

## ORIGINAL ARTICLE

## Lipocalin-2 protects the brain during inflammatory conditions

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Sepsis is a prevalent health issue that can lead to central nervous system (CNS) inflammation with long-term behavioral and cognitive alterations. Using unbiased proteomic profiling of over 100 different cytokines, we found that Lipocalin-2 (LCN2) was the most substantially elevated protein in the CNS after peripheral administration of lipopolysaccharide (LPS). To determine whether the high level of LCN2 in the CNS is protective or deleterious, we challenged *Lcn2*<sup>-/-</sup> mice with peripheral LPS and determined effects on behavior and neuroinflammation. At a time corresponding to peak LCN2 induction in wild-type (WT) mice injected with LPS, *Lcn2*<sup>-/-</sup> mice challenged with LPS had exacerbated levels of pro-inflammatory cytokines and exhibited significantly worsened behavioral phenotypes. To determine the extent of global inflammatory changes dependent upon LCN2, we performed an RNAseq transcriptomic analysis. Compared with WT mice injected with LPS, *Lcn2*<sup>-/-</sup> mice injected with LPS had unique transcriptional profiles and significantly elevated levels of multiple pro-inflammatory molecules. Several LCN2-dependent pathways were revealed with this analysis including, cytokine and chemokine signaling, nucleotide-binding oligomerization domain-like receptor signaling and Janus kinase-signal transducer and activator of transcription signaling. These findings demonstrate that LCN2 serves as a potent protective factor in the CNS in response to systemic inflammation and may be a potential candidate for limiting sepsis-related CNS sequelae.

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## INTRODUCTION

Sepsis is a life-threatening systemic host inflammatory response to infection.<sup>1</sup> The incidence of sepsis is on the rise and, depending on clinical criteria, can reach estimates of over 3 million new cases per year with ~20–50% mortality in the United States alone.<sup>2</sup> Sepsis is a continuum ranging from the initial systemic inflammatory response syndrome (SIRS) that can progress to sepsis, severe sepsis, to septic shock with or without multiple organ dysfunction.<sup>3</sup> A generalized increase in several pro-inflammatory cytokines is observed during SIRS progression to septic shock. Sepsis is associated with innate immune activation and inflammatory cytokine secretion that can damage organs when expressed at high enough levels. An emerging idea in sepsis is that while activation of innate immunity is necessary to combat the pathogen, over-activation is deleterious.

Although peripheral infections typically cause sepsis,<sup>4,5</sup> alterations in central nervous system (CNS) function are evident and can result in sepsis-associated delirium or long-term dementia.<sup>6,7</sup> For example, a landmark prospective study of 1194 patients found that elderly severe sepsis survivors had at >3-fold risk for subsequent cognitive impairment.<sup>8</sup> Bacterial infection commonly causes sepsis<sup>4</sup> and is modeled in animals using cecal ligation puncture or lipopolysaccharide (LPS) or bacterial injection *in vivo*.<sup>9</sup> Animal sepsis models also have CNS involvement as evidenced by increased tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, monocyte chemoattractant protein-1, and CXCL10 (ref. 10) in the brain, increased microgliosis, and blood–brain barrier disruptions, partially mediated by pericyte detachment.<sup>11,12</sup> Behavioral and cognitive alterations have also been noted following peripheral LPS challenge in rodents.<sup>13,14</sup> Therefore, understanding molecules and cellular mediators that modulate the inflammatory

response during sepsis is critical for preventing not only mortality but also sepsis-associated CNS sequelae.

## MATERIALS AND METHODS

## Animals

C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) and *Lcn2*<sup>-/-</sup> mice on a pure C57BL/6J background<sup>15</sup> were housed under standard laboratory conditions in ventilated cages on 12-h light:dark cycles in a specific pathogen-free environment. Three- to 4-month-old males and females were used. No differences in the overall effects were noted between sexes and therefore data were pooled together. For LPS studies, animals were injected with 2  $\mu\text{g g}^{-1}$  LPS (0111:B4; Sigma, St Louis, MO, USA) intraperitoneally (i.p.). All animals were included in the study and mice were randomly assigned to be injected with saline or LPS within each cage. The numbers per group differed based on the type of assay. We utilized  $N=3-4$  for RNAseq,  $N=3-12$  depending on the type of biochemical analyses, and  $N=12$  per group for behavioral studies as indicated in each of the figure legends. For behavioral studies, the observer was blinded and additional bias was prevented by use of automated tracking with the AnyMaze software (Stoelting, Wood Dale, IL, USA; no scoring or subjective measures were used). The investigator was blinded until all behavioral analysis was completed. Animal protocols were reviewed and approved by Mayo Clinic Institutional Animal Care and Use Committee.

## Statistics

Sample sizes were based on published literature and in-house data on variance (behavioral data, cytokine responses, RNAseq). All data were normally distributed and did not significantly differ between groups. Data were analyzed using Prism statistical software (La Jolla, CA, USA) with statistical testing methods indicated in the figure legends. All data with  $P < 0.05$  were considered significant.

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**RESULTS**

Peripheral LPS challenge induces LCN2 expression in the CNS

To identify key mediators of CNS inflammation in the context of sepsis/SIRS-like conditions without confounds related to pathogen clearance mechanisms, we injected peripheral LPS and analyzed brain cytokine expression using proteomic profiling arrays to measure 111 different cytokines. Phosphate buffered saline perfused brains were harvested from wild-type (WT) mice injected i.p. with either 2  $\mu\text{g g}^{-1}$  LPS or saline 24 h post challenge. Hemi-brain fractions pooled from  $N=2$  mice per group were run on proteome profiler cytokine array membranes. Of the detectable proteins in the brain, Lipocalin-2 (LCN2) was by far the top hit of candidate LPS-induced proteins, with over a 140-fold induction (Figure 1a, Supplementary Figure 1A, and Supplementary Materials and methods).

LCNs are a diverse family of secreted, structurally related proteins that typically bind small, hydrophobic molecules and have a role in reducing bacterial outgrowth and mortality.<sup>15,16</sup> To determine the kinetics of LCN2 induction after peripheral LPS challenge, WT or *Lcn2*<sup>-/-</sup> mice were injected i.p. with 2  $\mu\text{g g}^{-1}$  LPS. LCN2 protein levels were detected by both western blotting (Figure 1b) and enzyme-linked immunosorbent assay (Figure 1c) from phosphate buffered saline perfused brains. As expected, LCN2 was not detected in *Lcn2*<sup>-/-</sup> mice; however, in WT mice, LCN2 was significantly elevated twofold at 2 h (mean  $\pm$  s.e.m., 1270  $\pm$  161 pg mg<sup>-1</sup>) and ninefold at 4 h (5537  $\pm$  328 pg mg<sup>-1</sup>) compared with 0-h baseline (615  $\pm$  169 pg mg<sup>-1</sup>). Notably, LCN2 levels were markedly elevated at the later time points relative to baseline with a 27-fold increase at 8 h (17 057  $\pm$  1077 pg mg<sup>-1</sup>) and a 67-fold increase at 24 h (41 398  $\pm$  4469 pg mg<sup>-1</sup>). A similar pattern of induction was also noted in the peripheral serum (Figure 1d). Western blotting of cerebrospinal fluid also

demonstrated the tremendous levels of LCN2 protein induced after peripheral LPS (Supplementary Figure 2).

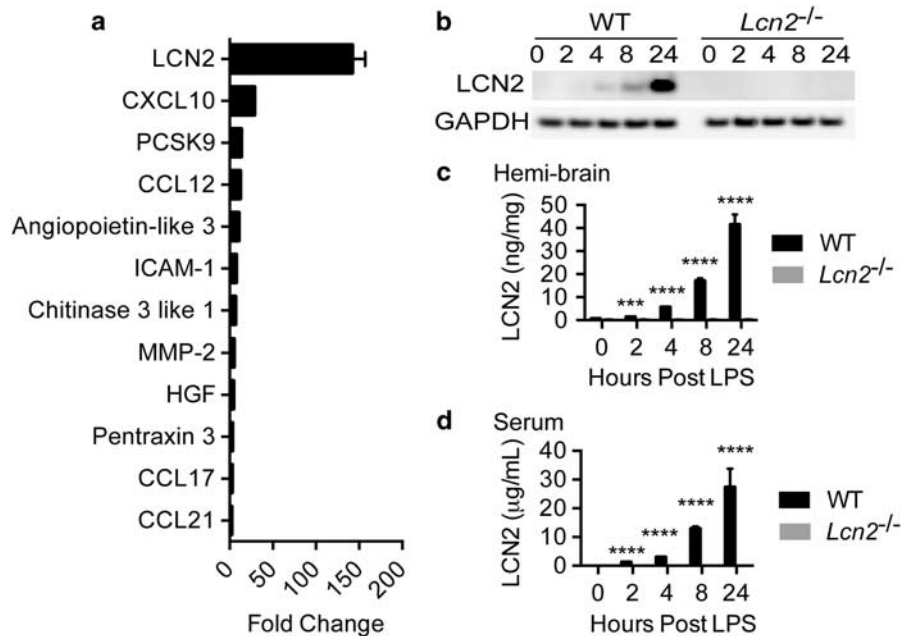
Increased CNS inflammation in the absence of LCN2

In the CNS, LCN2 deficiency has been associated with both increased and decreased inflammation depending on the model system.<sup>17–20</sup> To examine the role of LCN2 in a SIRS/sepsis-like system, we assessed the impact of LCN2 on neuroinflammation following systemic LPS challenge. We found no significant difference in IL-1 $\beta$  levels (Figure 2a) between LPS injected WT and *Lcn2*<sup>-/-</sup> mice at any time point examined. However, at both 8 and 24 h, when LCN2 is robustly expressed, both TNF $\alpha$  (Figure 2b) and IL-6 (Figure 2c) levels were significantly elevated in *Lcn2*<sup>-/-</sup> mice relative to WT controls.

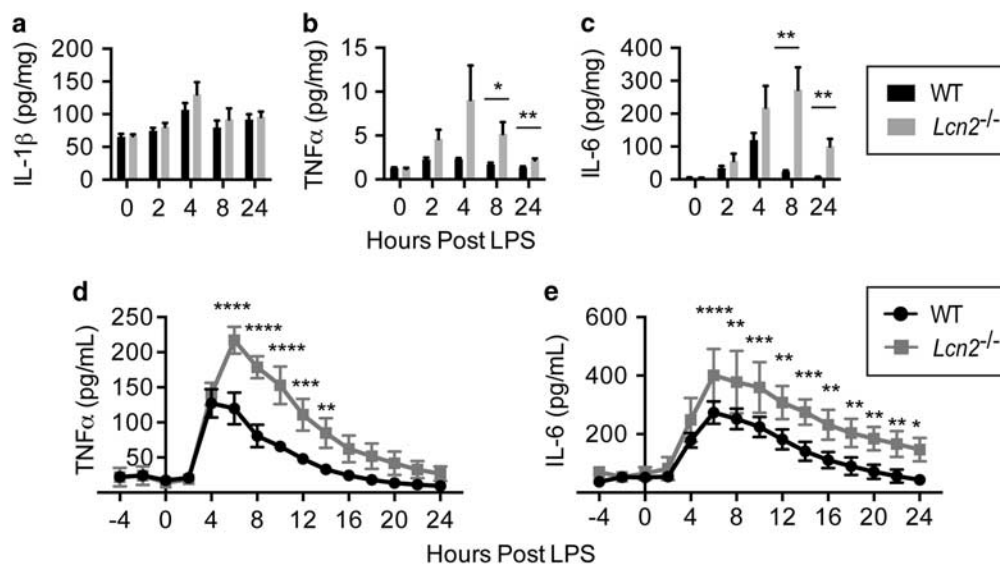
Microdialysis has been utilized to examine induction of proteins circulating in the brain interstitial fluid.<sup>21</sup> As the brain parenchyma is bathed in interstitial fluid, we assessed TNF $\alpha$  and IL-6 induction after systemic LPS challenge using a microdialysis probe inserted into the hippocampal region, an important area required for learning and memory. *Lcn2*<sup>-/-</sup> mice had significantly elevated levels of TNF $\alpha$  and IL-6 in the interstitial fluid starting at 6 h following peripheral LPS injection relative to controls, whereas before that point (that is,  $\leq 4$  h post injection), we saw no alterations in neuroinflammation (Figure 2d and e).

Loss of LCN2 exacerbates behavioral deficits resulting from systemic inflammation

As peripheral LPS and inflammation can impact behavior,<sup>13,14</sup> we investigated whether loss of LCN2 modifies LPS-induced behavioral phenotypes. WT and *Lcn2*<sup>-/-</sup> mice were examined by open field assay for activity and anxiety-like behaviors. Although other



**Figure 1.** Lipocalin-2 (LCN2) is markedly induced in the brain following systemic lipopolysaccharide (LPS) challenge. (a) Central nervous system (CNS) samples from wild-type (WT) mice injected intraperitoneally (i.p.) with saline or 2  $\mu\text{g g}^{-1}$  LPS were analyzed using a cytokine blot array for simultaneous measurement of 111 different cytokines. Shown is the mean  $\pm$  s.d. of the fold change of LPS-challenged WT mice from two separate pools ( $N=2$  mice per pool) for the detectable proteins on the membrane. LCN2 levels in the hemi-brain lysates of WT or *Lcn2*<sup>-/-</sup> mice were examined by (b), western blot of Tris-buffered saline (TBS) fractions for LCN2 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and (c, d) enzyme-linked immunosorbent assay (ELISA) at 0, 2, 4, 8 and 24 h post i.p. injection with 2  $\mu\text{g g}^{-1}$  LPS (mean  $\pm$  s.e.m.) in (c), hemi-brain lysates or (d) serum. LCN2 ELISA levels were significantly elevated in WT mice at 2, 4, 8 and 24 h post injection compared with 0-h baseline (one-way analysis of variance (ANOVA), Dunnett *post-hoc*, \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ). LCN2 was undetectable in *Lcn2*<sup>-/-</sup> mice.  $N=7$  per condition for each time point from  $N=2-3$  experiments.



**Figure 2.** Lipocalin-2 (LCN2) modulates neuroinflammation during systemic inflammation. Protein levels of (a) interleukin-1β (IL-1β), (b) tumor necrosis factor-α (TNFα) and (c) IL-6 were examined by enzyme-linked immunosorbent assay (ELISA) from hemi-brain lysates at 0, 2, 4, 8 and 24 h post intraperitoneal (i.p.) injection with saline or 2 μg g<sup>-1</sup> lipopolysaccharide (LPS; mean ± s.e.m.) in wild-type (WT) and *Lcn2*<sup>-/-</sup> mice. Both (b) TNFα and (c) IL-6 were significantly increased in *Lcn2*<sup>-/-</sup> mice relative to WT mice at 8 and 24 h post injection, but not at earlier time points (*t*-test between genotypes, \**P* ≤ 0.05, \*\**P* ≤ 0.01). *N* = 7–8 animals per time point per condition from *N* = 2 experiments. (d, e) Hippocampal interstitial fluid (ISF) was collected from WT and *Lcn2*<sup>-/-</sup> mice 6 h before and 24 h post i.p. injection with 2 μg g<sup>-1</sup> LPS using *in vivo* microdialysis. Levels of (d) TNFα and (e) IL-6 from ISF fractions pooled in 2-h increments were measured by cytokine bead array (mean ± s.e.m.). *N* = 3 per group. TNFα and IL-6 were significantly elevated in *Lcn2*<sup>-/-</sup> mice compared with WT mice in the ISF after LPS injection (two-way analysis of variance (ANOVA), repeated measures, Sidak *post-hoc t*-test \**P* ≤ 0.05, \*\**P* ≤ 0.01, \*\*\**P* ≤ 0.001, \*\*\*\**P* ≤ 0.0001).

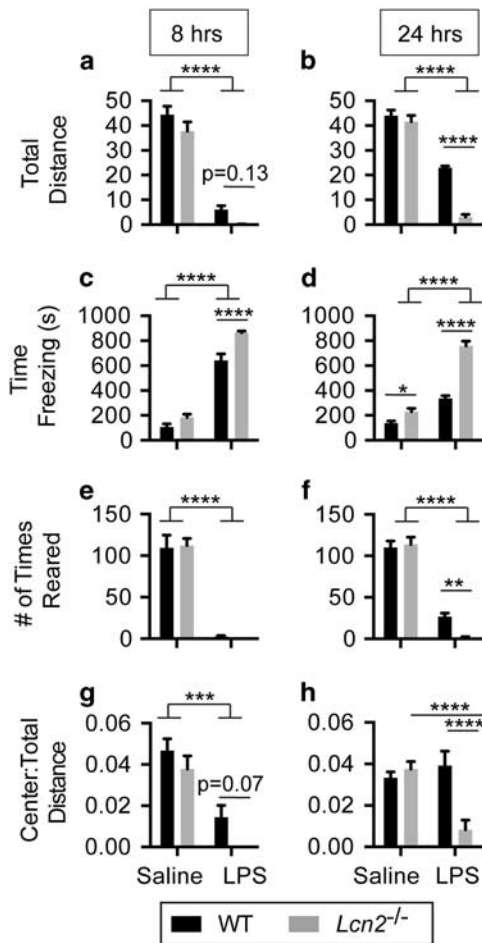
behavioral tests, such as elevated plus maze, are typically used for anxiety read outs, as we are examining behavior at specific times post LPS challenge, we selected open field assay as the readout as it yields information regarding both mobility and anxiety-like measurements. At 8 h post LPS injection, a strong LPS effect was observed in WT and *Lcn2*<sup>-/-</sup> groups (Figures 3a, c, e and g, Supplementary Figure 3A) with significant changes in activity (total distance, # of times reared) and anxiety-like behavior (time freezing, center:total distance) observed in both groups. Despite the robust LPS effect in WT mice, *Lcn2*<sup>-/-</sup> mice had a significant increase in time spent freezing at 8 h compared with WT controls. Moreover, at 24 h post LPS injection, when LCN2 concentrations are increased over 60-fold from basal levels in WT mice (Figures 1b and c), striking behavioral differences between WT and *Lcn2*<sup>-/-</sup> mice were observed (Figures 3b, d, f and h; Supplementary Figure 3B). LPS-challenged *Lcn2*<sup>-/-</sup> mice had significant and profound increases in time spent freezing and decreases in total distance traveled, rearing behavior and center:total distance parameters relative to LPS-challenged WT controls and saline injected controls (Figures 3b, d, f and h).

Loss of LCN2 does not impact gliosis observed after LPS challenge  
 To test whether the behavioral effects observed in *Lcn2*<sup>-/-</sup> mice after LPS injection were attributed to altered gliosis, we examined microglial activation by immunohistology for IBA1, a marker for microglia. IBA1 analysis revealed increased activated morphology observed with LPS, but no effect of *Lcn2* genotype (Figure 4a). In addition, reverse transcriptase-quantitative PCR analysis of mRNA transcript levels of *Aif1* (gene encoding IBA1) at 24 h post injection demonstrated a significant LPS, but not genotype, effect (Figure 4b). Examination of glial fibrillary acidic protein by western blot showed similar expression across all conditions (Figures 4c and d) suggesting similar levels of astrocyte reactivity.

LCN2 significantly modulates CNS responses to peripheral LPS challenge

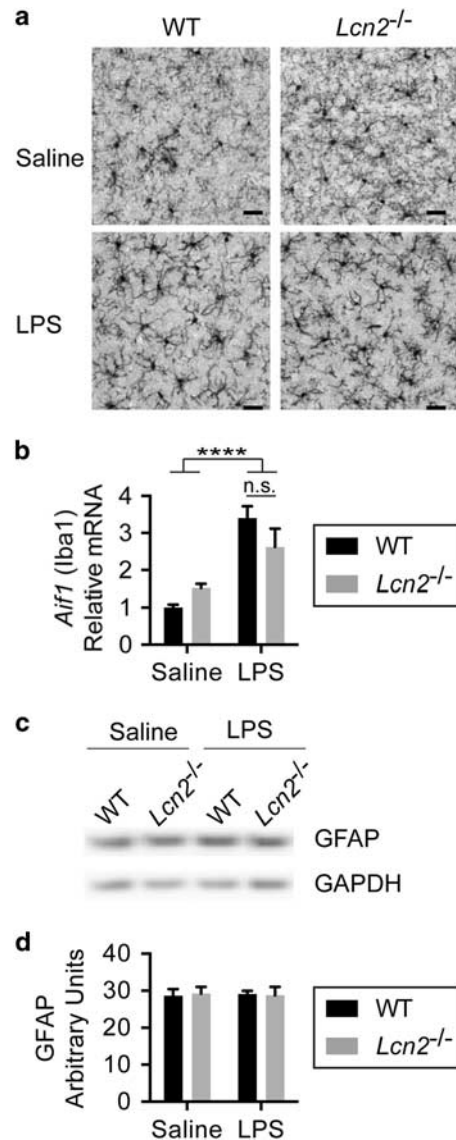
As we did not detect robust LCN2-dependent alterations in gliosis at 24 h (Figure 4), we hypothesized that earlier time points when LCN2 is robustly induced may be critical in determining LCN2-mediated pathways that shape the CNS response to systemic inflammation. To reveal potential LCN2 modulated pathways after peripheral LPS challenge, an RNAseq transcriptomic analysis was conducted from phosphate buffered saline perfused hemi-brain samples of WT and *Lcn2*<sup>-/-</sup> mice at 8 h post LPS or saline i.p. injection (saline-WT, LPS-WT, saline-*Lcn2*<sup>-/-</sup> and LPS-*Lcn2*<sup>-/-</sup>). This represents a time when LCN2 protein is highly induced in the CNS (~27-fold) and robust neuroinflammation is observed in LPS-*Lcn2*<sup>-/-</sup> mice relative to LPS-WT mice (Figures 2b–e). Comparisons between saline-WT and LPS-WT mice revealed that *Lcn2* was the highest conserved transcript induced by LPS in the CNS by > 650-fold (*Saa3* was more highly induced but is not conserved between mouse and human, Supplementary Table I). Principal component analysis to visually display group differences across a large data set showed that transcriptomes from saline-WT and saline-*Lcn2*<sup>-/-</sup> mice are fairly similar (Figure 5a). In fact, after applying a filter of > 2 for fold change and a corrected *P*-value of < 0.01, only one transcript, *Cercam*, was significantly altered by loss of LCN2 (*P* = 2.8E–4, fold change 2.24 for saline-WT vs saline-*Lcn2*<sup>-/-</sup>). In contrast, principal component analysis plots demonstrate two distinct and clearly separated clusters of LPS-WT and LPS-*Lcn2*<sup>-/-</sup> mice that also separate from saline injected groups, accounting for the majority of variability in this analysis (principal component #1 = 37.3%). Unsupervised hierarchical clustering also illustrated the strong, distinct transcriptomic signatures were observed between LPS-WT and LPS-*Lcn2*<sup>-/-</sup> mice (Figure 5b).

Metacore pathway analysis comparing LPS-WT vs LPS-*Lcn2*<sup>-/-</sup> transcriptomes revealed large changes in pathway maps including immune responses to IL-10 signaling, innate immune responses to RNA viral infection, CCL2 signaling, as well as nuclear factor-κB



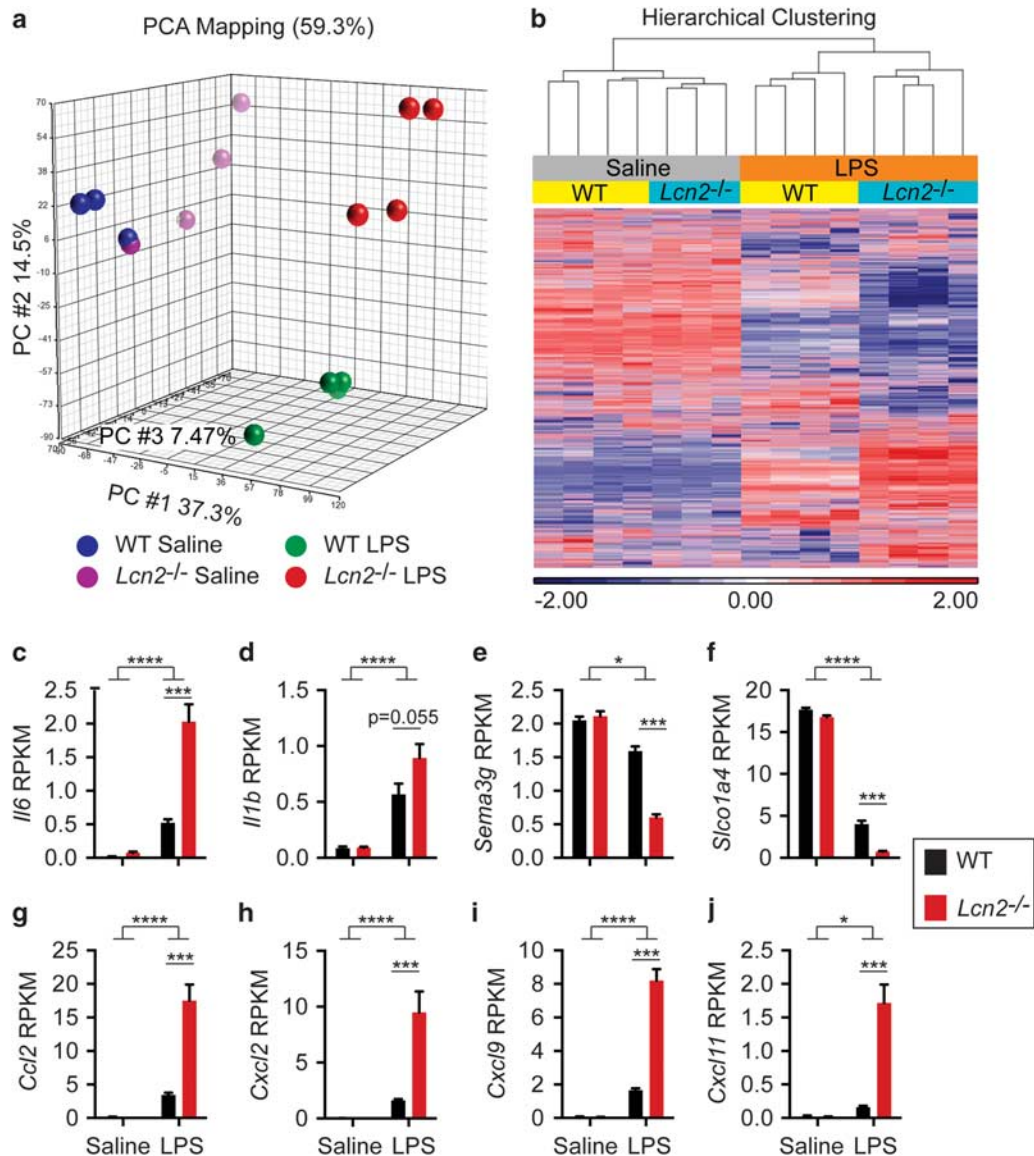
**Figure 3.** Loss of Lipocalin-2 (LCN2) results in exaggerated behavioral alterations during a peripheral inflammatory challenge. Saline or 2  $\mu\text{g g}^{-1}$  lipopolysaccharide (LPS) intraperitoneally (i.p.) injected wild-type (WT) or *Lcn2*<sup>-/-</sup> mice were assessed using open field assay (OFA). Quantification of behavioral tracking measurements acquired over a 15 min period at (a, c, e, g) 8 h or (b, d, f, h) 24 h post injection. Loss of LCN2 significantly altered (a) time freezing at 8 h and (b, d, f, h) all measures at 24 h post injection (two-way analysis of variance (ANOVA), Fisher's LSD *post-hoc* t-test, \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ).  $N = 9$  (8 h) or  $N = 12$  (24 h) per condition from  $N = 2$  experiments.

activation pathways (Supplementary Table II). Gene ontology processes analysis also yielded many pathways involved in immunity including: defense response, response to other organisms, external biotic stimulus, cytokine responses and immune responses (Supplementary Table III). Notably, the third top Metacore process networks altered between LPS-WT and LPS-*Lcn2*<sup>-/-</sup> brain transcriptomes revolved around chemotaxis networks (Supplementary Table IV). Examination of the top 20 transcripts that were robustly and significantly induced using a corrected  $P$ -value cutoff of  $P < 0.01$ , in LPS-*Lcn2*<sup>-/-</sup> relative to LPS-WT mice showed that over 25% of these transcripts were chemokines (Supplementary Table V). We have shown (Figures 2a and c) that pro-inflammatory cytokines IL-1 $\beta$  and IL-6 protein levels are differentially modulated with only IL-6 being significantly altered in *Lcn2*<sup>-/-</sup> brain lysates at 8 h post LPS challenge. In accordance with these results, we observed that *Il1b* transcript was significantly elevated in both LPS-WT and LPS-*Lcn2*<sup>-/-</sup> brain transcriptomes with ~1.7-fold more in *Lcn2*<sup>-/-</sup> brain but was nominally significant (corrected  $P$ -value = 0.055). However,



**Figure 4.** Lipocalin-2 (LCN2) does not markedly alter gliosis following systemic inflammation. Wild-type (WT) and *Lcn2*<sup>-/-</sup> mice were injected with saline or 2  $\mu\text{g g}^{-1}$  lipopolysaccharide (LPS) intraperitoneally (i.p.) and examined at 24 h post injection by (a) IBA1 immunohistochemistry for IBA1<sup>+</sup> microglia (representative images from  $N = 5$  per group, scale bar 25  $\mu\text{m}$ ), (b) reverse transcriptase-quantitative PCR (RT-qPCR) of *Aif1* (Iba1) hemi-brain mRNA levels (mean  $\pm$  s.e.m. from  $N = 11$  mice per group analyzed by two-way analysis of variance (ANOVA), Tukey *post-hoc* t-tests, \*\*\*\* $P \leq 0.0001$  for main effect of LPS), (c, d) western blot analysis for glial fibrillary acidic protein (GFAP) levels in the hemi-brain lysates. (d) Quantitation of GFAP intensity was analyzed by two-way ANOVA, Tukey *post-hoc* t-tests and revealed no significant differences between groups. Shown is the mean  $\pm$  s.e.m. from 9 to 12 mice per group.

*Il6* transcript was significantly higher in LPS-*Lcn2*<sup>-/-</sup> mice compared with LPS-WT mice (Figures 5c and d). Although a few transcripts were downregulated over twofold in LPS-*Lcn2*<sup>-/-</sup> mice relative to LPS-WT mice, such as *Sema3g* and *Slco1a4* (Figures 5e and f and Supplementary Table VI), we found that the majority of the chemokines detected were robustly elevated after LPS challenge in the absence of LCN2. Chemokines such as *Ccl2*, *Cxcl2*, *Cxcl9*, and *Cxcl11* were among the top transcripts that were



**Figure 5.** Lipocalin-2 (LCN2) significantly modulates central nervous system (CNS) transcriptomes following peripheral lipopolysaccharide (LPS) challenge. RNAseq of hemi-brains 8 h after intraperitoneal (i.p.) injection of saline or  $2 \mu\text{g g}^{-1}$  LPS from phosphate buffered saline perfused wild-type (WT) or *Lcn2*<sup>-/-</sup> mice. (a) Principal component analysis (PCA) and (b) hierarchical clustering and heat map analysis of RNAseq data from  $N = 3-4$  animals per group is shown. (c-j) Reads per kilobase of transcript per million mapped reads (RPKM) values from RNAseq of select transcripts are shown with genome-wide corrected *P*-values. Both (c) *Il6* and (d) *Il1b* displayed transcriptome changes that mirrored results obtained by examination of protein expression. Additional transcriptional changes between LPS-WT and LPS-*Lcn2*<sup>-/-</sup> mice that met the cutoff criteria of a fold change > 2-fold and a corrected *P*-value of < 0.01 using analysis of variance (ANOVA) analysis showed transcripts that were (e-f) further downregulated such as (e) *Sema3b*, (f) *Slco1a4*, or upregulated such as (g) *Ccl2*, (h) *Cxcl2*, (i) *Cxcl9*, (j) *Cxcl11* by LPS in the absence of LCN2.  $N = 3-4$  samples per group, (ANOVA), corrected *P*-values, \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ .

exacerbated in the *Lcn2*<sup>-/-</sup> brain response to peripheral LPS challenge by direct comparison of LPS-WT and LPS-*Lcn2*<sup>-/-</sup> transcriptomes and by interaction analysis (Figures 5g-j). In addition, other cytokines, such as *Cxcl10* and *Ccl7*, were robustly induced and were in the top 20 transcripts elevated in LPS-*Lcn2*<sup>-/-</sup> transcriptomes relative to LPS-WT mice (Supplementary Table V). Some transcripts were uniquely upregulated in LPS-*Lcn2*<sup>-/-</sup> brain tissue compared with all other groups, with some transcripts elevated by over fourfold such as *Timd4*, *Bcl2a1d*, *Hamp*, *Lif*, *Psors1c2*, *Wfikkn1* and *Osm* (Supplementary Figure 4). Several transcripts were also uniquely downregulated in LPS-*Lcn2*<sup>-/-</sup> brain tissue compared with all other groups, with some transcripts decreased by > 2-fold such as *Ptgd*, *Fmo2*, *Alx3* and *Scarna2* (Supplementary Figure 4).

## DISCUSSION

Sepsis is a prevalent health issue with potential life-threatening consequences. In addition, a subset of sepsis survivors present with life-altering cognitive and functional impairments.<sup>8,22</sup> Animal models of sepsis have demonstrated that systemic challenge can lead to CNS inflammation and behavioral and cognitive dysfunction.<sup>13,14,23</sup> Therefore, it is critical to understand how CNS abnormalities arising from systemic inflammation are regulated in order to develop future therapeutic strategies. Here, we show that LCN2 is robustly induced in the CNS following peripheral LPS challenge and acts in an anti-inflammatory manner.

LCN2 acts as an acute phase mediator in the CNS. Similar to what has been observed by Ip et al.,<sup>24</sup> we demonstrated that LCN2 is already significantly induced in the CNS at 4 h post LPS

injection; however, the major induction period for LCN2 protein in brain tissue occurs later with increases of over 27-fold and 67-fold by 8 and 24 h post LPS injection, respectively. In the absence of LCN2, we see marked increases in TNF $\alpha$  and IL-6 levels in the CNS following peripheral LPS injection. Although we cannot rule out that some LCN2 protein may passively or actively enter the CNS compartment to modulate some of the effects observed in our studies, it is likely that the bulk of the effects are due to CNS-derived LCN2 given the large increases in both protein and transcript levels. However, future studies with conditional knock-out mice could unequivocally address this issue. In contrast to our findings, Jang *et al.*<sup>25</sup> found that at 4 h after peripheral LPS injection, *Lcn2*<sup>-/-</sup> mice had decreased brain IL-12, TNF $\alpha$  and IL-23 mRNA transcripts, suggesting a pro-inflammatory role for LCN2 in the CNS, possibly due to examination of transcript levels compared with our protein analysis. Notably, we observed that differences in neuroinflammation in both hemi-brain lysate and interstitial fluid were coordinated with when LCN2 is robustly induced (that is, 8 or 24 h post LPS injection) and not at earlier time points (that is, 2 or 4 h) when relatively little LCN2 protein was being produced (Figure 1). Similar to our CNS findings of an anti-inflammatory role for LCN2, examination of the periphery has shown elevations in TNF $\alpha$  and/or IL-6 in serum or peripherally derived cells from *Lcn2*<sup>-/-</sup> mice and increased apoptosis in the spleen after *in vivo* or *ex vivo* LPS challenge.<sup>26</sup> LCN2 has also been implicated in peripheral macrophage deactivation by altering IL-10 and IL-6 levels after *S. pneumoniae* infection.<sup>27</sup> Taken together, these data demonstrate that LCN2 acts as an important modulator of both peripheral and CNS responses during systemic LPS-induced inflammation.

Sepsis has been associated with both behavioral and cognitive changes in humans.<sup>28,29</sup> Importantly, sepsis survivors have been shown to have an increased risk for the development of dementia with an odds ratio as high as 3.34.<sup>8,22</sup> Although loss of *Lcn2* has been associated with increased anxiety, depressive-like behavior and altered cognitive function during basal states,<sup>30</sup> we did not observe differences in saline injected controls. However, under inflammatory conditions, LCN2 is important for minimizing both total activity and anxiety-like deficits caused by systemic LPS challenge as a model of SIRS/sepsis-like conditions (Figure 3). This suggests that LCN2 is an important modulator of behavioral outcomes, potentially through modulation of pro-inflammatory cytokine production, which have previously been shown to alter behavioral and/or cognitive function.<sup>31,32</sup>

Functionally, LCN2 has numerous biological roles including iron sequestration and homeostasis,<sup>15,33,34</sup> cell survival,<sup>35–37</sup> apoptosis/cell death<sup>34,38–40</sup> and macrophage deactivation.<sup>27</sup> Using RNAseq, we show that in the absence of immune activation or other insult, baseline differences in CNS transcriptomes are minimal between WT and *Lcn2*<sup>-/-</sup> mice. This suggests that LCN2 likely has a negligible role in CNS homeostasis. However, under conditions of systemic inflammation, CNS transcriptomes are vastly altered by the presence of LCN2. Notably, numerous chemokines, including *Ccl2*, *Cxcl2*, *Cxcl9* and *Cxcl10* (Figure 5 and Supplementary Table V) were highly induced in LPS-treated *Lcn2*<sup>-/-</sup> relative to LPS treated WT mice. This suggests that LCN2 also functions to mitigate CNS chemokine responses following peripheral inflammation. In contrast to our results, *in vitro* studies have suggested that LCN2 exposure can induce *Cxcl10* from primary microglia, astrocytes, neurons and an endothelial cell line. In the same study, the *Cxcl10* transcript response was blunted using a cortical stab wound model in *Lcn2*<sup>-/-</sup> mice. Although the differences in our results may be ascribed to differences that emerge from *in vitro* vs *in vivo* settings and different model systems, it is possible that effects of LCN2 are truly context dependent.

Although we have shown LCN2 to act in an anti-inflammatory manner on both a transcript and protein level in the context of neuroinflammation following LPS challenge, the role of LCN2 in

models of CNS inflammation remains controversial. LCN2 has been shown to have opposing effects in experimental autoimmune encephalomyelitis models of neuroinflammation,<sup>17,18</sup> acts in a pro-inflammatory manner during ischemia<sup>41</sup> and has little impact in a model of West Nile viral encephalitis despite robust induction.<sup>19</sup> These data suggest that the role of LCN2 in neuroinflammation may be sensitive to the inflammatory context, timing and levels of expression. Other cellular populations that can be associated with inflammation, including endothelial cells and choroid plexus, express LCN2.<sup>42,43</sup> Furthermore, detection of 24p3R (*Slc22a17*), the LCN2 receptor, suggests that the choroid plexus may also directly respond to LCN2 and peripheral LPS challenge has been shown to modulate choroid plexus chemokine production, including CCL2, one of the candidate genes determined in our RNAseq that is impacted by LCN2.<sup>24,44,45</sup> Further work is necessary to determine if the mechanisms for LCN2 action in this context are driven by modulation of vascular or choroid plexus function. Regardless, our results indicate that high levels of LCN2 alone are not sufficient to harm the CNS. As conflicting roles in inflammation have been ascribed to LCN2 in different systems, great caution should be used when developing therapeutics centered on LCN2 modulation. Utilization of LCN2 therapy may be hindered by the surrounding disease context, which may impact whether LCN2 acts in a pro- or anti-inflammatory manner, and LCN2 therapeutics may prove to be detrimental if conditions are complicated by sepsis-like conditions arising from bacterial infections or other peripheral inflammatory states. Our results suggest that LCN2 may be a cerebrospinal fluid biomarker for sepsis and that therapeutics that target LCN2 may prevent the CNS damage and cognitive impairments that occur after sepsis-like conditions.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## REFERENCES

- Angus DC, van der Poll T. Severe sepsis and septic shock. *N Engl J Med* 2013; **369**: 840–851.
- Gaieski DF, Edwards JM, Kallan MJ, Carr BG. Benchmarking the incidence and mortality of severe sepsis in the United States. *Crit Care Med* 2013; **41**: 1167–1174.
- Riedemann NC, Guo R-F, Ward PA. The enigma of sepsis. *J Clin Invest* 2003; **112**: 460–467.
- Mayr FB, Yende S, Angus DC. Epidemiology of severe sepsis. *Virulence* 2014; **5**: 4–11.
- Mayr FB, Yende S, Linde-Zwirble WT, Peck-Palmer OM, Barnato AE, Weissfeld LA *et al.* Infection rate and acute organ dysfunction risk as explanations for racial differences in severe sepsis. *JAMA* 2010; **303**: 2495–2503.
- Ebersoldt M, Sharshar T, Annane D. Sepsis-associated delirium. *Intensive Care Med* 2007; **33**: 941–950.
- Widmann CN, Heneka MT. Long-term cerebral consequences of sepsis. *Lancet Neurol* 2014; **13**: 630–636.
- Iwashyna TJ, Ely EW, Smith DM, Langa KM. Long-term cognitive impairment and functional disability among survivors of severe sepsis. *JAMA* 2010; **304**: 1787–1794.
- Starr ME, Saito H. Sepsis in old age: review of human and animal studies. *Aging Dis* 2014; **5**: 126–136.

- 10 Erickson MA, Banks WA. Cytokine and chemokine responses in serum and brain after single and repeated injections of lipopolysaccharide: multiplex quantification with path analysis. *Brain Behav Immun* 2011; **25**: 1637–1648.
- 11 Semmler A, Hermann S, Mormann F, Weberpals M, Paxian SA, Okulla T et al. Sepsis causes neuroinflammation and concomitant decrease of cerebral metabolism. *J Neuroinflammation* 2008; **5**: 38.
- 12 Nishioku T, Dohgu S, Takata F, Eto T, Ishikawa N, Kodama KB et al. Detachment of brain pericytes from the basal lamina is involved in disruption of the blood-brain barrier caused by lipopolysaccharide-induced sepsis in mice. *Cell Mol Neurobiol* 2009; **29**: 309–316.
- 13 Weberpals M, Hermes M, Hermann S, Kummer MP, Terwel D, Semmler A et al. NOS2 gene deficiency protects from sepsis-induced long-term cognitive deficits. *J Neurosci Off J Soc Neurosci* 2009; **29**: 14177–14184.
- 14 Bossù P, Cutuli D, Palladino I, Caporali P, Angelucci F, Laricchiuta D et al. A single intraperitoneal injection of endotoxin in rats induces long-lasting modifications in behavior and brain protein levels of TNF- $\alpha$  and IL-18. *J Neuroinflammation* 2012; **9**: 101.
- 15 Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. *Nature* 2004; **432**: 917–921.
- 16 Flower DR. The lipocalin protein family: structure and function. *Biochem J* 1996; **318**(Pt 1): 1–14.
- 17 Nam Y, Kim J-H, Seo M, Kim J-H, Jin M, Jeon S et al. Lipocalin-2 deficiency ameliorates experimental autoimmune encephalomyelitis: the pathogenic role of lipocalin-2 in the central nervous system and peripheral lymphoid tissues. *J Biol Chem* 2014; **289**: 16773–16789.
- 18 Berard JL, Zarruk JG, Arbour N, Prat A, Yong VW, Jacques FH et al. Lipocalin 2 is a novel immune mediator of experimental autoimmune encephalomyelitis pathogenesis and is modulated in multiple sclerosis. *Glia* 2012; **60**: 1145–1159.
- 19 Noçon AL, Ip JPK, Terry R, Lim SL, Getts DR, Müller M et al. The bacteriostatic protein lipocalin 2 is induced in the central nervous system of mice with west Nile virus encephalitis. *J Virol* 2014; **88**: 679–689.
- 20 Rathore KI, Berard JL, Redensek A, Chierzi S, Lopez-Vales R, Santos M et al. Lipocalin 2 plays an immunomodulatory role and has detrimental effects after spinal cord injury. *J Neurosci Off J Soc Neurosci* 2011; **31**: 13412–13419.
- 21 Ulrich JD, Burchett JM, Restivo JL, Schuler DR, Verghese PB, Mahan TE et al. *In vivo* measurement of apolipoprotein E from the brain interstitial fluid using microdialysis. *Mol Neurodegener* 2013; **8**: 13.
- 22 Guerra C, Linde-Zwirble WT, Wunsch H. Risk factors for dementia after critical illness in elderly medicare beneficiaries. *Crit Care Lond Engl* 2012; **16**: R233.
- 23 Granger JJ, Ratti P-L, Datta SC, Raymond RM, Opp MR. Sepsis-induced morbidity in mice: effects on body temperature, body weight, cage activity, social behavior and cytokines in brain. *Psychoneuroendocrinology* 2013; **38**: 1047–1057.
- 24 Ip JPK, Noçon AL, Hofer MJ, Lim SL, Müller M, Campbell IL. Lipocalin 2 in the central nervous system host response to systemic lipopolysaccharide administration. *J Neuroinflammation* 2011; **8**: 124.
- 25 Jang E, Lee S, Kim J-H, Kim J-H, Seo J-W, Lee W-H et al. Secreted protein lipocalin-2 promotes microglial M1 polarization. *FASEB J Off Publ Fed Am Soc Exp Biol* 2013; **27**: 1176–1190.
- 26 Srinivasan G, Aitken JD, Zhang B, Carvalho FA, Chassaing B, Shashidharamurthy R et al. Lipocalin 2 deficiency dysregulates iron homeostasis and exacerbates endotoxin-induced sepsis. *J Immunol, Baltimore MD 1950* 2012; **189**: 1911–1919.
- 27 Warszawska JM, Gawish R, Sharif O, Sigel S, Doninger B, Lakovits K et al. Lipocalin 2 deactivates macrophages and worsens pneumococcal pneumonia outcomes. *J Clin Invest* 2013; **123**: 3363–3372.
- 28 Semmler A, Widmann CN, Okulla T, Urbach H, Kaiser M, Widman G et al. Persistent cognitive impairment, hippocampal atrophy and EEG changes in sepsis survivors. *J Neurol Neurosurg Psychiatry* 2013; **84**: 62–69.
- 29 Rosendahl J, Brunkhorst FM, Jaenichen D, Strauss B. Physical and mental health in patients and spouses after intensive care of severe sepsis: a dyadic perspective on long-term sequelae testing the Actor-Partner Interdependence Model. *Crit Care Med* 2013; **41**: 69–75.
- 30 Ferreira AC, Pinto V, Dá Mesquita S, Novais A, Sousa JC, Correia-Neves M et al. Lipocalin-2 is involved in emotional behaviors and cognitive function. *Front Cell Neurosci* 2013; **7**: 122.
- 31 Chen J, Song Y, Yang J, Zhang Y, Zhao P, Zhu X-J et al. The contribution of TNF- $\alpha$  in the amygdala to anxiety in mice with persistent inflammatory pain. *Neurosci Lett* 2013; **541**: 275–280.
- 32 Terrando N, Monaco C, Ma D, Foxwell BMJ, Feldmann M, Maze M. Tumor necrosis factor- $\alpha$  triggers a cytokine cascade yielding postoperative cognitive decline. *Proc Natl Acad Sci USA* 2010; **107**: 20518–20522.
- 33 Yang J, Goetz D, Li JY, Wang W, Mori K, Setlik D et al. An iron delivery pathway mediated by a lipocalin. *Mol Cell* 2002; **10**: 1045–1056.
- 34 Devireddy LR, Gazin C, Zhu X, Green MR. A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. *Cell* 2005; **123**: 1293–1305.
- 35 Mori K, Lee HT, Rapoport D, Drexler IR, Foster K, Yang J et al. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. *J Clin Invest* 2005; **115**: 610–621.
- 36 Mishra J, Mori K, Ma Q, Kelly C, Yang J, Mitsnefes M et al. Amelioration of ischemic acute renal injury by neutrophil gelatinase-associated lipocalin. *J Am Soc Nephrol* 2004; **15**: 3073–3082.
- 37 Tong Z, Wu X, Ovcharenko D, Zhu J, Chen C-S, Kehrler JP. Neutrophil gelatinase-associated lipocalin as a survival factor. *Biochem J* 2005; **391**(Pt 2): 441–448.
- 38 Naudé PJW, Nyakas C, Eiden LE, Ait-Ali D, van der Heide R, Engelborghs S et al. Lipocalin 2: novel component of proinflammatory signaling in Alzheimer's disease. *FASEB J Off Publ Fed Am Soc Exp Biol* 2012; **26**: 2811–2823.
- 39 Bi F, Huang C, Tong J, Qiu G, Huang B, Wu Q et al. Reactive astrocytes secrete lcn2 to promote neuron death. *Proc Natl Acad Sci USA* 2013; **110**: 4069–4074.
- 40 Lee S, Lee J, Kim S, Park J-Y, Lee W-H, Mori K et al. A dual role of lipocalin 2 in the apoptosis and deramification of activated microglia. *J Immunol Baltimore, MD 1950* 2007; **179**: 3231–3241.
- 41 Wang G, Weng Y-C, Han X, Whaley JD, McCrae KR, Chou W-H. Lipocalin-2 released in response to cerebral ischaemia mediates reperfusion injury in mice. *J Cell Mol Med* 2015; **19**: 1637–1645.
- 42 Egashira Y, Hua Y, Keep RF, Iwama T, Xi G. Lipocalin 2 and blood-brain barrier disruption in white matter after experimental subarachnoid hemorrhage. *Acta Neurochir Suppl* 2016; **121**: 131–134.
- 43 Marques F, Rodrigues A-J, Sousa JC, Coppola G, Geschwind DH, Sousa N et al. Lipocalin 2 is a choroid plexus acute-phase protein. *J Cereb Blood Flow Metab Off J Int Soc Cereb Blood Flow Metab* 2008; **28**: 450–455.
- 44 Marques F, Sousa JC, Coppola G, Falcao AM, Rodrigues AJ, Geschwind DH et al. Kinetic profile of the transcriptome changes induced in the choroid plexus by peripheral inflammation. *J Cereb Blood Flow Metab Off J Int Soc Cereb Blood Flow Metab* 2009; **29**: 921–932.
- 45 Hasegawa-Ishii S, Inaba M, Umegaki H, Unno K, Wakabayashi K, Shimada A. Endotoxemia-induced cytokine-mediated responses of hippocampal astrocytes transmitted by cells of the brain-immune interface. *Sci Rep* 2016; **6**: 25457.



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