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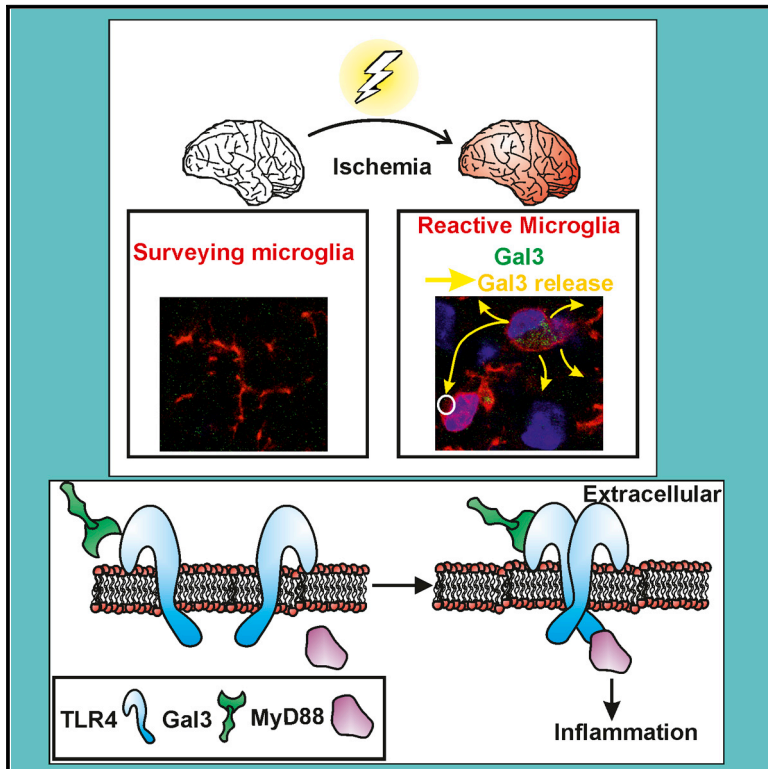
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Graphical Abstract



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In Brief

In this publication, Burguillos et al. demonstrate how galectin-3 (Gal3) released from reactive microglia cells can activate other surrounding immune cells in a paracrine manner by binding to and activating Toll-like receptor 4 (TLR4). This finding could explain the propagation of the inflammatory response once the initial stimulus is gone.

Highlights

- Gal3 acts as an endogenous TLR4 ligand with a Kd value around 1 μ M
- Gal3 can initiate a TLR4-dependent inflammatory response in microglia
- Gal3 is required for complete activation of TLR4 upon LPS treatment
- Gal3-TLR4 interaction is confirmed in vivo and in stroke patients



Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation

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SUMMARY

Inflammatory response induced by microglia plays a critical role in the demise of neuronal populations in neuroinflammatory diseases. Although the role of toll-like receptor 4 (TLR4) in microglia's inflammatory response is fully acknowledged, little is known about endogenous ligands that trigger TLR4 activation. Here, we report that galectin-3 (Gal3) released by microglia acts as an endogenous paracrine TLR4 ligand. Gal3-TLR4 interaction was further confirmed in a murine neuroinflammatory model (intranigral lipopolysaccharide [LPS] injection) and in human stroke subjects. Depletion of Gal3 exerted neuroprotective and anti-inflammatory effects following global brain ischemia and in the neuroinflammatory LPS model. These results suggest that Gal3-dependent-TLR4 activation could contribute to sustained microglia activation, prolonging the inflammatory response in the brain.

INTRODUCTION

The inflammatory response driven by microglia is a key element in brain ischemia (Lambertsen et al., 2012) and in neurodegenerative disorders (Burguillos et al., 2011; Saijo and Glass, 2011). Toll-like receptors (TLRs), like other pattern recognition recep-

tors (PRRs), are critical for the response to inflammatory agents (Hennessy et al., 2010). Since its discovery in 1996, the TLR family member TLR4 has attracted particular attention in several inflammatory diseases, including CNS pathologies (Buchanan et al., 2010; Lemaitre et al., 1996). Pharmacological inhibition of TLR4 and transgenic mice lacking the TLR4 gene exhibit neuroprotection in conditions of experimental stroke (Caso et al., 2007; Hyakkoku et al., 2010; Suzuki et al., 2012). Despite extensive research, only very few endogenous ligands for TLR4 have been described so far (Chen and Nuñez, 2010).

Galectins represent a protein family with at least 15 members that have significant sequence similarity in their carbohydrate-recognition domain (CRD) and bind to β -galactosides with varying affinities and specificities (Barondes et al., 1994; Leffler et al., 2004). Galectins are classified into three subgroups (1) proto, (2) chimera, and (3) tandem repeat based on their molecular architecture. The proto-type and tandem-repeat-type families comprise proteins with one and two CRDs on a single polypeptide chain, respectively (Kasai and Hirabayashi, 1996).

Galectin-3 (Gal3) is the only known member of the chimera-type family comprising a C-terminal CRD and N-terminal non-CRD for carbohydrate binding and increased self-association, respectively (Lepur et al., 2012). Gal3 is known to be involved in the inflammatory response, and its expression is increased in microglial cells upon various neuroinflammatory stimuli as, for instance, the process of ischemic injury (Lalancette-Hébert et al., 2012; Satoh et al., 2011a, b; Wesley et al., 2013). Gal3 can be found in the cytoplasm, nucleus, and membranes (Shimura et al., 2004) and can be released into the extracellular

space upon certain stimuli such as lipopolysaccharide (LPS) (Li et al., 2008) and interferon γ (IFN- γ) (Jeon et al., 2010). The different subcellular localizations of Gal3 together with its possible posttranslational modifications are likely to affect the function of Gal3 and explain why rather contradictory effects have been reported, e.g., pro- versus anti-apoptotic (Nakahara et al., 2005) and pro- versus anti-inflammatory (Jeon et al., 2010; MacKinnon et al., 2008). As an example of this duality of function, it has been reported that Gal3 deficiency aggravates the neuronal damage in the adult mouse brain following transient focal brain ischemia, due to a reduced signaling of insulin-like growth factor receptor in microglia (Lalancette-Hébert et al., 2012), whereas in a transgenic mouse model of amyotrophic lateral sclerosis (ALS), the lack of Gal3 increases the inflammatory response (Lerman et al., 2012). In contrast, in a model of global brain ischemia, microglial Gal3 was suggested to contribute to neuronal death in the CA1 subregion of the hippocampus (Satoh et al., 2011a, b) as well as contribute to the inflammation and severity in experimental autoimmune encephalitis (Jiang et al., 2009).

Previous studies have focused on the relationship between Gal3 and members of the TLR family such as TLR2. For example, in differentiated macrophages, Gal3 can form a complex with TLR2 and thereby improves the inflammatory response against *C. Albicans* (Jouault et al., 2006). In addition, it has been suggested that Gal3 can act as co-receptor, presenting the *Toxoplasma gondii* glycosylphosphatidylinositols (GPIs) to TLR2 and TLR4 on macrophages (Debierre-Grockiego et al., 2010). Furthermore, an interaction between Gal3 and LPS, a known TLR4 ligand, has been reported as well (Li et al., 2008; Mey et al., 1996). Gal3 and TLR4 are both considered to be independent actors in the initiation and progression of the inflammatory response after brain ischemia. In this study, we demonstrate that Gal3 can act as an endogenous ligand for TLR4. We show that Gal3 can induce, per se, a TLR4-dependent inflammatory response as well as contribute to the full activation of this receptor upon binding to other proinflammatory stimuli, such as LPS.

RESULTS

Gal3 Affects Downstream TLR-Signaling Pathways in Microglia

We first set out to determine the effect of Gal3 on the TLR-mediated signaling pathways. To achieve this, we took advantage of an array that monitors the expression of 84 genes involved in the TLRs intracellular signaling pathways. BV2 microglia cells were exposed to endotoxin-free (as confirmed by *Limulus* amoebocyte lysate assay) soluble Gal3 (referred henceforth as sGal3) for 6 hr. In addition, because Gal3 can be rapidly internalized by cells and thereby activate intracellular signaling pathways, we used a so-called “immobilized form” of Gal3 (referred to as iGal3) that only can interact with proteins on the cell surface (e.g., receptors). Due to Gal3’s high hydrophobicity of its N terminus part, it can bind to plastic, allowing the exposure of both domains: its CRD and also its N-terminal site (Sörme et al., 2002). Cell culture wells were coated overnight at 4°C with 100 μ g/ml of Gal3 and washed three times with PBS to remove unbound Gal3. BV2 microglial

cells were then seeded in these Gal3-coated plastic wells for 6 hr before harvesting them. Cells seeded on non-coated wells for 6 hr were used as a negative control. LPS (1 μ g/ml) added to the cell culture medium for 6 hr was used as a positive control for TLR4 activation.

Thus, BV2 microglia cells were treated with sGal3, iGal3, or LPS and gene expression of the TLRs-signaling pathway investigated. As shown in Figure 1, sGal3 or iGal3 treatment results in statistically significant changes in gene expression as compared to untreated cells. Both induction and repression in gene expression can be observed after either of these treatments. Remarkably, there was significant overlap in microglial gene expression related to TLR4 signaling in responses to either Gal3 or LPS (Figures S1A and S1B).

Gal3 Binds to TLR4 through Its CRD

Next, we explored the possibility of a direct physical interaction between Gal3 and TLR4. Using confocal microscopy, Gal3 and TLR4 were found to be colocalized in BV2 cells 1 hr after adding sGal3 (Figure 2A). Under these conditions, TLR4 was immunoprecipitated and Gal3 was found to be part of the resulting immune complexes (Figure 2B).

Gal3 interaction with glycoproteins is complex, and the initial binding of the CRD often triggers a subsequent self-association of Gal3, sometimes resulting in crosslinking and precipitation (Lepur et al., 2012). This self-association also involves the canonical carbohydrate recognition site in the CRD but also the N-terminal non-CRD domain of Gal3, which makes it much more efficient, and is also required for most biological effects of Gal3.

The apparent affinity of the interaction between TLR4 and Gal3 was determined using microscale thermophoresis (MST). In MST, the thermophoretic mobility of a fluorescently labeled molecule in an infrared-laser-induced microscopic temperature gradient is recorded, yielding a fluorescence time trace from which a normalized fluorescence value (F_{norm}) is derived. Changes in the thermophoretic mobility of the molecule upon ligand binding manifest as shifts in the F_{norm} values and are used to quantify the affinity of the interactions (Seidel et al., 2013). Accordingly, binding of Gal3 to fluorophore-tagged TLR4 (at a constant concentration of about 120 nM) produced a clear shift in the recorded fluorescence time traces (Figure S2B) with increased F_{norm} values for the Gal3-TLR4 complex. The minimal and maximal F_{norm} values for the unbound and fully bound state of TLR4, respectively, were used to calculate the fraction of TLR4 bound at each Gal3 concentration. The resulting saturation binding curve (Figure 2C) shows that 50% of TLR4 is bound at about 1.5 μ M Gal3.

The presence of lactose, a competitive inhibitor of both Gal3 carbohydrate binding and self-association, completely abolished the interaction (purple data points in Figure 2C). Further evidence for the involvement of the Gal3 canonical carbohydrate-binding site was the fact that a mutant, Gal3 R186S, showed interaction with TLR4 at a much-higher concentration with 50% bound at about 45 μ M. This mutant reduces affinity of Gal3 for many glycoproteins and for the disaccharide LacNAc, which is the most common minimal galectin-binding moiety in glycoproteins (Lepur et al., 2012; Salomonsson et al., 2010a). The Gal3 CRD, lacking the N-terminal domain, also bound

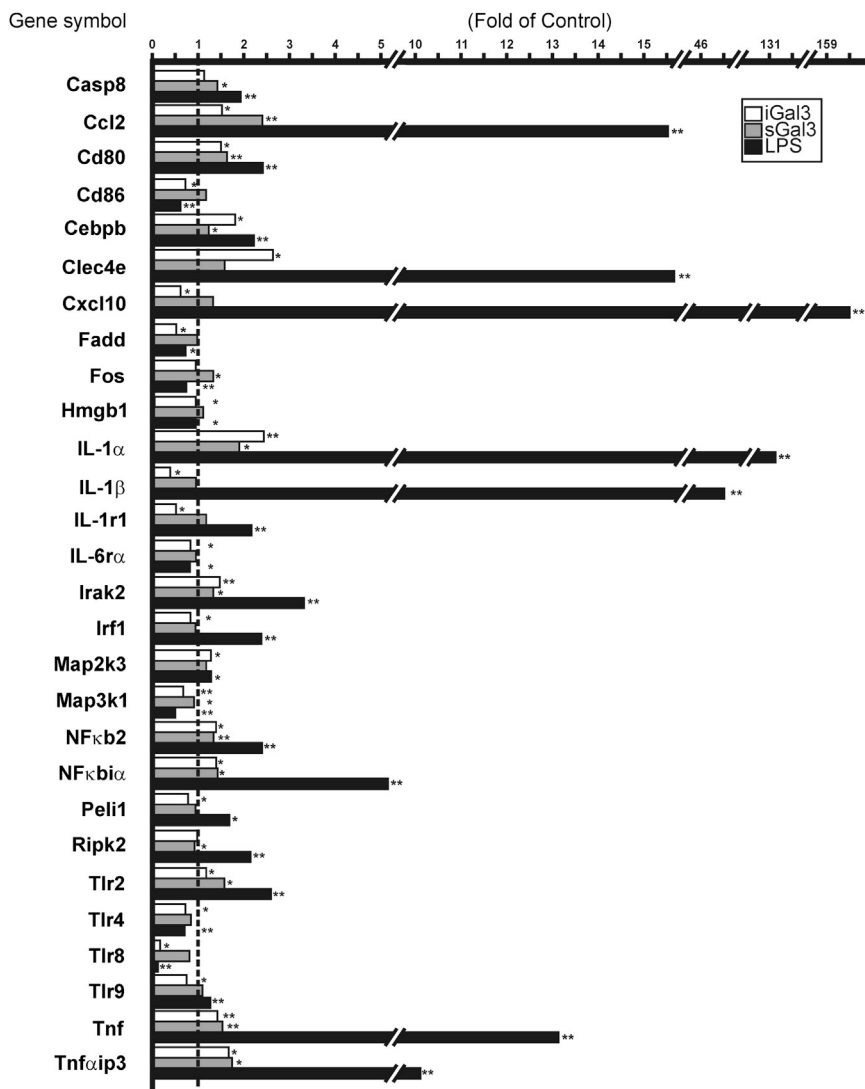


Figure 1. Expression Analysis of Genes Related to the TLR Family after Treatment with Gal3 and LPS

Gene expression array analysis of mRNA related to TLR activation in BV2 microglial cells upon sGal3 (1 μ M), iGal3 (100 μ g/ml coated well), and LPS (1 μ g/ml) treatment for 6 hr. Data are representative of three independent experiments and expressed as mean (n = 3). *p < 0.05; **p < 0.01. χ^2 analysis revealed similar up- or downregulation of Gal3 compared to LPS (*p < 0.05). See also Figure S1.

titrated with a range of TLR4 concentrations, the Hill coefficient for intact Gal3 was about 0.4, whereas for the CRD, it was again about 1 (Table S1). This indicates that intact Gal3 binds with apparent positive cooperativity and/or in an event with stoichiometry of greater than two Gal3 per TLR4, whereas the CRD binds in simple 1:1 interactions to one or more independent sites on TLR4.

Addition of Gal3 concentration $> \sim 1 \mu$ M to fluorescent TLR4 at 120 nM caused precipitation, as measured by removal of fluorescence by centrifugation of the samples before loading into capillaries that are used for the MST measurements (Figure S2A). This observation probably also explains the gradual fluorescence increase in un-centrifuged samples (Figure S2A) and the wavy line shapes of the fluorescence time traces recorded in the MST experiment (Figure S2B). However, the aggregation did not prevent obtaining highly reproducible binding curves that could be used for quantitative analysis of the interaction (Figure 2C).

TLR4 at about equal concentrations as intact Gal3 (red curve in Figure 2C).

To gain further evidence for Gal3-TLR4 interaction, we used fluorescence anisotropy as a separate independent technique. In this technique, the interaction of Gal3 with a fluorescein-tagged saccharide probe is inhibited by increasing concentrations of TLR4 and quantitatively analyzed, as has been done for many other inhibitors before (Lepur et al., 2012; Salomonsson et al., 2010b). The data are presented in the form of percent Gal3 bound to TLR4 to make them more easily comparable to the previous experiment (Figure 2D). This again demonstrated that TLR4 binds both Gal3 and Gal3 CRD, with 50% of Gal3 bound by about 1 μ M TLR4, and also shows that TLR4 competes for the canonical carbohydrate-binding site of Gal3.

The data also provided insight into TLR4-induced self-association of Gal3. The slope of the binding curve in Figure 2C, where fixed TLR4 is titrated with a range of Gal3 concentrations, was consistent with a Hill coefficient of above 2 for intact Gal3 but was about 1 for the CRD. In Figure 2D, where fixed Gal3 is

The different methods, hence, demonstrate that Gal3 interacts directly with TLR4 at physiologically relevant concentrations and also at the Gal3 concentrations (1 μ M) used in the cell experiment here.

All galectin family members have in common a canonical CRD with high-sequence homology. Galectin-1 and galectin-4 were chosen as examples of the proto and tandem repeat families, respectively, and they also bind to TLR4 in MST experiments but with lower apparent affinities of about 8 and 14 μ M, respectively, and Hill coefficients of about 1, indicating a lower cooperativity (Figure S2F).

TLR4 Contributes to Gal3 Proinflammatory Response

Contradictory reports suggest that Gal3 can play both proinflammatory and anti-inflammatory roles. Gal3 has been shown to elicit a proinflammatory (M1) response per se (Jeon et al., 2010) or amplify a pre-existent proinflammatory reaction (Devillers et al., 2013) in macrophages. Similarly, we have recently demonstrated that Gal3 is involved in the proinflammatory

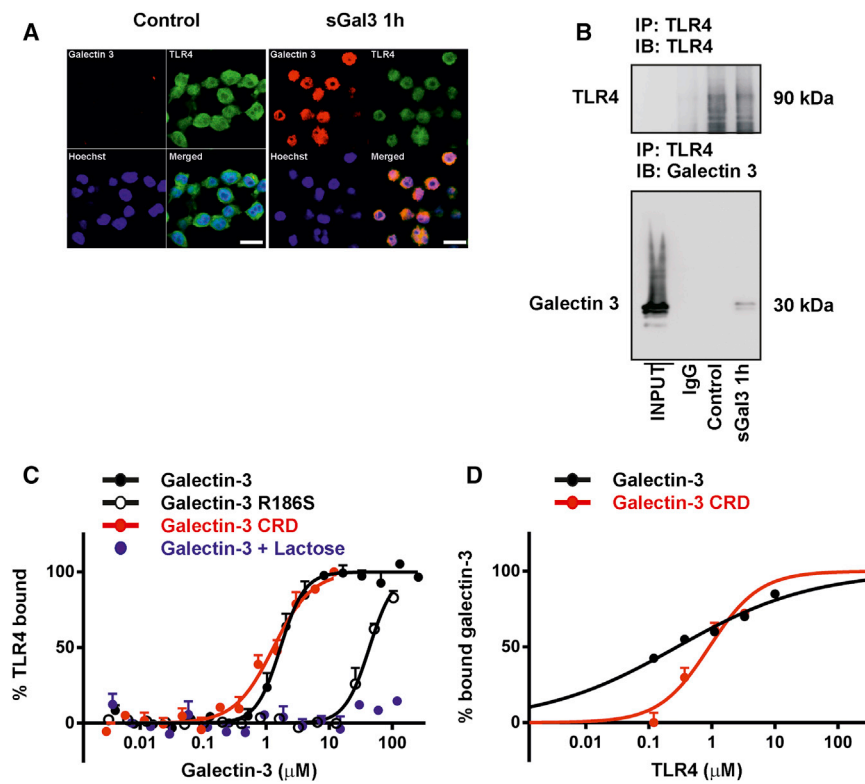


Figure 2. Gal3 Acts as a Ligand to TLR4

(A) Colocalization of Gal3 and TLR4 in BV2 cells after 1 hr exposure with sGal3 protein.

(B) Immunoblot showing the presence of Gal3 in an immune complex formed after pull-down of TLR4 in BV2 microglial cell line after being treated with 1 μ M of soluble Gal3 for 1 hr.

(C) Microscale thermophoresis was used to analyze the direct binding of TLR4 to Gal3, the Gal3 CRD, Gal3 R186S, and Gal3 in the presence of inhibitory lactose (40 mM). Whereas the concentration of fluorescently labeled TLR4 was kept constant, the non-labeled proteins were titrated (x axis), and the minimal and maximal F_{norm} values of the unbound and bound state of TLR4, respectively, were used to calculate percent TLR4 bound to Gal3 (y axis).

(D) Fluorescence anisotropy was used to analyze the potency of TLR4 (x axis) to inhibit binding of Gal3 (0.2 μ M) proteins to a fluorescent saccharide probe (0.02 μ M).

The measured values were used to calculate the percent of Gal3 bound to TLR4 (y axis). The scale bar for (A) represents 15 μ m. Data points in (C) and (D) are averaged from two to six measurements for each of the different conditions and binding curves obtained by non-linear regression to the Hill equation, with EC_{50} and Hill coefficient as variables and minimum (0%) and maximum (100%) constrained; numerical results are given in Table S1. Values are expressed as mean \pm SE. See also Figure S2 and Table S1.

response triggered by α -synuclein in microglial cells (Boza-Serrano et al., 2014). Other studies have, however, suggested that Gal3 is involved in the alternative activation of macrophages and microglia (Hoyos et al., 2014; MacKinnon et al., 2008). In order to clarify the effect of Gal3 per se on microglial cells, BV2 cells were treated with sGal3 and several phenotypical markers were analyzed, including the expression of inducible nitric oxide synthase (iNOS) (M1 phenotype), CD206, TGF- β , Ym 1/2, arginase-1 activity (M2 phenotype), and CD45 (phosphatase that can inhibit the proinflammatory response; Starossom et al., 2012). We observed that sGal3 treatment induced iNOS expression (Figures 3B and 3C) and an overall trend to decrease the different M2 markers, although only arginase activity and CD206 expression reached statistical significance (Figures S3A and S3B). These data support the view that Gal3 stimulates a proinflammatory M1 phenotype in microglia.

The similarities between the changes in gene expression induced by Gal3 and LPS, which acts as a TLR4 ligand (Figure 1), and the physical interaction between Gal3 and TLR4 made us think that Gal3 could be inducing a TLR4-dependent inflammatory response. To explore this possibility, the expression of TLR4 was silenced in BV2 microglial cells using small interfering RNA (siRNA) (Figure 3A). Interestingly, silencing of TLR4 in BV2 cells leads to a reduction in the iNOS protein expression upon LPS, sGal3, and iGal3 treatments (Figures 3B–3E), suggesting that these stimuli share a common TLR4-dependent signaling pathway. The silencing of MyD88, a downstream protein triggered by activation of TLR4, shows as well a

decrease in iNOS expression upon sGal3 treatment in BV2 cells (Figures S3C and S3D). To validate the TLR4 dependency of the Gal3 response, the release of several cytokines (i.e., TNF- α and interleukins [IL-1 β , IL-4, IL-5, IL-10, and IL-12]) were investigated in primary microglia cultures derived from wild-type and TLR4 knockout mice upon sGal3 and iGal3 treatment. The release of the above-mentioned cytokines was found to be increased upon both types of Gal3 treatment in wild-type microglia (Figures 3F–3K). In contrast, the increases in cytokines released upon Gal3 treatments were abrogated in primary microglial cells originating from TLR4 knockout mice (Figures 3F–3K), demonstrating that TLR4 is essential for Gal3-induced cytokine release. In the case of IL-10 and TNF- α , we observed that their decrease is not complete in TLR4 knockout mice, which suggests also that Gal3 may be interacting also with other receptors other than TLR4 such as for example TLR2 (Jouault et al., 2006).

Gal3 Promotes Caspase-3/7 and Caspase-8 Activities in the Absence of Cell Death

We recently uncovered that the orderly activation of caspase-8 and caspase-3/7 contributes to the activation of microglia by several proinflammatory stimuli including LPS (Burguillos et al., 2011; Venero et al., 2011). Because both Gal3 and LPS can act as TLR4 ligands, we next examined whether Gal3 induces the activation of these caspases. Indeed, both sGal3 (Figure 4A) and iGal3 treatments (Figure 4B) induced DEVDase activity (caspase-3/7 activation) and IETDase activity (caspase-8 activation)

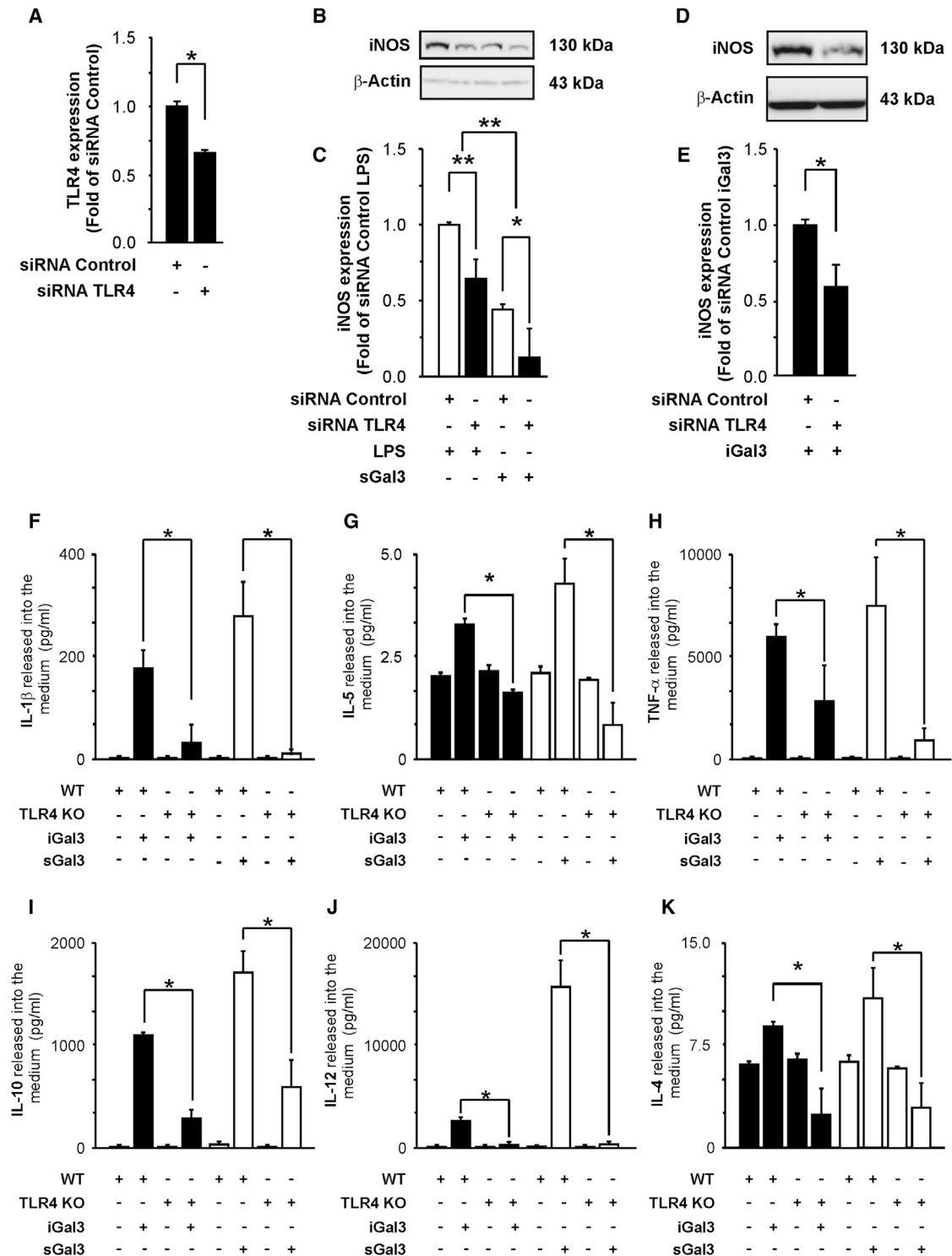


Figure 3. Gal3-Induced Inflammatory Response Is Dependent on TLR4

(A) TLR4 mRNA levels are downregulated after its knockdown.

(B–K) Knocking down TLR4 decreases iNOS expression after LPS, sGal3 (B and C), and iGal3 (D and E) treatment for 6 hr. Cytokine profile in wild-type (WT) primary microglia versus TLR4 knockout (TLR4 KO) primary microglia cells after 24 hr treatment with sGal3 and iGal3 (F–K).

Data are expressed as mean ± SD (A–E; n = 4) and mean ± SEM (F–K; n = 4). *p < 0.05; **p < 0.01. See also Figure S3.

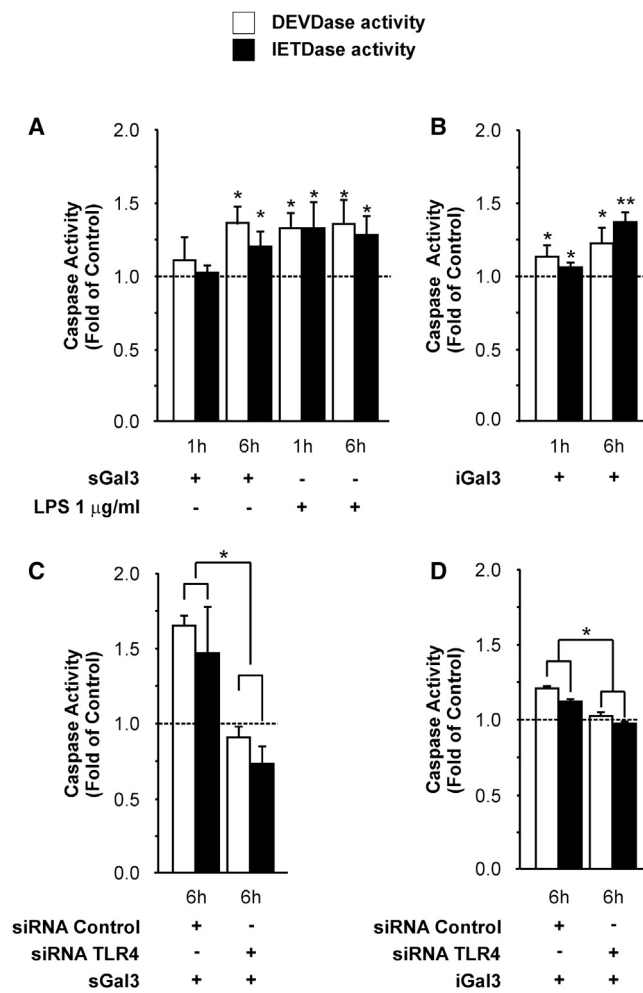


Figure 4. Gal3 Treatment Induces Caspase-3/7 and Caspase-8 Activities in a TLR4-Dependent Manner

(A and B) Analysis of caspase 3/7 (DEVDase) and caspase 8 (IETDase) activities at 1 hr and 6 hr treatment with 1 μ M of sGal3, LPS (1 μ g/ml), A), and iGal3 (B) in BV2 microglial cells. LPS treatment was used as a positive control for caspase 3/7 and 8 activation under inflammatory conditions.

(C and D) The increase of DEVDase and IETDase activity after sGal3 and iGal3 treatment is abolished when TLR4 is knocked down.

Data are expressed as mean \pm SEM (n = 3). *p < 0.05; **p < 0.01. See also Figure S4.

as early as 6 hr after sGal3 and 1 hr after iGal3 treatment. In accordance with the TLR4 dependency of Gal3 response, silencing of TLR4 expression using siRNA abrogated the increase of both caspase-3/7 and caspase-8 activities after either sGal3 (Figure 4C) or iGal3 (Figure 4D) treatment.

We previously demonstrated that the TLR4-dependent activation of these caspases during microglia activation did not lead to cell death (Burguillos et al., 2011). We confirm here the absence of apoptotic cell death upon Gal3 treatment using a panel of methods (Figures S4A–S4D). Some reports indicated that Gal3 can affect the cell cycle (Lin et al., 2002). However, we did not find any alteration in the cell cycle after Gal3 treatment (Figures S4E and S4F).

Released Gal3 Is Essential for Full Microglial Response upon LPS Stimulation

Several proinflammatory stimuli, including LPS, have been shown to induce the release of Gal3 in macrophages and glial cells (Jeon et al., 2010; Li et al., 2008). This urged us to investigate whether endogenous Gal3 could play a paracrine role in the response triggered by an inflammatory stimulus. In culture, we observed a time- and dose-dependent release of Gal3 protein from BV2 microglia cells in response to LPS exposure (Figure S5A). After LPS treatment, Gal3 and TLR4 were also found to colocalize in BV2 cells (Figure S5B). Furthermore, the amount of Gal3 found to co-immunoprecipitate with TLR4 was directly proportional to the dose of LPS used (Figure 5A).

To study the contribution of Gal3 in the response of microglia cells to a LPS stimulus, we decided to inhibit it through two different approaches: (1) Gal3 expression was suppressed using siRNAs in BV2 cells (Figure S5C) and (2) a Gal3 blocking antibody was used to neutralize the effects of released Gal3. We observed that both methods prevented LPS-induced iNOS expression at 6 hr and 24 hr (Figures 5B–5D). To validate Gal3 effect over the inflammatory response upon LPS stimulus, the release of several proinflammatory cytokines were checked in wild-type and Gal3 knockout primary microglial cell cultures, confirming the BV2 cell data, with reduced inflammatory response in Gal3 knockout microglia (Figure 5E).

We also analyzed the effect of Gal3 inhibition in terms of IETDase and DEVDase activities in response to LPS treatment; the Gal3 siRNA knockdown has an inhibitory effect on both activities, especially at 24 hr (Figures S5D and S5E). Collectively, these results demonstrate that Gal3 indeed contributes to the response of microglia cells to LPS stimulus.

In Vivo Interaction between Gal3 and TLR4 and Its Contribution to the Inflammatory Response Induced by LPS

At this point, we wanted to validate our in vitro observation in vivo and explore whether Gal3-TLR4 interactions could be observed in the brains of mice 24 hr after LPS injection into the substantia nigra, an established model of neuroinflammation (Castaño et al., 2002; Herrera et al., 2000). First, we established an in vivo rat brain microdialysis approach to detect released Gal3 in the ventral mesencephalon in response to intranigral LPS injection. We discovered that Gal3 is released in the substantia nigra 24 hr after LPS injection (Figure 6A). We further used TLR4, Iba-1, and Gal3 immunohistochemistry and observed colocalization of the three markers in several cells in the same region after LPS injection in mice (Figure 6B). We confirmed colocalization of Gal3 and TLR4 in microglial cells by using double heterozygous Cx3cr1GFP/+Ccr2RFP/+ mice, where GFP is expressed only in microglial cells and RFP in monocytes (Figure S6F). We performed fluorescence resonance energy transfer (FRET) analysis, using TLR4-FITC as a donor and Gal3 Texas Red as an acceptor, and an interaction between TLR4 and Gal3 proteins was demonstrated at 24 hr following injection of LPS in the substantia nigra (Figure 6C). Our in vitro investigations suggest that the absence of Gal3 is associated with reduced inflammation upon LPS stimulus. We decided to compare the neuroinflammatory response after intranigral LPS injection in wild-type and Gal3

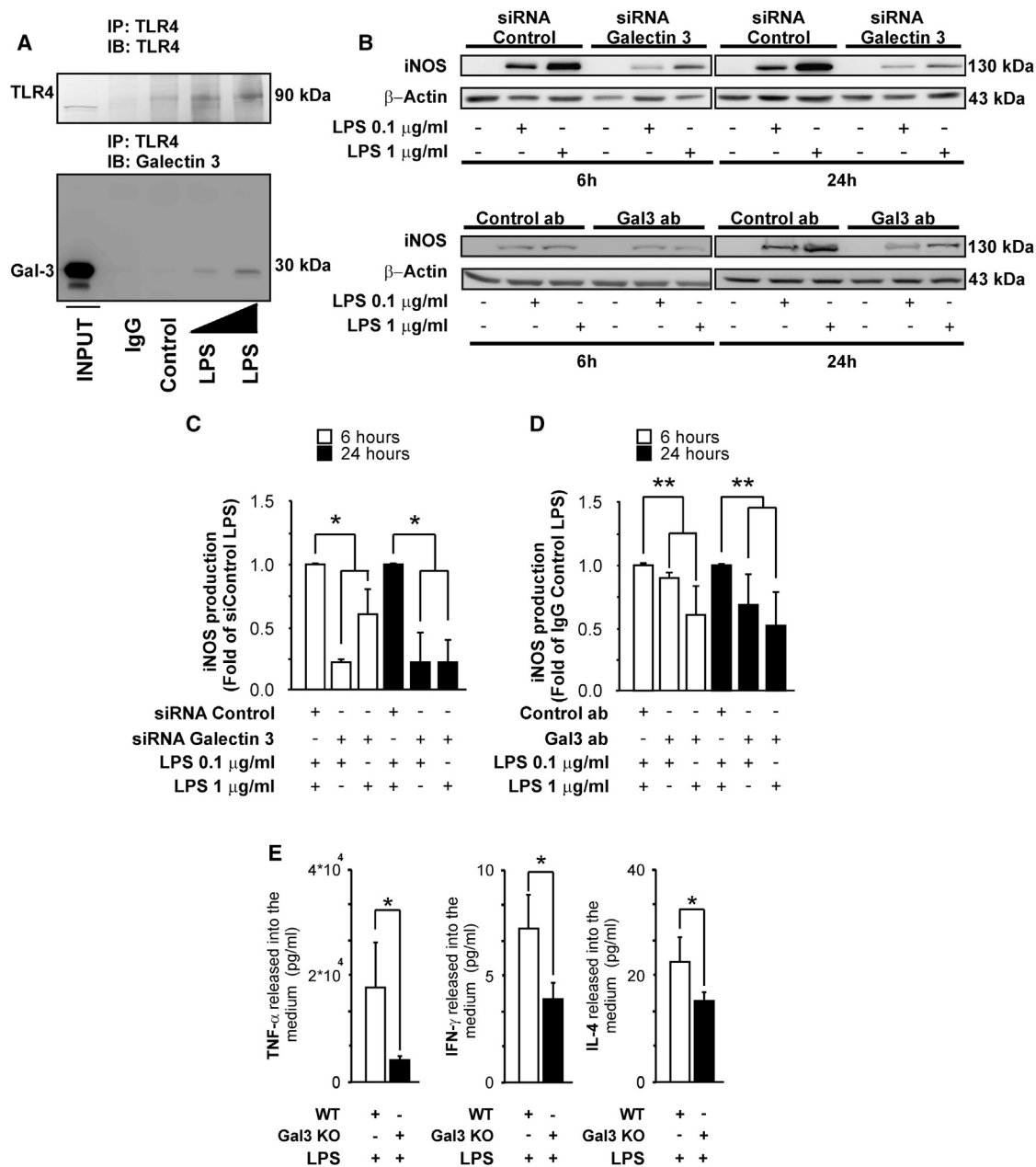


Figure 5. Released Gal3 Enhances the Inflammatory Response after LPS Treatment in a Dose- and Time-Dependent Manner In Vitro

(A) Immunoblot showing the presence of Gal3 in immune complexes formed after pull-down of TLR4 in BV2 microglial cell line after being treated with LPS (0.1 μg/ml and 1 μg/ml) for 24 hr.

(B–D) Reduced iNOS expression upon LPS treatment for 6 hr and 24 hr in BV2 cells transfected with siRNA-targeting Gal3 as compared to BV2 cells transfected with siRNA control and after co-treatment of LPS with a neutralizing antibody against Gal3 as compared to the same amount of the same isotype of IgG as a negative control.

(E) Cytokine profile in WT primary microglia versus Gal3 knockout (Gal3 KO) primary microglia cells after 12 hr treatment with LPS 0.1 μg/ml (E). a Data are expressed as mean ± SEM (n = 4). *p < 0.05; **p < 0.01. See also Figure S5.

knockout mice. We found a significant decrease in the expression of proinflammatory markers IL-1β and IL-6, (Figure 6D) which is consistent with the reduced numbers of reactive microglia/macrophages (Figures 6E and 6F) and proliferating microglia (Iba1 and BrdU double-positive cells; Figures S6A and S6B) in

the Gal3 knockout mice as compared to wild-type mice after LPS treatment. As a consequence of this ameliorated inflammatory response in Gal3 knockout mice, there was a clear neuroprotection of the dopaminergic system 7 days after LPS injection (sham WT animals: 1,467 ± 304, LPS WT animals: 711 ± 128,

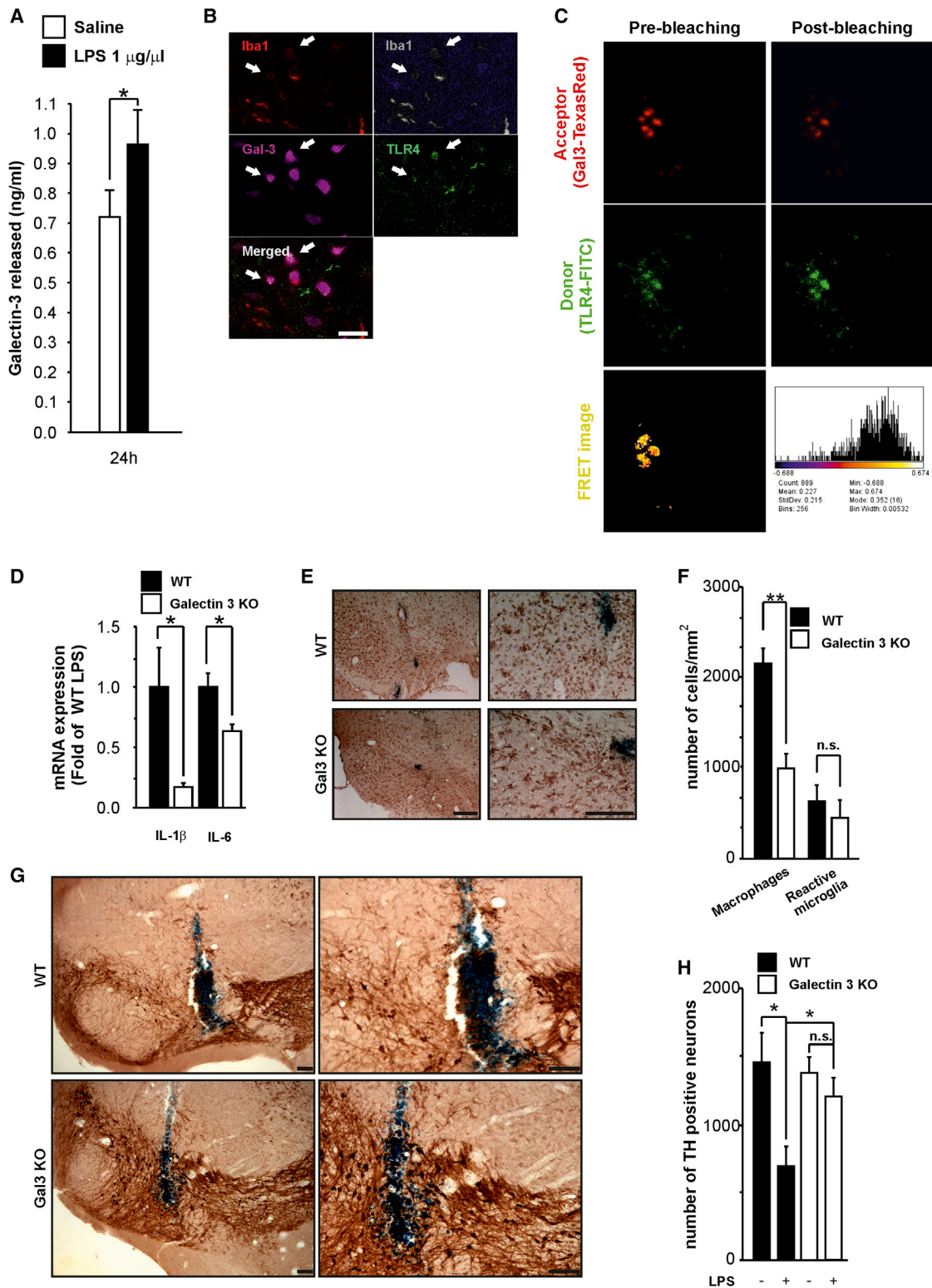


Figure 6. Gal3 Colocalizes with TLR4 and Contributes to the Inflammatory Response Induced by LPS Injection in the Substantia Nigra

(A) Measurement of Gal3 release 24 hr after LPS injection compared to saline injection in the substantia nigra.

(B) Increased expression and colocalization of Gal3 (purple), Iba1 (in red and also using range indicator filter in gray), and TLR4 (green) 24 hr upon LPS injection in the substantia nigra.

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and LPS Gal3 KO: $1,213 \pm 130$; Figures 6G and 6H), with a clear decrease in the number (Figure S6C) and M1 polarization phenotype (measured as CD16/32 expression; Figures S6D and S6E) of the microglial population.

Gal3 Contribution to the Inflammatory Response Induced in Global Brain Ischemia Model

Our next step was to assess the importance of Gal3 in a mouse model of global cerebral ischemia that mimics the brain damage caused by cardiac arrest. For this reason, wild-type and Gal3 knockout mice were used, and we found an increase of the survival of the hippocampal neurons in the mice lacking Gal3 (3,100 NeuN-positive neurons in sham; $1,415 \pm 774$ in wild-type and $1,868 \pm 658$ in ischemia-treated animals; Figures 7A and 7B). The increase in the neuronal survival in the Gal3 knockout mice was linked to a lower inflammatory response in terms of hippocampal Iba1 protein expression (Figures 7C and 7D). Mice lacking Gal3 showed a lower body weight reduction (Figure 7E) following ischemia. Also, mice lacking Gal3 show a tendency (although not statistically significant) of ameliorated memory deficits in the hippocampal-dependent Y-maze test 1 week after ischemia (Figure 7F).

Gal3 Interacts with TLR4 in Human Brain Tissue

The expression of Gal3 and TLR4 was also investigated in postmortem brain tissue from patients who had suffered and died from cardiac arrest. High expression of both Gal3 and TLR4 was observed in the ischemia-damaged brain tissue as compared to age-matched controls (Figures S7A–S7D). Both markers were found to be present, suggesting colocalization (Figure S7D). Finally, FRET signal between Gal3 and TLR4 could also be detected in cells in human stroke brain (Figures S7E–S7G).

DISCUSSION

In this study, we show that, under conditions of acute brain inflammation, there is release of endogenous Gal3, which subsequently binds to and stimulates microglial TLR4, thus eliciting a proinflammatory M1 response in the brain. Furthermore, released Gal3 appears necessary to elicit a full-blown activation of microglia in response to proinflammatory stimuli such as LPS.

In ischemia/stroke, microglial cells are highly activated around the site of a brain injury, where they typically express high levels of Gal3 (Inácio et al., 2011; Lalancette-Hébert et al., 2012), a protein known to be a potent immunomodulator in neuroinflammatory disorders. The inflammatory role of Gal3 in brain ischemia appears to be diverse, conceivably depending on the specific neuroinflammatory conditions. This is most likely due to several

factors such as the type of ischemic insult, its timing, and the subcellular localization of Gal3, as well as the immunological status of the individual. In neuroinflammatory models of ALS, Gal3 can induce an anti-inflammatory response (Lerman et al., 2012), whereas in experimental autoimmune encephalomyelitis, Gal3 can exacerbate the disease by reinforcing the inflammatory response (Jiang et al., 2009).

Other members of the galectin family of proteins, despite significant sequence homologies and shared functional capabilities, exert diverse and even sometimes opposite effects on several biological processes. For instance, galectin-1 and galectin-9 illustrate the variety of effects of the galectin family during the inflammatory response. Indeed, galectin-1 can deactivate “classically activated microglia” through binding to the CD45 phosphatase, increasing the microglial surface’s retention time of this glycoprotein and increasing its inhibitory function over the inflammatory response (Starossom et al., 2012). In contrast, galectin-9, acting as a ligand for Tim-3, can trigger a proinflammatory response in naive resting immune cells (such as dendritic cells) and synergizes with the TLR-signaling pathway (Anderson et al., 2007). Here, we show that Gal3 acts as a ligand for TLR4 under the described conditions and at a given time of cellular activation/differentiation. This is driven by CRD-mediated engagement of Gal3 to TLR4-attached carbohydrates (Figure 2). Our data indicate that the CRDs of the other galectin subclasses are capable of binding to TLR4, albeit with lower apparent affinities (Figure S2F; Table S1). The fine specificity that varies between different galectins has been already thoroughly discussed (Carlsson et al., 2007; Salomonsson et al., 2010a; Stowell et al., 2008). This paper focuses on the role of Gal3 in the TLR4-mediated microglial activation. The biological effect of other galectins as TLR4 ligands should be addressed in future studies.

TLR4 is considered to be a key player in the innate inflammatory response, but most of the studies performed in the field of TLR4 are based on LPS administration. Although great advances have been achieved using LPS as TLR4 ligand, its physiological relevance is more related to sepsis than to sterile inflammation (Chen and Nuñez, 2010). To support this, we observe a quantitative difference between LPS and Gal3 in the gene expression profile, most likely because of the low LPS Kd value toward TLR4 (range of nM; Akashi et al., 2003) as compared with Gal3 (range of μM). The results show not only a quantitative but also qualitative difference in the gene expression response after LPS or Gal3 treatment (Figures 1 and S1), which suggests a different TLR4 downstream response depending on the stimulus. In the past years, considerable efforts have been made to identify endogenous ligands that can activate TLR4. As a result, some proteins—i.e., heat shock protein (HSP)-70 and high mobility group box 1 (HMGB1)—and glycosaminoglycans such as hyaluronan

(C) FRET of Gal3 and TLR4 after 24 hr treatment of LPS in substantia nigra.

(D) Comparison of IL-1 β and IL-6 mRNA expression by qPCR between WT LPS-injected mice versus LPS-injected Gal3 knockout mice.

(E and F) Comparison of the levels of macrophages (i.e., Iba-1+ cells with amoeboid morphology) and reactive microglia (i.e., Iba-1+ cells with thick processes) directly in the vicinity of the injection site in WT mice and in Gal3 knockout mice.

(G and H) Quantification of TH⁺ dopaminergic neurons in the nigra 7 days after LPS injection in WT and Gal3 knockout mice.

Data are expressed as mean \pm SEM in (D; $n = 4$), mean \pm SD in (F; $n = 4$), and mean \pm SD in (H; $n = 4$). White arrows show colocalization of the three markers. The percent of FRET is represented as a color bar besides the FRET picture. The scale bar for (B) and (E) represents 27 μm . * $p < 0.05$. White arrows in (B) represent colocalization of the three markers. See also Figure S6.

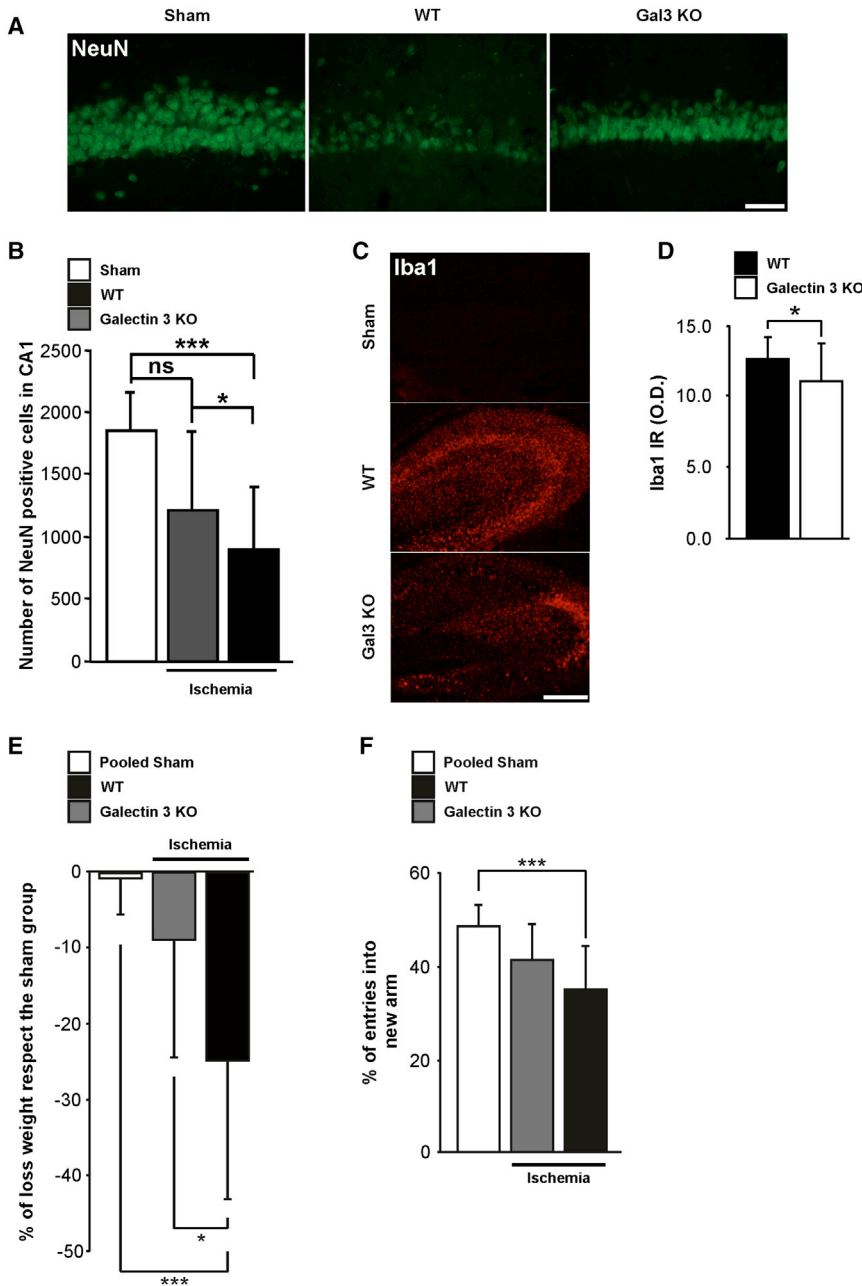


Figure 7. Gal3 Deficiency Ameliorates Microglial Activity, Neuronal Cell Death, and Memory Impairment following Global Brain Ischemia in Mice

(A and B) Representative picture (A) and quantification (B) of viable NeuN+ pyramidal neurons in hippocampal CA1 (A) subregion in sham, Gal3 knockout, and WT mice 8 days following global brain ischemia (B).

(C and D) Reduced inflammatory response in hippocampus measured by Iba1 immunoreactivity in Gal3 knockout mice compared to WT mice.

(E and F) Body weight (E) and memory impairment assessed by the Y-maze behavioral test (F) measurements.

Values are expressed as mean \pm SEM (n = 4) in (B), (D), (E), and (F). The scale bar for (A) represents 50 μ m and for (C) represents 372 μ m. See also Figure S7.

the elevated production and release of Gal3 by microglia under ischemia/stroke condition. These findings indicate that Gal3 can play a decisive role in the expansion and enforcement of the inflammatory response and might potentially contribute to the long-term inflammatory response. New therapies specifically targeting Gal3 released from microglia could counteract some of the deleterious effects resulting from ischemia/stroke.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection, and Reagents

Murine microglial BV2 cell line was cultured as described (Bocchini et al., 1992). Cells were maintained in 10% FCS in DMEM and reduced to 2%–5% FCS while performing the experiments. Transfection of BV2 cells was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendation. LPS (from *Escherichia coli*, serotype 026:B6) and staurosporine were purchased from Sigma-Aldrich. Recombinant Gal3 production and Gal3R186S mutant were prepared as described (Salomonsson et al., 2010a). The purity of Gal3 and mutants proteins were determined by the *Limulus* amoebocyte lysate assay (Charles River Laboratories), and only endotoxin-free proteins were used. The recombinant proteins used for MST and fluorescence anisotropy were obtained from R&D Systems, and the catalog numbers are provided in the Supplemental Experimental Procedures. Non-targeting control, TLR4, and Gal3 siRNAs were obtained from Dharmacon. A complete list of siRNA sequences, primers, and antibodies are provided in the Supplemental Experimental Procedures. In order to study the effect of the released Gal3 over the sustained inflammatory response, cells were treated with 3 μ g/ml of anti-Gal3 antibody or IgG as a negative control, together with LPS for 24 hr, and the inflammatory response checked.

Gal3-null mutant mice (Colnot et al., 1998; C57BL/6 background) were obtained from Dr. K. Sävman/Gothenburg University and housed and bred at Lund University and the Center of Production and Animal Experimentation.

Animals and Surgery

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have been shown to be TLR4 ligands, as reviewed in Chen and Nuñez (2010).

In summary, we demonstrate that (1) Gal3 can be actively released into the extracellular compartment by activated microglial cells, (2) Gal3 binds directly to TLR4 at physiological concentrations, (3) Gal3 itself activates TLR4 and is capable of activating surrounding microglia, (4) Gal3 amplifies the typical TLR4-dependent proinflammatory response, including caspase-mediated inflammation (Burguillos et al., 2011; Venero et al., 2011), and (5) TLR4/Gal3 interaction occurs in the brain of stroke patients as evidenced by FRET analysis. The discovery that Gal3 can act as a TLR4 ligand brings further importance to

The Gal3 $-/-$ and $+/+$ genotyping was performed as described in [Doverhag et al. \(2010\)](#).

Double heterozygous Cx3cr1GFP/+Ccr2RFP/+ mice were generated as previously described in [Mizutani et al., 2012](#) from CX3CR1-GFP knockin and CCR2-red fluorescent protein (RFP) knockin reporter mice ([Jung et al., 2000](#); [Saederup et al., 2010](#)).

Male albino rats weighing 270–320 g were used for Gal3 microdialysis after LPS injection.

Animals had free access to food and water. Experiments were performed in accordance with the Guidelines of the European Union Council (86/609/EU), following Spanish and Swedish regulations and approved by the Ethical Committee for Animal Research (ethical permit numbers M303-09 and N248/13).

In order to model the brain damage following cardiac arrest with successful cardiopulmonary resuscitation, global ischemia was induced in mice ([Olsson et al., 2003](#); [Deierborg et al., 2008](#)). In brief, mice were first anesthetized with 5% isoflurane in oxygen. Thereafter, the anesthesia was maintained at 2% isoflurane (IsobaVet; Schering-Plough Animal Health). A small cut parallel to the trachea was made. The common carotid arteries were isolated and encircled with a thin silk thread to allow occlusion with a micro-aneurysm clip. Ischemia was induced for 13 min. The wound was then sealed with a few absorbable stitches before the anesthesia was discontinued. During the surgery, the body temperature was monitored and controlled by a heating pad and infrared lamp to keep the mice normothermic. The body temperature of the mouse was maintained around 37.5°C during the whole procedure. Mice were housed in an incubator at 34°C overnight in order to maintain normothermia. Sham mice were subjected to the same surgical protocol, except occlusion to the common carotid arteries. The person performing the surgery was blinded to the genotype of the animals.

Intranigral LPS injections (2 μ g in 1 μ l sterile saline) were made 1.2 mm posterior, 1.2 mm lateral, and 5.0 mm ventral to the lambda.

Twenty-four hours later, mice were transcardially perfused under deep anesthesia with 4% paraformaldehyde/PBS (pH 7.4). Brains were removed, cryoprotected in sucrose, and frozen in isopentane at -15°C , and serial coronal sections (25 μ m sections) covering the substantia nigra were cut and further processed for immunohistochemistry.

Statistical Analysis

The differences between control and experimental groups were evaluated with one-way ANOVA with a Bonferroni's post hoc analysis. χ^2 test was used to analyze the up/downregulation of the genes presented in [Figure 1](#). Mann-Whitney test was used to analyze the NeuN-positive cells in [Figure 7B](#). $p < 0.05$ was considered as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.02.012>.

AUTHOR CONTRIBUTIONS

M.A.B. performed all the experiments except as otherwise noted. T.S, H.L., and A.A. determined the K_D between Gal1/Gal3/Gal4-TLR4 by MST and FA. qPCR was performed by A.G.-Q. E.K., M.A.B., M. Svensson, J.L.V., M. Santiago, M.J.O.-M., and T.D. performed the surgery and dissecting of the animal brains. M. Santiago and N.V. performed the microdialysis. A.M.O. and K.B. performed and analyzed the experiments in the Cx3cr1GFP/+Ccr2RFP/+ double-heterozygous mice. M.A.B., T.D., A.B.-S., and M. Svensson performed primary cell culture experiments and cytokine analysis. E.S. and H.L. produced and prepared the protein. B.J. collaborated in the confocal imaging analysis. M. Svensson and T.D. performed the behavioral tests. E.E. did the neuropathology analysis of the individuals with stroke and control cases. A.P. prepared tissue and participated in the morphological assessment of human brain specimens. L.A. was involved in the study design. M.A.B., J.L.V., B.J., and T.D. designed the study and analyzed and interpreted the data. All authors discussed the results and commented on or edited the manuscript. The first draft of the

paper was written by M.A.B. and T.D. B.J., and J.L.V. discussed the results and commented on or edited the manuscript.

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