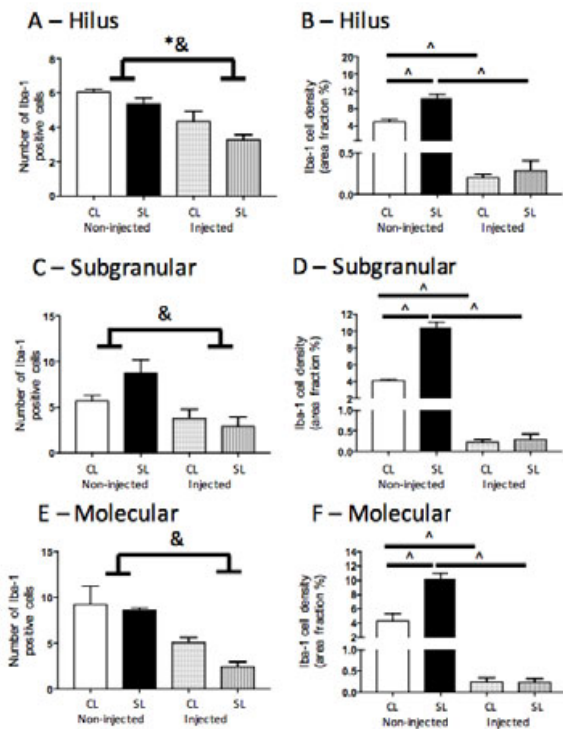
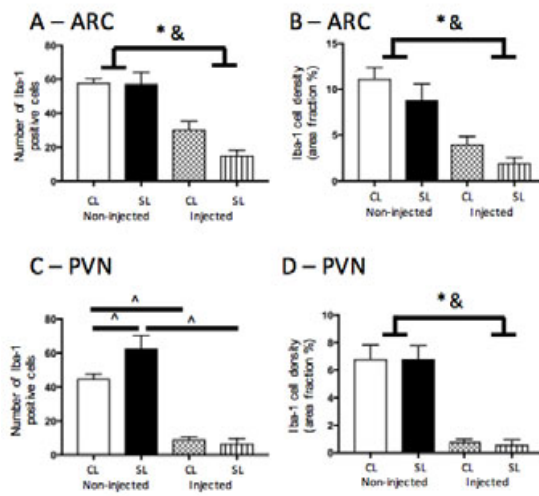


Figure 8De Luca *et al.* 2017**Figure 9**De Luca *et al.* 2017