

Lipocalin-2 in the Inflammatory Activation of Brain Astrocytes

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ABSTRACT: Lipocalin-2 (LCN2), a secretory protein, regulates diverse cellular processes such as cell death/survival, cell migration/invasion, cell differentiation, iron delivery, inflammation, insulin resistance, and tissue regeneration. Recently, we reported that LCN2 is secreted by brain astrocytes under inflammatory conditions and that it promotes apoptosis, morphological changes, and migration in astrocytes both in vitro and in vivo. Activated astrocytes release LCN2 not only to induce the morphological transformation associated with reactive astrocytosis, but also to promote their own death. Under inflammatory conditions, activated astrocytes also show functional dichotomy similar to the M1/M2 phenotypes of microglia and macrophages. LCN2 is thought to be a chemokine inducer and an autocrine promoter of the classical proinflammatory activation of astrocytes. This article summarizes the current knowledge regarding the role of astrocyte-derived LCN2 as a proinflammatory mediator in the central nervous system and discusses LCN2's role in neuroinflammatory disorders.

KEY WORDS: lipocalin-2; astrocytes; neuroinflammation; central nervous system; chemokine; polarization

ABBREVIATIONS: LCN2: lipocalin-2; CNS: central nervous system; BBB: blood-brain barrier; GFAP: glial fibrillary acidic protein; S100B: S100 calcium binding protein B; NF- κ B: nuclear factor- κ B; NGAL: neutrophil gelatinase-associated lipocalin

I. INTRODUCTION: ROLE OF ASTROCYTES IN CNS HEALTH AND DISEASE

Astrocytes are the most abundant glial cell type in the central nervous system (CNS). They modulate synaptic transmission, provide metabolic and trophic support to neurons, maintain blood–brain barrier (BBB) integrity, and regulate neuronal–glial communications. Functional impairment of these cells contributes to diverse pathological conditions in the CNS.¹ Although glial cells play pivotal roles in the normal functioning and survival of neurons, in their reactive state, these cells may be toxic to neurons.² Astrocytes become reactive and undergo a process termed reactive astrocytosis in response to

an array of CNS insults.^{3–5} Reactive astrocytes are characterized by hypertrophic nuclei and cell bodies as well as a distinct pattern of long and thick cellular processes. Expression of several molecules, including glial fibrillary acidic protein (GFAP), S100 calcium binding protein B (S100B), and nuclear factor- κ B (NF- κ B), is upregulated during reactive astrocytosis.⁶ Reactive astrocytosis is stimulated by cytokines and chemokines released from damaged microglia and neurons. The JAK-STAT pathway has been shown to be one of the principal pathways mediating reactive astrocytosis. Stat3^{-/-} mice show inhibited migration of astrocytes and decreased motor recovery in response to spinal cord injury.⁷ Moreover, JAK2-STAT3 pathway-mediated induction of CXCL10 in astrocytes promotes migration of different CNS cells such as

astrocytes themselves, microglia, and neurons.⁸ These results suggest that reactive astrocytes orchestrate the migration of neurons and glia to the site of injury for the maintenance of CNS homeostasis. Astrocytes actively participate in the complex regulation of neuroinflammation. Reactive astrocytes are considered as a crucial mediator of neuroinflammation and the subsequent neurological dysfunction.⁹ Astrocytes in response to diverse stimuli can produce distinct pro- or anti-inflammatory molecules. Generally, astrocytes play a critical role in neuroinflammation via extensive interaction with microglia.¹ Reactive astrocyte-derived proinflammatory molecules are often associated with enhanced inflammatory responses following traumatic injury in the CNS and peripheral nervous system (PNS).^{10,11} Chronically activated astrocytes of proinflammatory phenotypes show the reduced expression of the genes involved in neuronal support and neuronal signaling, which ultimately contributes to neuronal dysfunction and cognitive decline in Alzheimer's disease.¹²

II. LIPOCALIN-2 IN THE CNS

Lipocalin 2 (LCN2), a member of the lipocalin protein family, binds or transports lipids and other small hydrophobic molecules.^{13,14} LCN2 was first described as neutrophil gelatinase-associated lipocalin (NGAL)¹⁵ and is also known as 24p3¹⁶ or 24-kDa superinducible protein (SIP24).¹⁷ Previous studies have suggested that LCN2 protects neutrophil gelatinase from auto-degradation.¹⁸ LCN2 also functions as an acute-phase protein.¹⁹ LCN2 binds to two cell-surface receptors: brain type organic cation transporter (24p3R) and megalin.^{20,21} 24p3R is expressed constitutively and is present in particularly high levels in kidney epithelial cells,²¹ brain microglia,²² astrocytes,²³ and neurons.²⁴ Megalin is expressed in a wide variety of tissues and cell types, such as brain capillaries, choroid plexus, ependymal cells of lateral ventricles, neural progenitors in embryonic mouse spinal cord, neural tube floor plate cells, oligodendrocytes, retinal ganglion cells, cultured astrocytes, and neurons.²⁵ LCN2 has been implicated in a diverse array of cellular processes

including cell death/survival,^{21,26,27} cell migration/invasion,^{28,29} cell differentiation,^{30,31} iron delivery,^{21,30,32,33} inflammation,¹⁹ insulin resistance,³⁴ and tissue regeneration.^{35–37} Moreover, Flo et al. reported that *Lcn2*-deficient mice have an increased susceptibility to bacterial infections owing to a failure of iron sequestration by LCN2, indicating a critical role for LCN2 in protection against bacterial infection.³² Although several reports have acknowledged the important role of LCN2 in the pathophysiology of various peripheral tissues/organs, little is known about its contribution to the maintenance of CNS health and disease. Jha et al. recently reviewed the diverse functional role of LCN2 played in the context of CNS health and disease.²⁵ Emerging evidence has outlined LCN2 as a chemokine inducer and an autocrine mediator of reactive astrocytosis^{23,38} (Figures 1 and 2). In addition, LCN2 has been reported to modulate several biobehavioral responses including pain hypersensitivity,^{39–41} emotional behaviors,^{42,43} cognitive functions,^{42,44,45} depression,^{44,46} neuronal excitability,⁴³ and anxiety.²⁵

A. LCN2 REGULATES MORPHOLOGY AND MIGRATION OF ASTROCYTES

LCN2 has been proposed as an autocrine mediator of reactive astrocytosis based on the following roles of LCN2 in the regulation of astrocyte death, morphology, and migration:²³ (1) a forced increase or decrease in the expression of LCN2 regulated astrocyte sensitivity to cell death positively or negatively, respectively; (2) recombinant LCN2 protein mimicked the effect of LCN2 cDNA transfection or adenoviral expression; (3) LCN2 expression and secretion was induced by inflammatory insults in astrocytes; and (4) LCN2 protein induced GFAP expression and morphological changes in astrocytes consistent with the hallmarks of reactive astrocytosis *in vivo* (Figure 1). Further studies have elucidated the molecular mechanisms underlying LCN2-induced morphological changes and migration of astrocytes. The number and length of cellular processes of astrocytes, which increase during reactive astrocytosis, are increased following exposure to LCN2.²³ Similarly, forskolin and dibutyryl-cGMP have been shown to

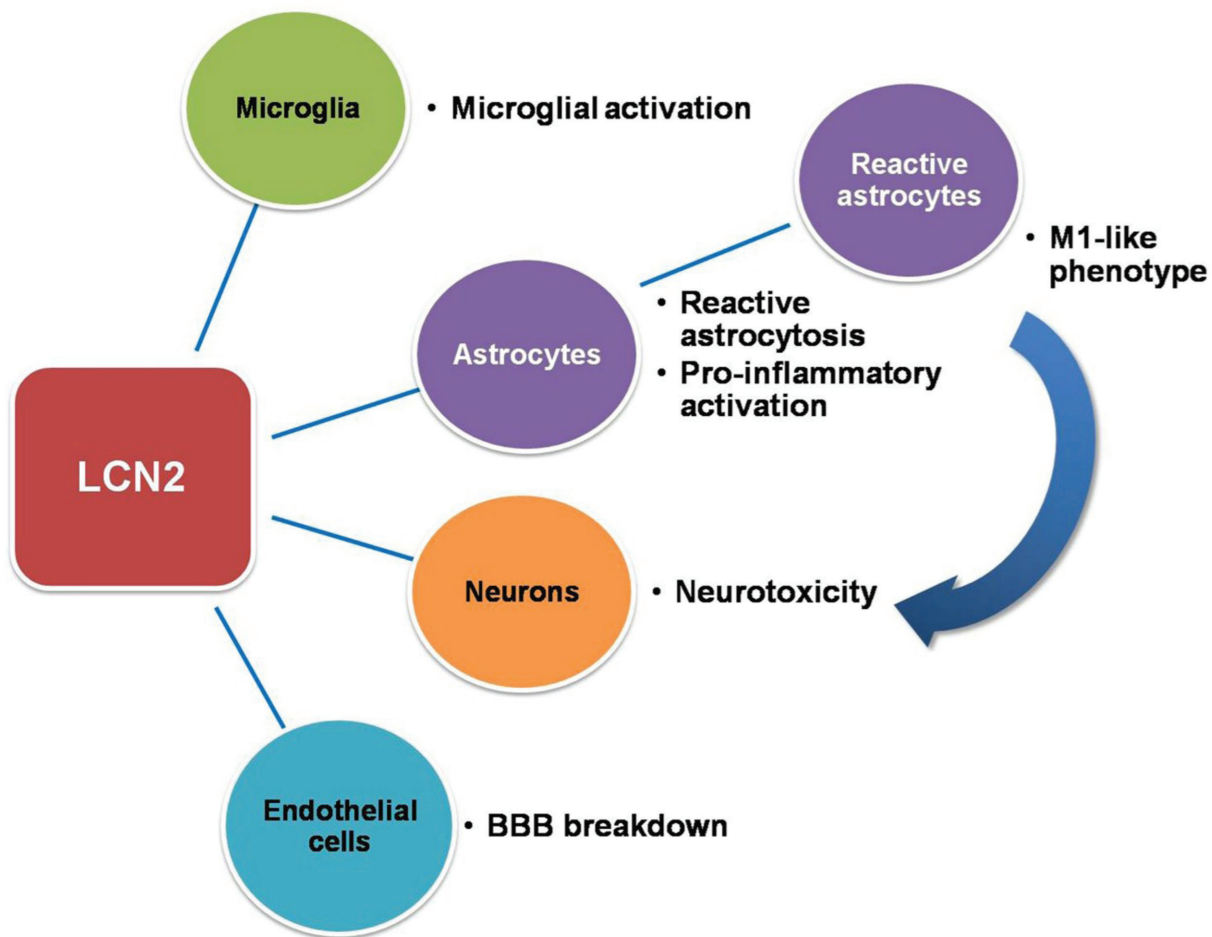


FIG. 1: LCN2 promotes neuroinflammation by modulating glial, neuronal, and endothelial responses. LCN2 activates glial cells (microglia and astrocytes), exerts neurotoxic effects, and increases BBB permeability by acting on endothelial cells. These effects of LCN2 on microglia, astrocytes, neurons, and endothelial cells amplify neuroinflammation. In particular, under inflammatory conditions, LCN2 shifts the phenotype of astrocytes towards M1-like classical activation to further augment neuroinflammation.

induce alterations in astrocyte morphology resembling those induced by LCN2. LCN2-induced morphological changes and cell migration are supported by immunofluorescence analysis and cell migration assays. LCN2 increases GFAP immunoreactivity and phalloidin staining in astrocytes. Additionally, LCN2-induced phenotypic changes in astrocytes have been shown to occur through the Rho–Rho kinase (ROCK)–GFAP pathway, which is positively regulated by nitric oxide and cGMP.²³ These types of morphological changes in astrocytes are similar to those occurring in reactive astrocytosis,^{47–49} and

they indicate a role of LCN2 in mediating reactive astrocytosis *in vivo*. In addition to the induction of morphological changes, LCN2 enhances the capability of astrocytes to migrate via chemokine induction (Figure 2). In particular, CXCL10 expression and secretion by astrocytes is highly upregulated by LCN2. CXCL10 plays a pivotal role in LCN2-induced cell migration, as indicated by both *in vitro* wound healing assays and Boyden chamber assays. Namely, LCN2-treated astrocyte-conditioned media accelerates the migration of microglia, neurons, and astrocytes themselves. This effect is mimicked or

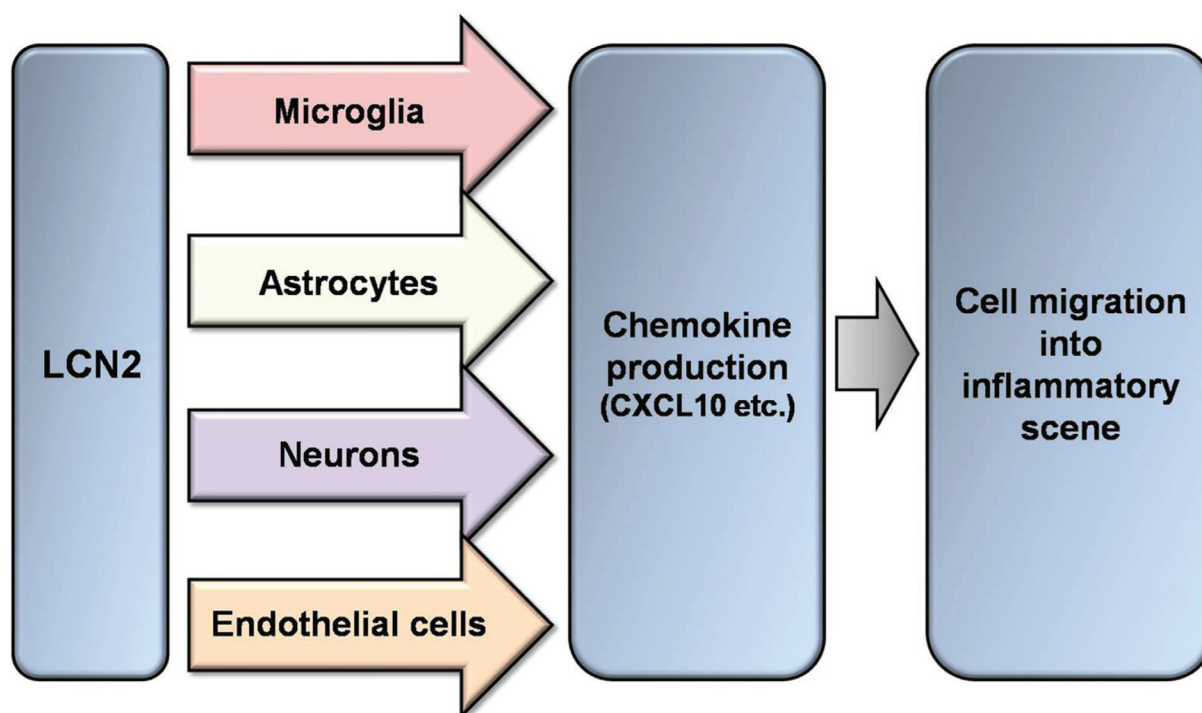


FIG. 2: LCN2 orchestrates CNS cell migration through chemokine induction. LCN2 expression is induced in response to injury, infection, and inflammatory stimuli. LCN2 upregulates chemokine expression in different CNS cell types, including microglia, astrocytes, neurons, and endothelial cells. These chemokines act in a paracrine or autocrine manner to facilitate cell migration to sites of inflammation.

blocked by recombinant CXCL10 protein and a CXCL10 neutralizing antibody, respectively. LCN2-induced expression of CXCL10 in astrocytes is mediated by the JAK2/STAT3 and IKK/NF- κ B pathway.

The functional role of LCN2 in reactive astrogliosis has been further examined using zebrafish and mouse models *in vivo*. *Lcn2* mRNA injection induced morphological changes of radial glia cells, leading to an increase in the number and thickness of Zrf-1⁺ radial glia processes in zebrafish embryos. Stab wound injury increased the expression of GFAP in mouse brain cortex, indicating astrocyte migration toward the stab injury site. Mice lacking LCN2 showed an impaired migration of astrocytes to the site of injury in neuroinflammation or injury models with a concomitant reduction in CXCL10 expression. Thus, LCN2 may be responsible for both morphological and migratory features of reactive phenotypes of astrocytes. These results suggest that activated astrocytes may secrete

LCN2 and CXCL10 under inflammatory conditions, providing evidence for the molecular link between LCN2 and reactive astrogliosis. A recent report by Lee et al.²³ suggests a dual functional role of LCN2 in astrocyte morphology and migration under inflammatory conditions. It also suggests that LCN2 affects glial cell biology as well as brain pathologies involving reactive astrogliosis. Because cell migration plays a pivotal role in development, wound healing, immune/inflammatory responses, and tumor metastasis,^{50–52} the effects of LCN2 on cell migration identified by Lee et al.²³ may broaden our understanding of these biological processes.

B. LCN2 REGULATES FUNCTIONAL POLARIZATION OF ASTROCYTES

A crucial role of LCN2 in the phenotypic polarization of cells has been well documented;²⁵ LCN2 regulates

macrophage polarization.^{53,54} Furthermore, LCN2 has been reported to promote M1 macrophage polarization in a mouse cardiac ischemia-reperfusion injury model.⁵⁵ Recent studies have established the role of LCN2 in M1-like polarization of brain microglia as well.^{56,57} Astrocytes have also been shown to exhibit M1/M2-like functional polarization, and LCN2 has been shown to be an autocrine promoter of the classical proinflammatory activation of astrocytes³⁸ (Figure 1). LCN2's functional role in this regard has been supported by the following evidence: (1) cultured astrocytes exhibited classical and alternative polarized phenotypes; (2) recombinant LCN2 protein treatment promoted the proinflammatory phenotypes of astrocytes; (3) LCN2 inhibited alternative activation by IL-4 in a mouse neuroinflammation model; and (4) lipopolysaccharide (LPS)-promoted classical proinflammatory activation of astrocytes was attenuated in *Lcn2*-deficient mice. In *in vitro* studies, LCN2 significantly inhibited IL-4-induced STAT6 phosphorylation, a canonical pathway involved in alternative anti-inflammatory activation, in astrocytes. LCN2 also inhibited the expression of alternative activation-related genes (Il-10, Arg1, Mrc1, Il-1ra, Fizz1, and Ym1) downstream of IL-4-STAT6, which supports the idea that LCN2 partially blocks the alternative activation of astrocytes. The role of LCN2 in the classical activation of astrocytes has also been evaluated *in vivo* by comparing *Lcn2* wild-type (WT) and knockout (KO) mice injected with LPS or IL-4. In LPS-injected WT mice, GFAP, LCN2, and iNOS were expressed in the cortex. GFAP expression was partly colocalized with LCN2 and iNOS expression after LPS injection. Both of these findings demonstrated that LCN2 induction occurs with the classical activation of GFAP⁺ astrocytes. A similar increase in GFAP expression was observed in the hippocampus after LPS injection, whereas a decrease was observed in *Lcn2*-KO mice. Furthermore, DAPI staining showed that LPS injection induced hippocampal injury, which was attenuated by *Lcn2* deficiency. As expected, the alternative activation marker ARG1 was expressed in astrocytes after IL-4 injection. However, the induction of ARG1 expression in cortical astrocytes was not significantly influenced by *Lcn2* deficiency. Furthermore,

the number of GFAP⁺ cells did not significantly change due to *Lcn2* deficiency. These histological findings confirm that the functional polarization of astrocytes can be induced in experimental animals and that LCN2 plays a central role in the classical activation of astrocytes.

In contrast to classical activation, alternatively activated astrocytes show distinct phenotypes with neuroprotective activity. To determine the role played by LCN2 in the regulation of these functional phenotypes, the phenotypes of astrocytes derived from WT and *Lcn2* KO mice have been compared. In cultured *Lcn2*-deficient astrocytes, the LPS/IFN- γ -induced increase in GFAP expression was diminished. Furthermore, neurotoxicity induced by classically activated astrocytes was reduced by *Lcn2* deficiency. The neurotoxic effects exerted by the transfer of astrocyte-conditioned media following LPS/IFN- γ stimulation and coculture of LPS/IFN- γ -stimulated astrocytes with cortical neurons was attenuated by *Lcn2* deficiency in astrocytes. The effects of IL-4 were not affected by the presence or absence of *Lcn2* in astrocytes. These results suggest that LCN2 is an autocrine mediator that determines the functionally polarized phenotypes of astrocytes in the presence of immune or inflammatory stimuli. Therefore, LCN2 appears to be a potential therapeutic target for reducing proinflammatory astrocytic activation and preventing related pathologies in the CNS.

III. CONCLUSIONS

LCN2 has been implicated in diverse cellular processes such as cell growth, death/survival, migration/invasion, and differentiation. Recently, we and several other investigators have reported that activated astrocytes may secrete LCN2 under inflammatory conditions. LCN2 seems to be a central mediator of reactive astrogliosis, establishing the molecular link between LCN2 and reactive phenotypes of astrocytes, which include morphological changes and enhanced migration. Moreover, astrocytes activated under neuroinflammatory conditions show classical or alternative activation, being respectively either neurotoxic or neuroprotective, depending on the

nature of immune or inflammatory microenvironments. Recent studies show a crucial role for LCN2 in astrocytes as a promoter of classical proinflammatory activation and an inhibitor of alternative activation phenotypes. These findings put forward LCN2 as a promising drug target that can be manipulated for the diagnosis, prognosis, and therapy of multiple CNS disorders with a strong inflammatory component.

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