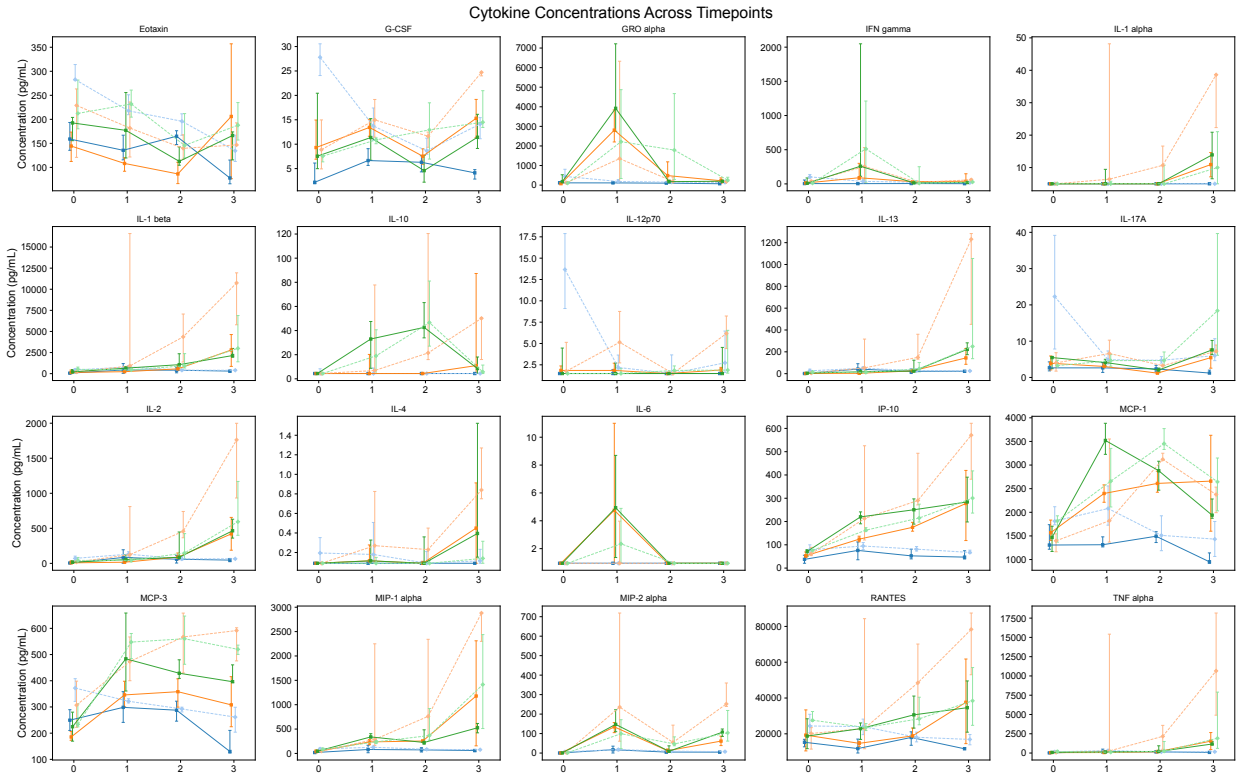
Supplementary Information

Cytokine Profiles Across Timepoints



Supplementary Figure 1 | Cytokine profiles across treatment timepoints. Cytokine concentrations measured using the ProcartaPlex assay at baseline (0), Day 1 (1), Day 3 (2), and Day 5 (3). Control animals are shown in blue, LPS-treated animals in orange, and LPS+VNS-treated animals in green. Sex is indicated by line style (males, solid; females, dashed).

Supplementary Analysis 1

Splenocytes – Effect of LPS

The effect of LPS revealed many significantly differentially expressed genes (DEGs) with 782 upregulated and 128 downregulated in the LPS group compared to controls ($p_{adj} < 0.05$, $\log_2$FoldChange > 1.5). Top upregulated transcripts indicate strong activation of inflammatory pathways, marked by induction of *Nos2*, *Lcn2*, *Pglyrp1*, *Cd177*, *S100a8/9*, and mast cell proteases (*Mcpt1l1, Mcpt3/4*), consistent with myeloid and neutrophil activation and antimicrobial defense. Multiple transcripts with established anti-inflammatory or regulatory roles, including *Il1r2*, *Tnip3*, *Acod1*, *Tnfaip6*, and *Crispld2*, suggest activation of feedback mechanisms that reduce excessive inflammation and promote inflammatory resolution. Additional metabolic and secretory regulators (*Mogat2*, *Alpl*, *Upp1*, *Hp*, *Oas1k*) point toward metabolic reprogramming to support proliferative and reparative processes in conjunction with genes linked to tissue remodeling and extracellular matrix turnover (*Mmp9/13/14*, *Scube1*, *Tnfaip6*). Top downregulated genes further indicate immune tolerance and metabolic reprioritization, with suppression of type I interferon and antiviral signaling (*Oas2*, *Ifi27*) and reduced lymphocyte activation and antigen presentation (*Il12b*, *Il15*, *Ccl24*, *Ccr3*, *Xcr1*).

To achieve a more integrated understanding of the underlying changes, we performed gene set enrichment analysis (GSEA)[1,2]. Analysis using the Molecular Signatures Database (MSigDB) Hallmark gene sets[3,4] showed strong enrichment of cell cycle and metabolic pathways, including G2M Checkpoint, E2F Targets, and MYC Targets, indicating strong proliferative signaling consistent with heightened myeloid expansion and increased immune cell turnover. Metabolic pathways such as Oxidative Phosphorylation, mTORC1 Signaling, and Unfolded Protein Response were also positively enriched, reflecting increased energy demand and biosynthetic activity during sustained immune activation. Consistent with these findings, enriched pathways from Gene Ontology (GO) Biological Process (BP) gene sets[5,6] similarly reflected increased cellular proliferation, mitochondrial respiration, protein synthesis, and endoplasmic reticulum stress.

Interestingly, several adaptive immune pathways were significantly downregulated, while humoral immune response pathways were modestly upregulated. Within the Hallmark collection[3,4], both Interferon Alpha/Gamma Response were negatively enriched following LPS treatment. GO pathways[5,6] associated with interferon production, T-cell activation, adaptive immune response, and cytokine-mediated signaling were also downregulated, suggesting a shift away from antigen-specific lymphocyte activity toward a predominantly innate, myeloid-driven inflammatory state.

Finally, the MSigDB Immunologic Signature gene sets[3,7] were analyzed to obtain cell-type-specific responses to cytokines. This analysis revealed upregulation of gene sets typically associated with exposure to pro-inflammatory cytokines including IL-1α/β, TNF-α, GM-CSF, IL-36a, IL-7, and IL-18, consistent with broad innate immune activation. Gene sets reflecting myeloid cell responses to anti-inflammatory cytokines such as IL-10 and IL-13 were also positively enriched, indicating concurrent engagement of counter-regulatory circuits. In contrast, multiple gene sets representing type I interferon-stimulated programs were negatively enriched, reinforcing the observation that prolonged LPS exposure in the spleen is characterized by strong innate inflammatory and proliferative activation accompanied by partial suppression of antiviral and adaptive lymphocyte-driven transcriptional programs.

Splenocytes – Effect of VNS

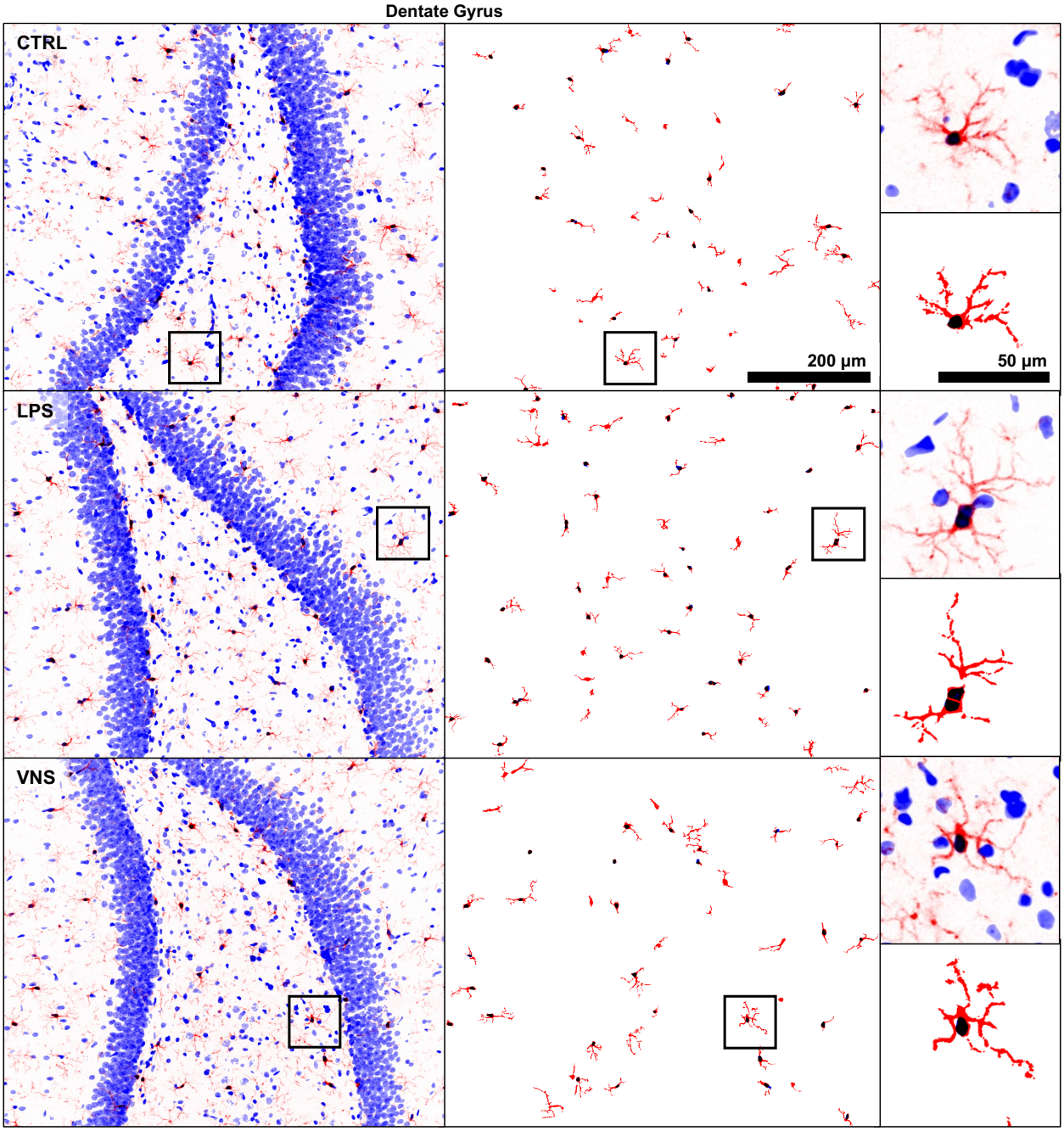
No individual DEGs met significance thresholds between LPS+shamVNS and LPS+VNS samples. However, GSEA[1,2] revealed a reversal of several pathways that were previously upregulated by LPS treatment. Within the Hallmark collection[3,4], key proliferative programs such as E2F/MYC Targets were significantly negatively enriched in the VNS group compared to LPS alone, indicating that VNS suppressed some elements of the hyperproliferative, metabolically activated state induced by sustained inflammation. The Interferon Alpha Response gene set was also further negatively enriched, suggesting that VNS continued to dampen antiviral signaling. GO BP enrichment[5,6] demonstrated coordinated suppression of biosynthetic, translational, and metabolic pathways, including DNA Replication, rRNA Metabolic Process, and Ribosome Biogenesis, supporting a shift toward reduced cellular growth and protein synthesis. The only pathway positively enriched by VNS was Vesicle Targeting to/from/within Golgi, potentially reflecting altered secretory trafficking rather than increased inflammatory signaling.

Analysis of MSigDB Immunologic Signature gene sets[3,7] demonstrated selective suppression of lymphocyte cytokine-response programs that has been upregulated following LPS exposure.

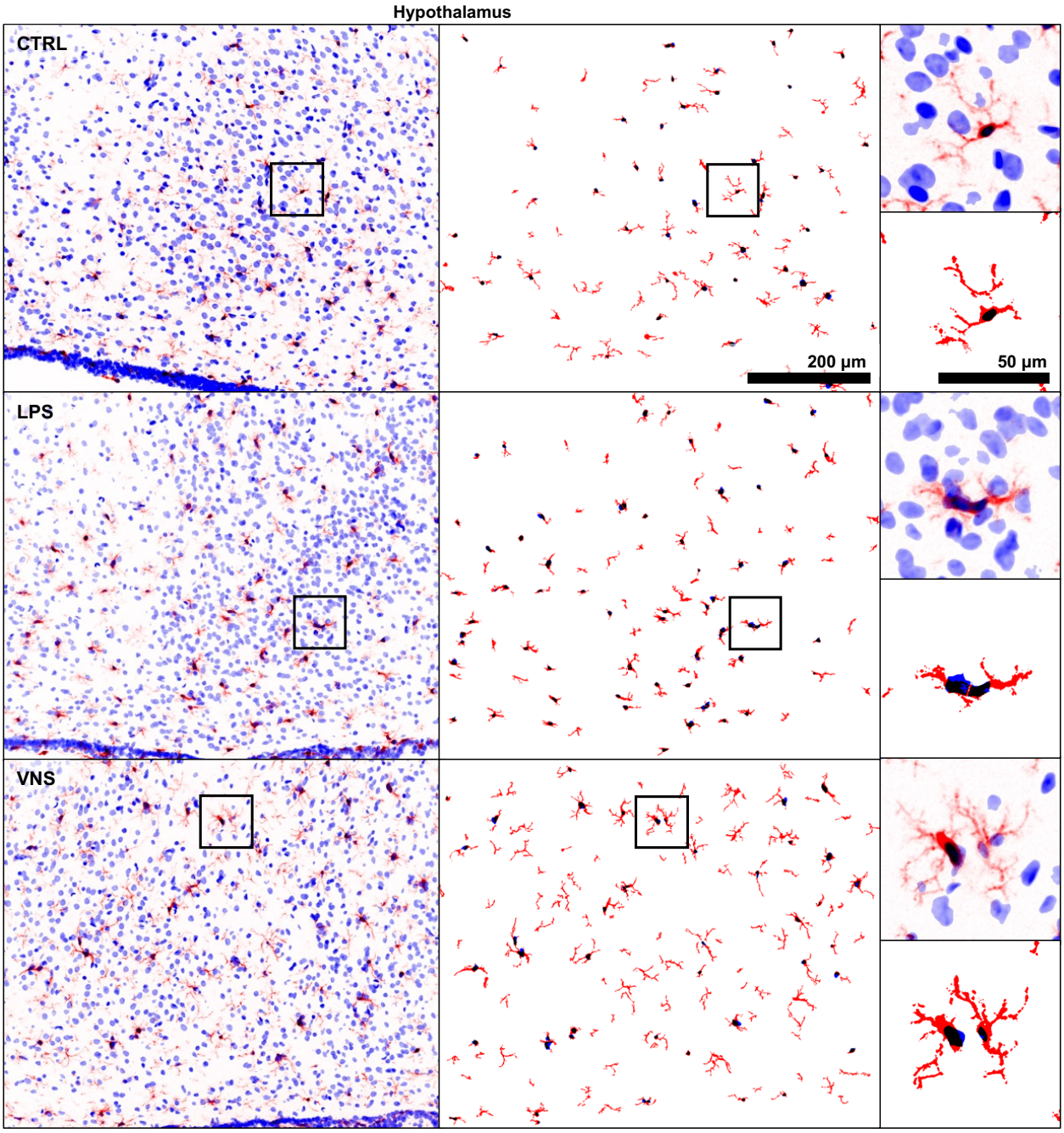
Specifically, multiple gene sets representing transcriptional responses of B and T cells to cytokines such as IL-4, IL-7, IL-15, IL-18, IL-36α were negatively enriched in the VNS condition. These gene sets typically include regulators of lymphocyte proliferation, survival signaling, and cytokine receptor-mediated STAT activation. Their coordinated downregulation indicates that VNS reduced adaptive immune cell sensitivity to cytokine stimulation rather than broadly silencing innate inflammatory pathways. Importantly, this suppression was moderate in magnitude and not universal across all immune signatures, suggesting a fine-tuning effect rather than global immunosuppression.

Gene set enrichment analysis[1,2] of splenocytes therefore indicates that VNS primarily reverses proliferative and metabolic activation induced by LPS treatment and secondarily dampens cytokine-responsive transcriptional programs in adaptive immune cells. Collectively, these results suggest a transcriptional shift toward a more metabolically restrained and energy conserving state, supporting the concept that the longer-term therapeutic action of VNS may lie not in blocking cytokine release outright, but in restoring homeostatic balance within immune signaling networks.

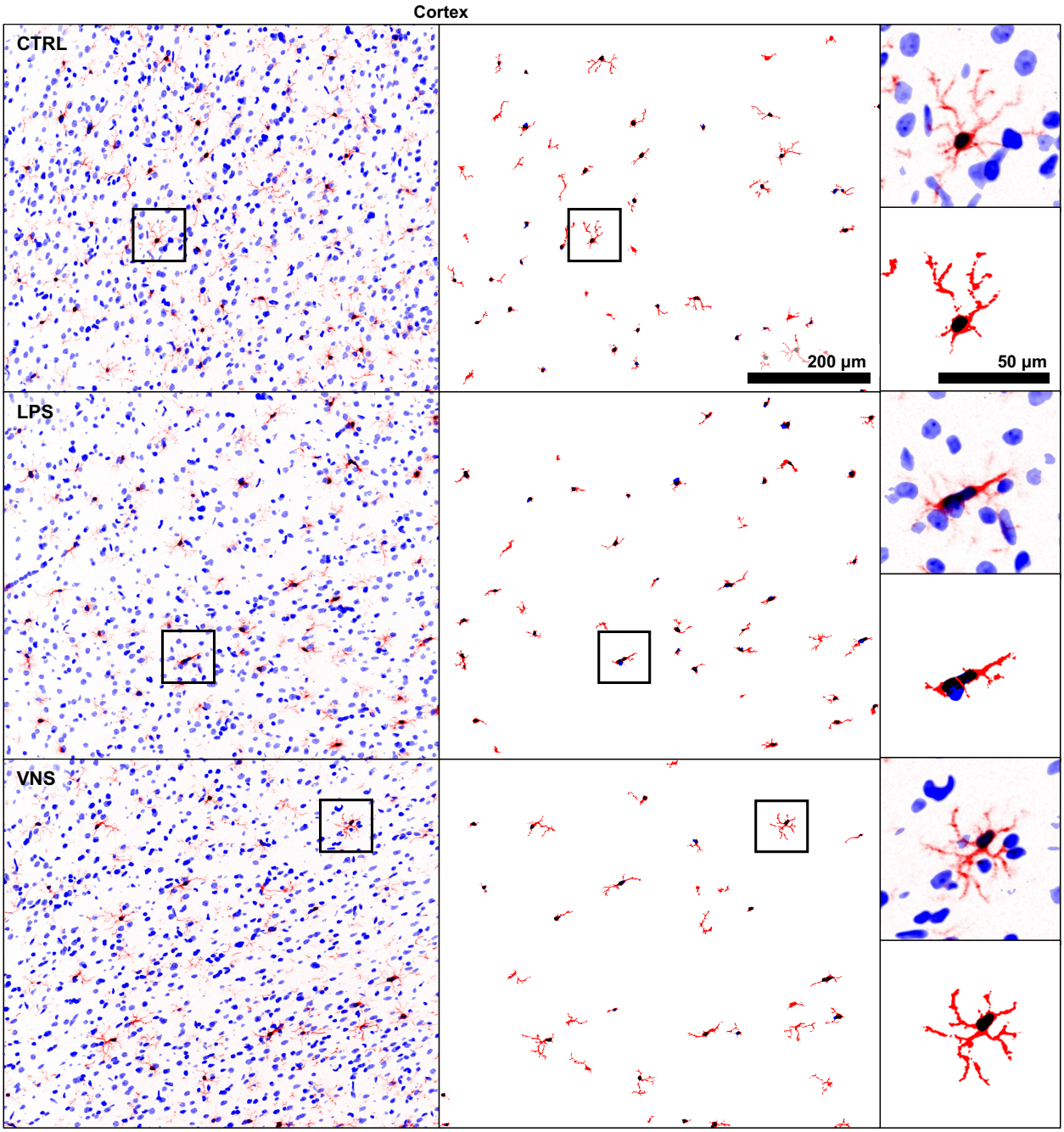
Representative Immunofluorescence Images in Additional Brain Regions



Supplementary Figure 2 | Microglial morphology and segmentation in the dentate gyrus. Representative confocal images of IBA1^{+} microglia (red) and DAPI^{+} nuclei (blue) in female rats. Enhanced-contrast images highlighting microglial processes are followed by corresponding segmented images used for quantitative analysis. Insets show magnified regions of interest.



Supplementary Figure 3 | Microglial morphology and segmentation in the hypothalamus. Representative confocal images of IBA1^{+} microglia (red) and DAPI^{+} nuclei (blue) in female rats. Enhanced-contrast images highlighting microglial processes are followed by corresponding segmented images used for quantitative analysis. Insets show magnified regions of interest.



Supplementary Figure 4 | Microglial morphology and segmentation in the motor cortex. Representative confocal images of IBA1$^+$ microglia (red) and DAPI$^+$ nuclei (blue) in female rats. Enhanced-contrast images highlighting microglial processes are followed by corresponding segmented images used for quantitative analysis. Insets show magnified regions of interest.

Supplementary Analysis 2

Cortex/Hippocampus – Effect of LPS

RNA sequencing showed that LPS significantly upregulated ($p_{adj} < 0.05$, $\log_2$FoldChange > 1.5) 57 genes in the cortex and 53 genes in the hippocampus. Thirty-two of these genes overlapped between the two regions and were primarily related to innate immune activation and inflammation (*C3, Il1b, Cxcl10, Cxcl13, Rac2, Pla2g2a*) and immune cell migration and adhesion (*Adgre1*, *Itgal*, *Selp*, *Sele*, *Serpine1*, *Lrg1*). Also included were negative regulators of immune signaling such as *Lrrc25*, *Cd72*, and *Bcl3*, suggesting engagement of compensatory anti-inflammatory controls alongside pro-inflammatory transcription.

Gene set enrichment analysis[1,2] revealed strong upregulation of immune-related and inflammatory pathways in both regions. Looking at overlapping responses, in the Hallmark gene sets[3,4], Allograft Rejection, Inflammatory Response, IL6/JAK/STAT3 Signaling, Complement, and Interferon Gamma Response were among the most significantly enriched. Consistent with these findings, GO BP[5,6] enrichment highlighted extensive upregulation of leukocyte activation, cytokine signaling, phagocytosis, and complement cascades. These results point to strong neuroimmune engagement and microglial activation following sustained LPS treatment. Notably, pathways associated with metabolism and biosynthetic activity were downregulated. Hallmark[3,4] and GO BP[5,6] analyses showed significant suppression of oxidative and energetic processes, including Oxidative Phosphorylation, Ribosome Biogenesis and ATP Synthesis Coupled Electron Transport, suggesting an inflammation-associated metabolic shift away from oxidative energy production.

Importantly, several pathways indicative of resolutory or protective mechanisms were also upregulated, including gene sets associated with production or signaling of IL-10, IL-4, IL-13, apoptotic cell clearance, and negative regulation of NF-κB activity. Enrichment of other negative regulation pathways for adaptive immune response and cytokine production suggest that although the brain mounts a strong pro-inflammatory response to sustained LPS exposure, anti-inflammatory and tissue-repair programs are simultaneously engaged, reflecting an intrinsic attempt to limit neuronal injury and promote inflammatory resolution.

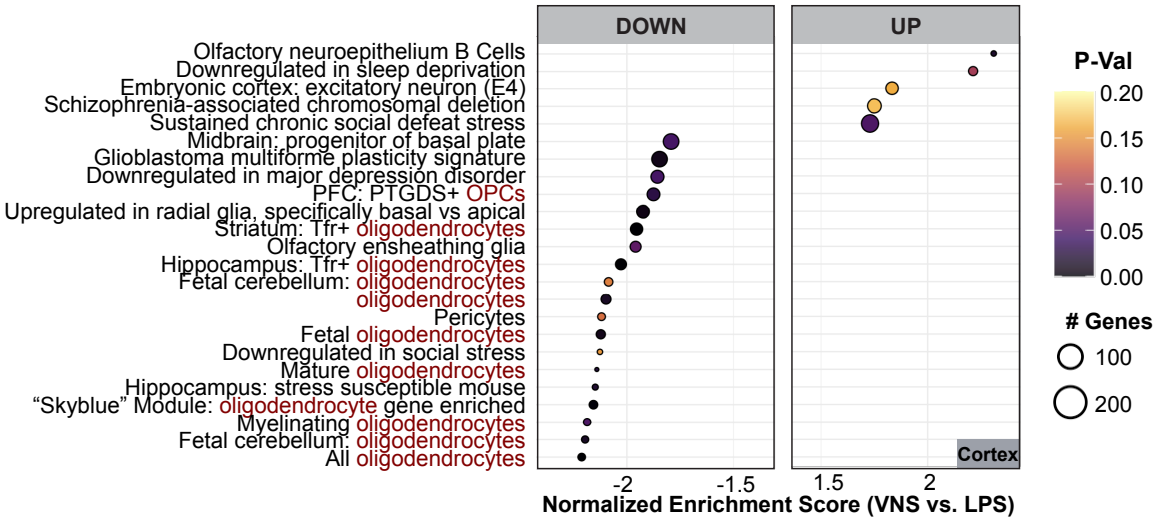
Complementary enrichment using a curated brain-specific gene set collection (Brain.GMT)[8] revealed broadly convergent transcriptional responses in both the cortex and hippocampus, characterized by glial and innate immune activity with concurrent attenuation of neuronal and oligodendrocyte-associated programs. Multiple independent microglial, macrophage, and monocyte reference gene sets were positively enriched in both regions, consistent with robust neuroimmune and innate inflammatory activation. Astrocytic reactivity and endothelial/vascular gene sets were similarly enriched, suggesting coordinated glial and cerebrovascular engagement. In contrast, neuronal, pyramidal glutamatergic, synaptic transmission, and mature oligodendrocyte/myelination gene sets were negatively enriched across both structures, supporting reduced neuronal metabolic and signaling activity alongside diminished myelin-associated transcriptional programs. While the overall directionality of enrichment was shared, the hippocampus demonstrated slightly stronger enrichment of stress- and glucocorticoid-responsive signatures, whereas the cortex showed comparatively greater suppression of synaptic and oligodendrocyte differentiation modules. Collectively, these findings suggest a regionally conserved but subtly graded shift toward immune-dominant transcriptional states with reduced neuronal and metabolic activity following sustained inflammatory exposure.

Direct comparison of cortex and hippocampus Hallmark enrichment profiles indicates that LPS induces a largely conserved neuroimmune transcriptional program across both regions, with only modest regional gradations rather than fundamentally distinct responses. The primary distinctions were quantitative rather than qualitative: the hippocampus tended toward relatively higher enrichment of proliferative and stress-responsive modules, whereas the cortex showed somewhat greater suppression of metabolic and synaptic programs. Together, these results indicate that sustained peripheral inflammatory challenge triggers a broadly uniform neuroimmune transcriptional response across brain regions, with subtle region-specific differences in energetic and structural remodeling.

Cortex/Hippocampus – Effect of VNS

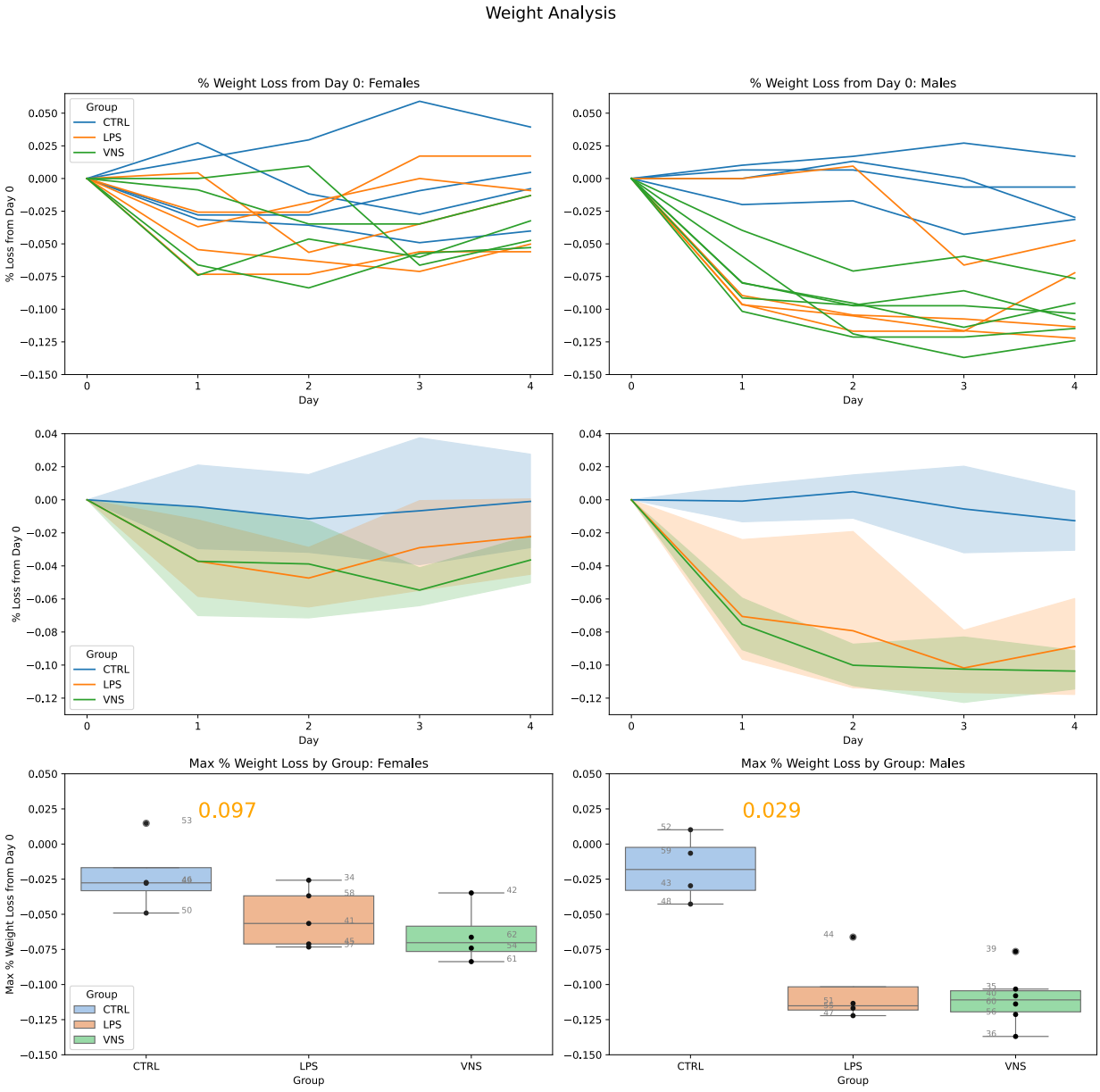
Treatment with VNS did not significantly alter individual gene expression in the context of sustained inflammation, as indicated by the absence of DEGs and lack of significantly enriched pathways in the Hallmark[3,4], GO[5,6], or Immunologic Signatures[3,7] collections. However, we also investigated a curated database for brain-related functional gene sets (Brain.GMT)[8] which revealed selective effects in the cortex but not hippocampus (Supplementary Figure 5).

In contrast to the broad inflammatory activation observed following LPS exposure, VNS did not significantly alter canonical immune or cytokine-associated gene sets. Instead, the cortex demonstrated coordinated negative enrichment of multiple independent oligodendrocyte lineage, myelination, and glial developmental gene sets alongside significant negative enrichment of transcriptional signatures associated with stress susceptibility and depressive phenotypes. These findings suggest that VNS preferentially modulates cortical glial and stress-responsive transcriptional networks rather than directly reversing inflammatory immune activation. No significant Brain.GMT enrichments were detected in the hippocampus, supporting relative regional stability and supporting a cortex-dominant neuromodulatory effect.



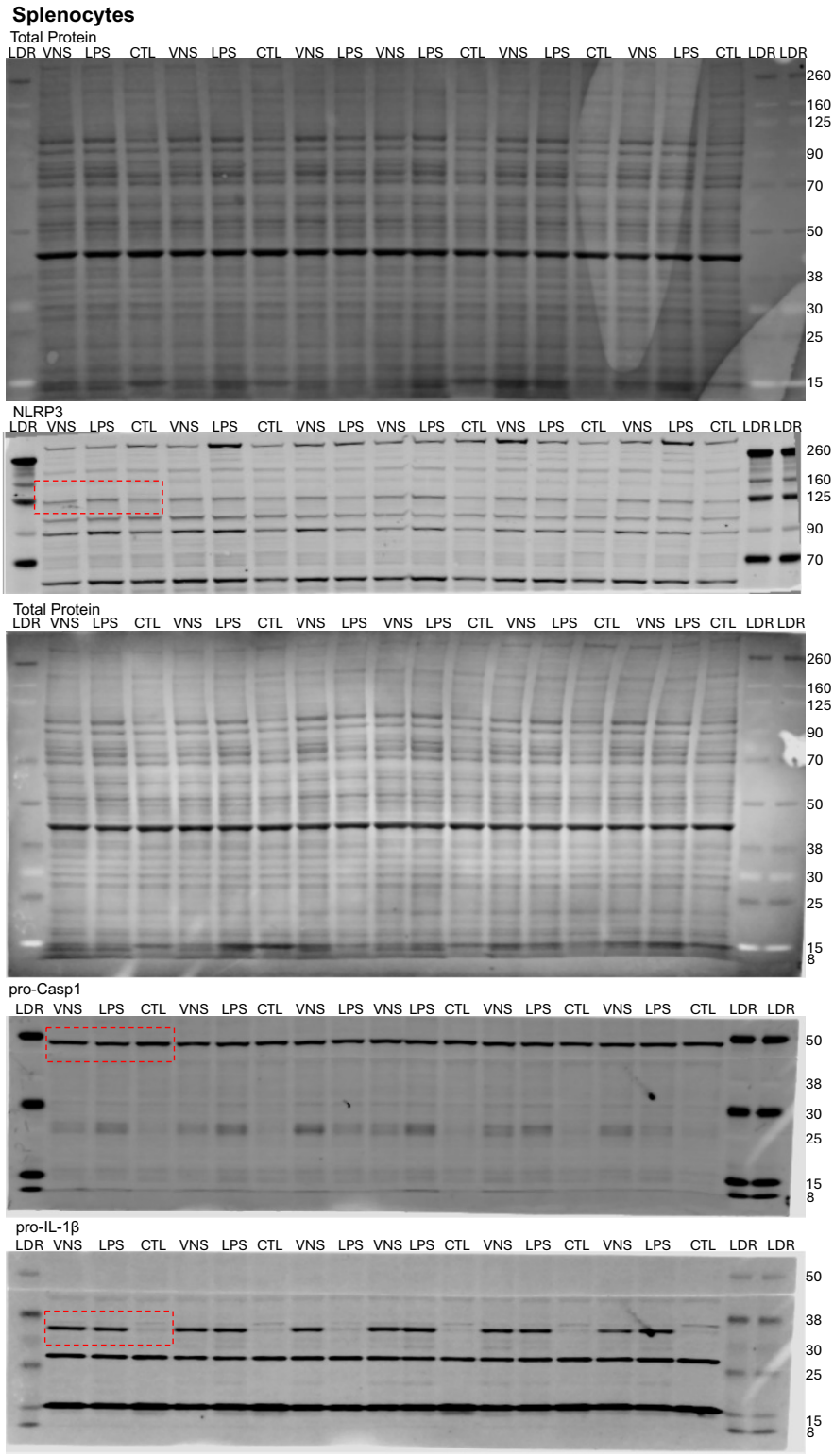
Supplementary Figure 5 | Brain.GMT gene set enrichment in cortex. Brain.GMT[8] gene sets significantly enriched ($p_{adj} < 0.05$) in the cortex of the VNS group compared with the LPS group, demonstrating selective downregulation of oligodendrocyte-associated and stress-susceptibility transcriptional programs.

Weight Loss During LPS Treatment

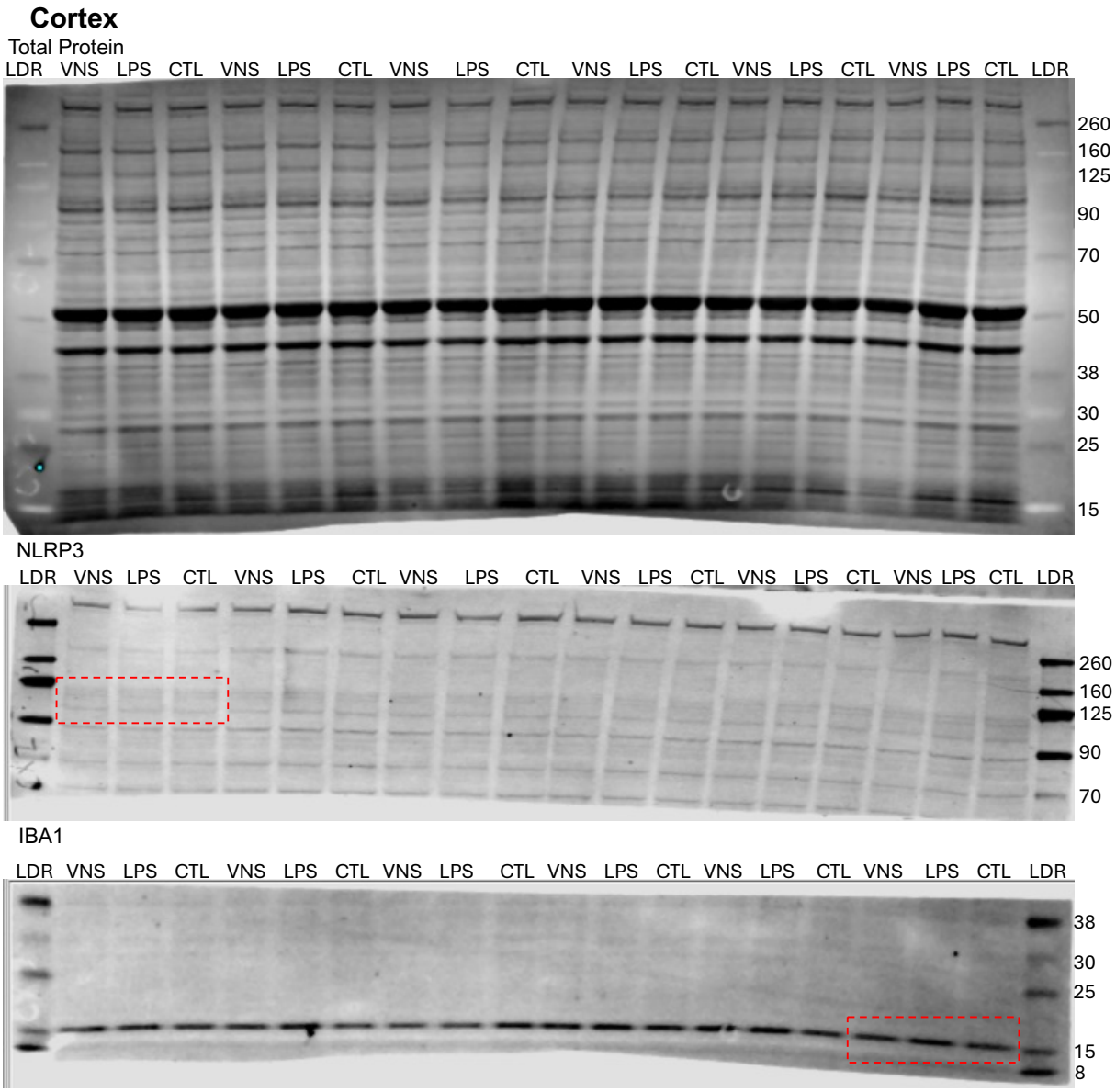


Supplementary Figure 6 | Weight loss during LPS treatment. Percent body weight change for each animal across the LPS treatment period and maximum percent weight loss observed within the treatment timeframe.

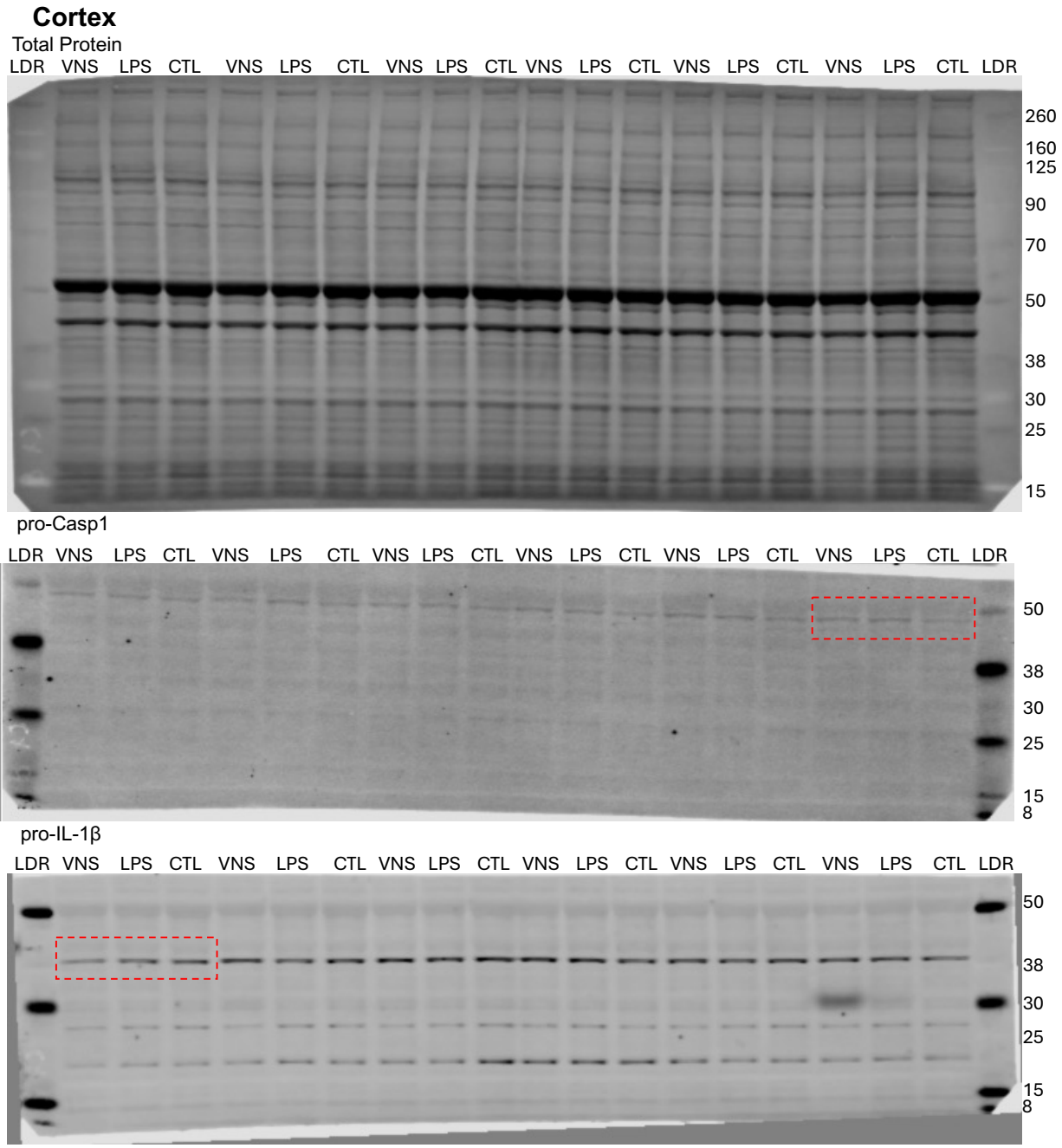
Full Western Blots with Total Protein Normalization



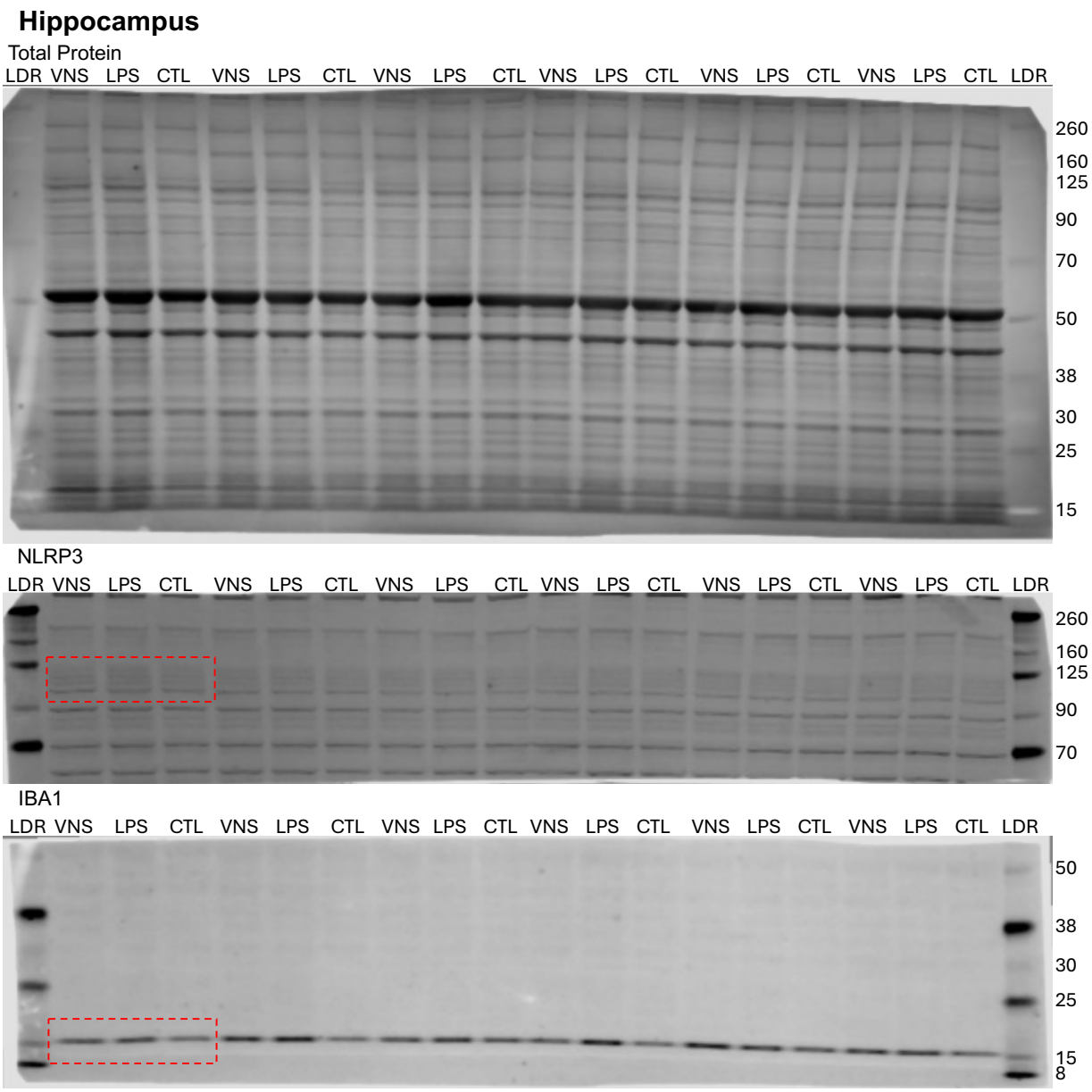
Supplementary Figure 7 | Full western blots of splenocyte lysates. Membranes show total protein normalization and full blots for NLRP3, pro-Caspase-1, and pro-IL-1β. Red boxes indicate the representative regions displayed in the main figures.



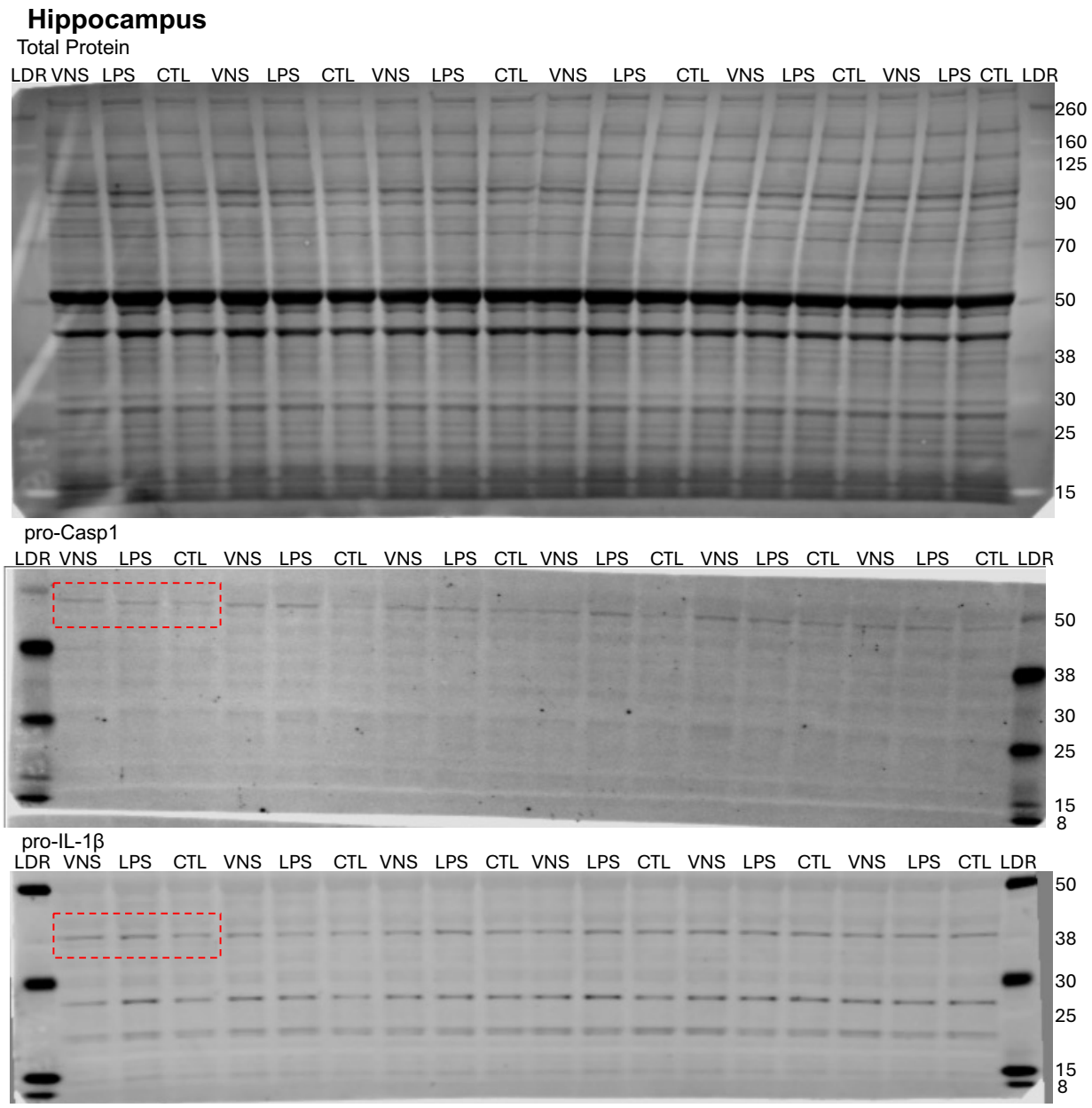
Supplementary Figure 8 | Full western blots of cortical lysates (NLRP3 and IBA1). Membranes show total protein normalization and complete blots. Red boxes indicate the representative regions displayed in the main figures.



Supplementary Figure 9 | Full western blots of cortical lysates (pro-Caspase-1 and pro-IL-1β). Membranes show total protein normalization and complete blots. Red boxes indicate the representative regions displayed in the main figures.



Supplementary Figure 10 | Full western blots of hippocampal lysates (NLRP3 and IBA1). Membranes show total protein normalization and complete blots. Red boxes indicate the representative regions displayed in the main figures.



Supplementary Figure 11 | Full western blots of hippocampal lysates (pro-Caspase-1 and pro-IL-1β). Membranes show total protein normalization and complete blots. Red boxes indicate the representative regions displayed in the main figures.

Supplementary References

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