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# Inflammatory signals induce the expression of tonicity-responsive enhancer binding protein (TonEBP) in microglia

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ABSTRACT

Tonicity-responsive enhancer (TonE) binding protein (TonEBP) is known as an osmosensitive transcription factor that regulates cellular homeostasis during states of hypo- and hypertonic stress. In addition to its role in osmoadaptation, growing lines of evidence suggest that TonEBP might have tonicity-independent functions. In particular, a number of studies suggest that inflammatory stimuli induce the expression and activation of TonEBP in peripheral immune cells. However, whether TonEBP is expressed in microglia, resident immune cells of the central nervous system, is unknown. Here we show that inflammatory signals induce the expression of TonEBP in microglia both *in vitro* and *in vitro*. In cultured primary microglia, treatment with lipopolysaccharide (LPS), interferon-γ, and interleukin 4 increased the expression of TonEBP. Moreover, we found that stereotaxic injection of LPS into the substantia nigra region of rat brain increased TonEBP expression in OX-42-positive cells. Furthermore, expression of TonEBP was induced in OX-42-positive cells in a rat model of transient middle cerebral artery occlusion. Together these results show that the expression of TonEBP is regulated by inflammatory signals in mammalian brain, suggesting that TonEBP might play a part during neuroinflammation.



## 1. Introduction

Microglia, the resident macrophages of the central nervous system (CNS), represent the major immune-competent cell type in the brain (Nayak et al., 2014). A major role of microglia is to survey the CNS environment and serve as first line defenders against infectious agents. Activation of microglia in response to noxious stimuli triggers inflammatory processes characterized by the secretion of pro-inflammatory mediators and the induction of several enzymes responsible for the production of reactive molecules. Fundamentally, immune system functions to protect the CNS from infection, tissue damage, or metabolic derangements, but if the condition causing acute inflammation persists for a prolonged period of time and transits to a chronic phage, neuroinflammation can be detrimental and contribute to the development and progression of a number of neurodegenerative diseases (Lee et al., 2009). Therefore, identifying the players and molecules as well as investigating the regulatory mechanisms of neuroinflammation are of crucial importance.

Tonicity-responsive enhancer binding protein (TonEBP), also known as nuclear factor of activated T-cells 5 (NFAT5), is an essential transcription factor involved in the maintenance of cellular homeostasis against hypo- or hypertonic osmotic stress (Halterman et al., 2012b; Miyakawa et al., 1999). Hypertonic condition is known to induce TonEBP expression, nuclear translocation and association with target genes that control osmoadaptation. In addition to the well-documented role of TonEBP in osmoadaptation, previous studies have implied that TonEBP might have tonicity-independent functions

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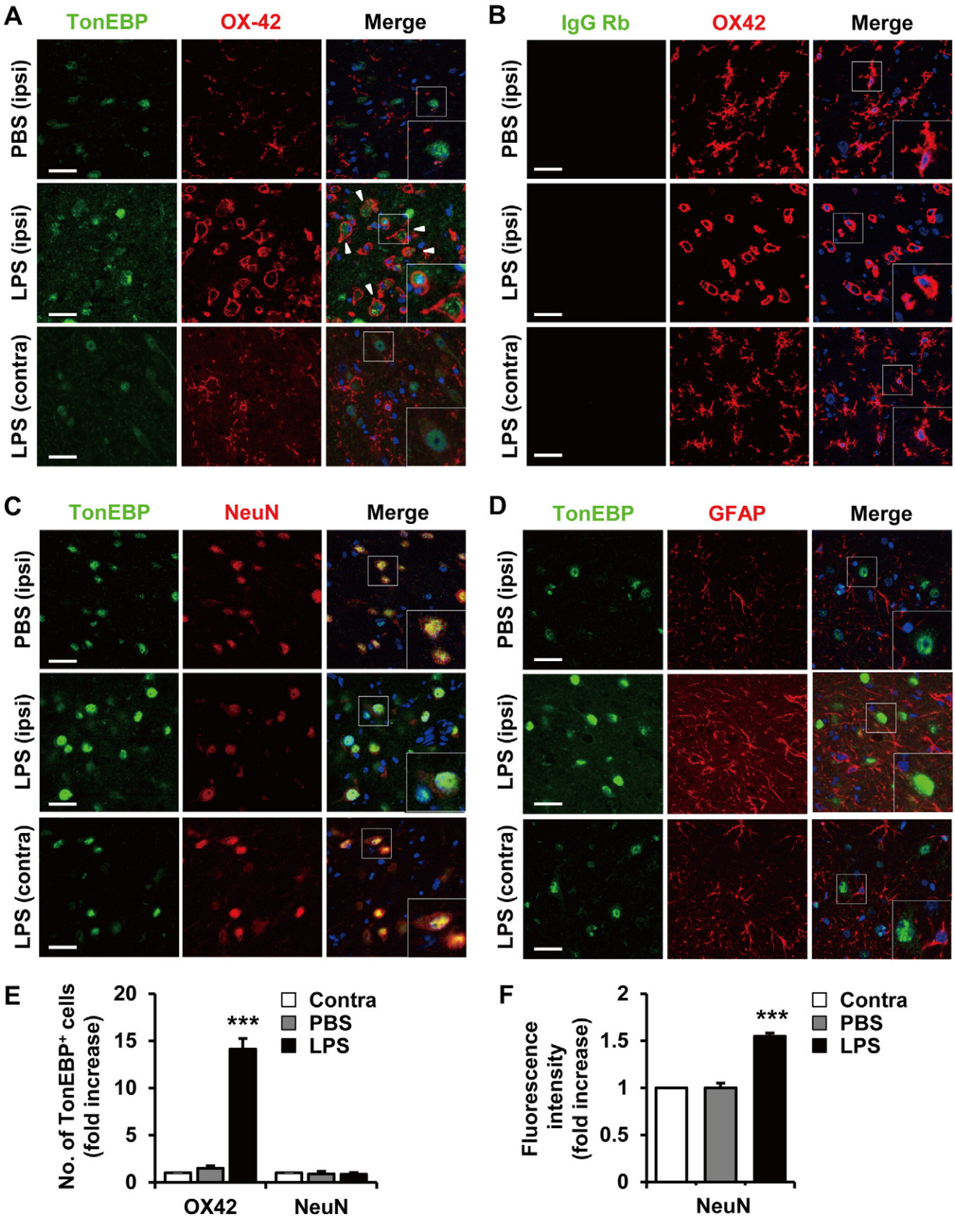
in a wide range of processes, such as inflammation, embryogenesis and development, tumor metastasis, and hepatic detoxification (Berga-Bolanos et al., 2010; Go et al., 2004; Halterman et al., 2012b; Levy et al., 2010; Lopez-Rodriguez et al., 2004). Notably, tonicity-independent signals, such as inflammatory stimuli, have been suggested to regulate TonEBP in several physiological and pathological conditions. In particular, elevated expression or activation of TonEBP has been reported in a number of inflammatory diseases including atherosclerosis,



diabetic nephropathy, and rheumatoid arthritis (Halterman et al., 2012a; Yang et al., 2006; Yoon et al., 2011). In a rodent model of atherosclerosis, haploinsufficiency of TonEBP (TonEBP+/−) suppressed atherosclerotic lesion formation, which was accompanied by impaired macrophage migration (Yoon et al., 2011), and decreased arthritis severity with limited inflammatory cell infiltration, joint destruction, and synovial hyperplasia (Halterman et al., 2012a). These results suggest that TonEBP might play a part in the regulation of inflammatory functions in immune cells.

Microglia in the brain exert functions similar to that of macrophages in the periphery. To the best of our knowledge, under both physiological and pathological conditions, expression of TonEBP in microglia has not been reported to date. In normal rat brain, TonEBP expression appears to be widespread and heterogeneous (Maallem et al., 2006), but previous studies have shown that TonEBP is expressed primarily in neurons rather than glial cells, and its localization is restricted in the nucleus. In rats subjected to acute or prolonged systemic hypertonicity, TonEBP expression increased in the nucleus (Loyher et al., 2004; Maallem et al., 2006). In addition to neurons, faint nuclear labeling of TonEBP was detected in non-neuronal cells in the brain, but only in a very minor proportion of astrocytes and oligodendrocytes. The level in such cells, however, did not increase in animals which received i.p. injections of hypersonic sucrose solution to induce hypertonicity (Loyher et al., 2004; Maallem et al., 2006). Notably, none of the TonEBP-expressing cells stained positive for markers of microglia in both normal isotonic animals and animals subjected to hypertonicity. In this study, we have examined if TonEBP expression is regulated by inflammatory signals in microglia both *in vivo* and *in vitro*. We found that intracranial injection of lipopolysaccharide (LPS) into the substantia nigra (SN) of rat brain and ischemic brain injury induced by middle cerebral artery infarction increased TonEBP expression in microglia *in vivo*. In addition, LPS as well as other inflammatory activators, such as interferon-γ and interleukin 4, increased the expression of TonEBP in primary cultures of microglia. These results suggest that in microglia, TonEBP expression is controlled by inflammatory signals and might play part during neuroinflammation.

## 2. Materials and methods

### 2.1. Animals

Sprague–Dawley rats (SD rat, 8-weeks-old) were acclimatized for 1 week under conditions of controlled temperature (22 ± 2 °C), constant humidity, and a 12-h light/dark cycle, and food and water were made available *ad libitum*. All surgical procedures were conducted according to the animal welfare guidelines approved by the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP(SE)-12-027).

### 2.2. Rat stereotaxic surgery

Female Sprague Dawley (SD) rats (230–280 g) were anesthetized by injection of chloral hydrate (400 mg/kg, i.p.), positioned in a stereotaxic apparatus and received a unilateral administration of phosphate buffered saline (PBS) or LPS (5 μg in 3 μl of PBS; Sigma-Aldrich) into the right SN (anteroposterior (AP) 5.1 mm, mediololateral (ML) 2.0 mm, dorsoventral (DV) 7.9 mm from bregma), according to the atlas of Paxinos and Watson (2005). All injections were performed by using a Hamilton syringe equipped with a 30 s gauge beveled needle attached to a syringe pump (KD Scientific, MA, USA). Infusions were performed at a rate of 0.5 μl/min for LPS or PBS as a control. After injection, animals were euthanized by cervical dislocation and brains were harvested at indicated time points.

### 2.3. Focal cerebral ischemia rat model induction and treatment

Focal cerebral ischemia was induced by MCAo using the intraluminal suture method, as described elsewhere (Lee et al., 2012). Briefly, rats were anesthetized with isoflurane in $N_2O$ and $O_2$ (70:30). MCAo was induced by inserting sutures (360 μm in thicknesses; Doccol Co., NM, USA) 19–20 mm from the bifurcation of the internal and external carotid arteries for 2 h. Reperfusion was achieved by retracting the sutures. In the sham-operated group, rats were subjected to identical procedure, except for the probe insertion length (10 mm). Rectal temperatures were monitored and controlled throughout surgery (37 ± 0.5 °C). Brains were dissected at 24 h after MCAo.

### 2.4. Tissue preparation

Animals were transcardially perfused with a saline solution containing 0.5% sodium nitrate and heparin (10 U/ml) and then fixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer. Brains were removed from the skull, post fixed overnight in buffered 4% paraformaldehyde at 4 °C, stored in a 30% sucrose solution at 4 °C until they sank, and were frozen sectioned on a sliding microtome to 30 μm-thick coronal sections. At least four sections per animal were tested and all sections were collected and processed for immunostaining, as described previously (Chung et al., 2012).

### 2.5. Double immunofluorescence staining

For double-immunofluorescence staining, tissue sections were processed as described elsewhere (Park et al., 2007). Briefly, sections were collected in PBS and sections were incubated in 0.2% Triton X-100 for 30 min and rinsed three times with 0.5% BSA. The sections were then incubated with the indicated primary antibodies (rabbit anti-TonEBP (Miyakawa et al., 1999), 1:1000; mouse anti-OX-42, 1:400 (Serotec, UK); mouse anti-NeuN, 1:1000 (Millipore, US); mouse anti-GFAP 1:1500 (Abcam, US)) overnight at 4 °C. After washing in PBS, the sections were incubated with secondary antibodies (FITC-conjugated rabbit anti-rat IgG, 1:1000; Texas Red-conjugated donkey anti-goat IgG, 1:1000 (Abcam, USA)) for 1 h at room temperature. Floating sections were mounted on gelatin-coated slides and dried for 1 h at room temperature. Slides were mounted by adding Vectashield medium (Vector Laboratories). Slides were imaged by using an LSM 700 confocal laser scanning microscope (Carl Zeiss, Germany).

### 2.6. Mesencephalic microglia culture

Primary microglia were prepared from the ventral mesencephalons of embryonic 14 day brains of SD rats, as described elsewhere (Kim et al., 2000) with some modifications. Briefly, tissues were triturated and dissociated cells were plated into 75-$cm^2$ T-flasks pre-coated with

**Fig. 1.** LPS induces TonEBP expression in OX-42-positive cells *in vivo*. PBS or LPS (5 μg in 3 μl of PBS) was injected unilaterally into the SN. After three days, rats were sacrificed, and brains were removed and sectioned for immunostaining, as described in the Materials and methods. The SN sections were co-immunostained with anti-OX-42 and anti-TonEBP (A), anti-OX-42 and rabbit immunoglobulin (IgG) (B), anti-NeuN and anti-TonEBP antibodies (C), or anti-GFAP and anti-TonEBP antibodies (D), as indicated. Arrowheads show the expression of TonEBP specifically in the nucleus of OX-42-positive cells. In both PBS- and LPS-injected brain sections, TonEBP was detected in NeuN-positive cells, but not in GFAP-positive cells. At least four animals were tested and similar results were obtained in all animals examined. Scale bar, 30 μm. Shown are representative images from the ipsilateral (LPS, *middle* panels) or contralateral side of LPS (LPS, *lower panels*) or ipsilateral side of PBS (PBS, *upper panels*) injection. (E) Quantification of the number of cells expressing TonEBP in SN sections injected with LPS or PBS. Cells expressing TonEBP in $OX\text{-}42^+$ or $NeuN^+$ cells were counted and values were presented as fold increase compared to the number of $TonEBP^+$ cells in SN sections of the contralateral side. Note that the number of $TonEBP^+$ cells markedly increases in $OX\text{-}42^+$ cells but not in $NeuN^+$ cells. (F) Quantification of fluorescence intensity of TonEBP signal in $NeuN^+$ cells. SN sections from rats injected with PBS or LPS were immunostained with anti-NeuN and anti-TonEBP antibodies and TonEBP signals were quantified in $NeuN^+$ cells. *** $p < 0.001$; Student's *t*-test *versus* contralateral side of PBS injection.

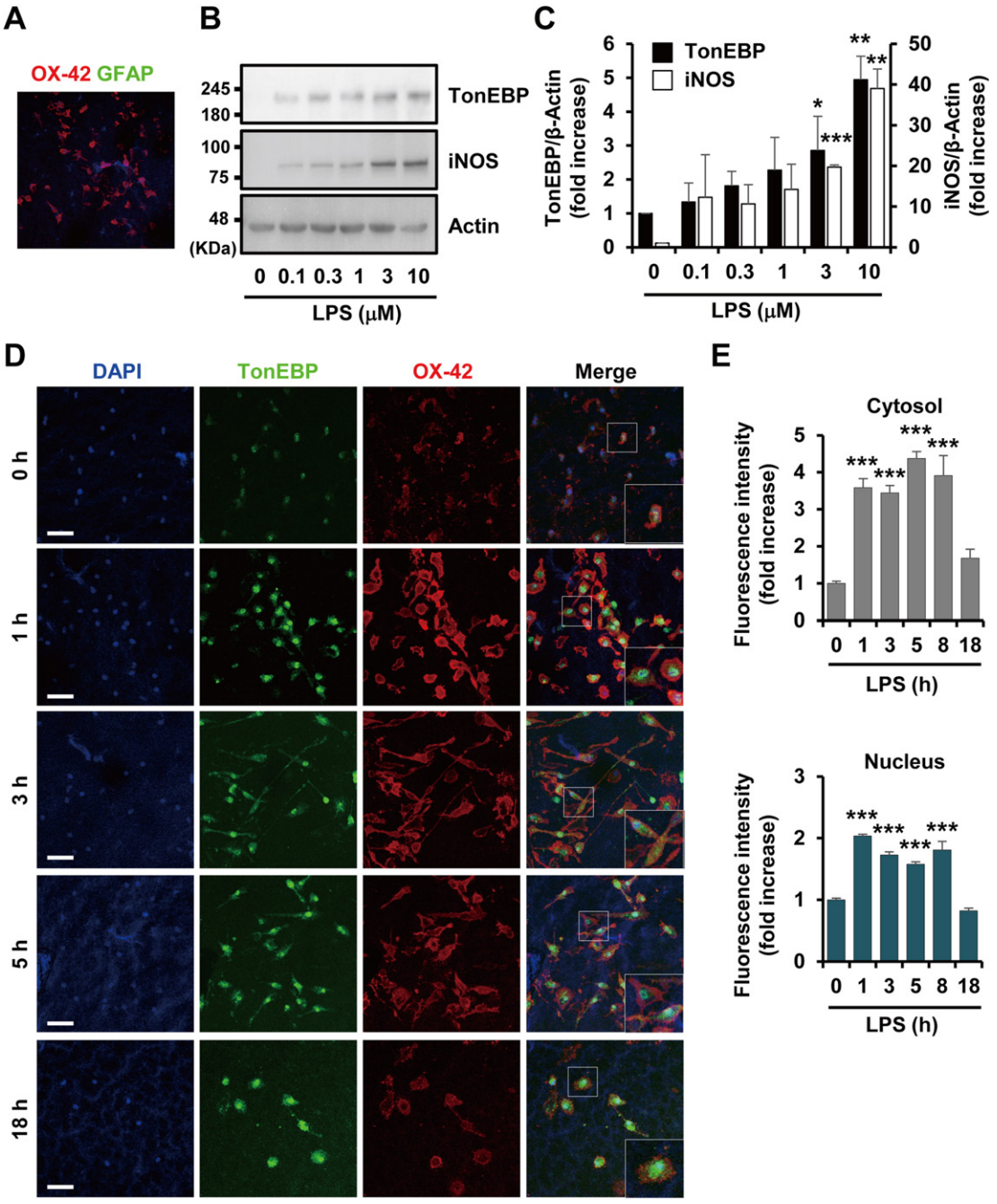



**Fig. 2.** TonEBP expression is increased in response to LPS treatment in primary cultures of microglia. Primary microglia were isolated from mesencephalon tissue of embryonic 14 day brain and cultured, as described in the Materials and methods. (A) Representative image of microglia culture at DIV18, immunostained with anti-OX-42 and anti-GFAP antibodies. Typical cultures contained few $GFAP^+$ cells. At DIV 18, different doses of LPS were treated, as indicated, and cell lysates were subjected to Western blot analysis using anti-TonEBP, iNOS, or β-actin antibodies. Note that LPS induced the expression of TonEBP as well as iNOS in microglia in a concentration-dependent fashion. Representative immunoblots (B) and quantification (C) from three independent experiments are shown. (C, D) Microglia were treated with LPS (10 μM) for different periods of time (0, 1, 3, 5, 18 h), as indicated. Cells were fixed and stained for TonEBP and OX-42. TonEBP expression was increased in both cytosol and nucleus after treatment of LPS. Shown are representative images (D) and quantification of TonEBP signals in the cytosol and nuclei (E) from three independent experiments. Scale bar, 50 μm. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA, followed by Newman–Keuls post-hoc test *versus* vehicle control.

poly-D-lysine at a density of $1 \times 10^7$ cells/flask, and then maintained in DMEM supplemented with 10% FBS. When the mixed cell cultures reached confluency in approximately 2–3 weeks post plating, microglia were separated from the mixed primary culture by gentle shaking and tapping of the flasks. Detached cells were applied to a nylon mesh to remove astrocytes and microglia were then seeded as appropriate for each experimental analysis (see below). Cells were grown in MEM supplemented with 10% FBS. LPS was serially diluted in the medium before treatment to minimize changes in osmolality. After 24 h, LPS was treated, as indicated, and cells were subjected to Western blot analysis or immunocytochemistry. For NaCl treatment, we added 25 μl of 25-fold concentrated NaCl solution to 475 μl of culture media to increase NaCl concentration to 75 mM.

### 2.7. Western blot analysis

Primary microglia were plated into 60 mm culture plates pre-coated with poly-D-lysine at a density of $5 \times 10^5$ cells/well. After treatment

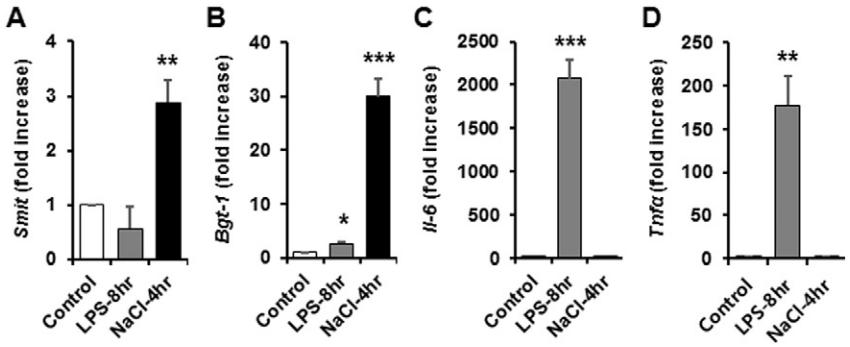



**Fig. 3.** Analysis of immunomodulatory and osmomodulatory gene expression in response to LPS and NaCl treatment. Primary microglia were isolated from mesencephalon tissue of embryonic 14 day brain and cultured, as described in the Materials and methods. At DIV 18, microglia were treated with LPS (10 μM, 8 h) or NaCl (75 mM, 4 h), as indicated, and were subjected to qRT-PCR. mRNA levels of *sodium/myoinositol cotransporter* (*smit*) (A), *sodium/chloride/betaine cotransporter* (*bgt-1*) (B), *interleukin-6* (*il-6*) (C), and *tumor necrosis factor-alpha* (*tnf-α*) (D) were examined and normalized against the level of β-actin. Note that LPS increased mRNA levels of *il-6* and *tnf-α*, but had no effect on *smit* and only marginally increased *bgt-1*. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Student's *t*-test *versus* vehicle control.

with differing concentrations of LPS, recombinant IFN-γ (Novus, US), or recombinant IL-4 (PeproTech, KR) at 24 h after plating, cells were washed with PBS. Cell lysates were prepared by adding 1 × SDS loading buffer (60 mM Tris–HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue), followed by vigorous shaking and boiling. Protein extracts were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% skim milk in TBS-T (10 mM Tri-HCl (pH 7.5), 10 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and probed with the indicated antibodies (TonEBP; (Miyakawa et al., 1999), 1/3000; iNOS (Santa Cruz Biotechnology, Inc.), 1/1000; Actin, (Abcam), 1/5000). Signal was developed with the Enhanced Chemiluminescence (ECL) detection system (Millipore).

### 2.8. Immunocytochemistry

Primary microglia were plated onto 12 mm coverslips pre-coated with poly-D-lysine at a density of $1 \times 10^5$ cells/coverslip. Cells were fixed with 4% paraformaldehyde, washed twice with 0.1% bovine serum albumin (BSA) in PBS, and then permeabilized in 0.2% Triton X-100 for 30 min. To compare fluorescence intensity all sections were immunostained side-by-side. Primary antibodies (rabbit anti-TonEBP, 1:1000; mouse anti-OX-42, 1:400, mouse anti-GFAP, 1:1500) were applied for 1 h at room temperature. After washing in 0.1% BSA/PBS, secondary antibodies (FITC-conjugated rabbit anti-rat IgG, 1:200; Texas Red-conjugated donkey anti-goat IgG, 1:200) were applied for 1 h at room temperature. After three times of washing in 0.1% BSA/PBS, coverslips were mounted with Vectashield medium (Vector Laboratories). Samples were imaged by using an LSM 700 confocal laser scanning microscope (Carl Zeiss, Germany). To quantify fluorescence intensity in the cytosol and the nucleus in response to LPS treatment, all identifiable cells from three independent experiments were photographed and

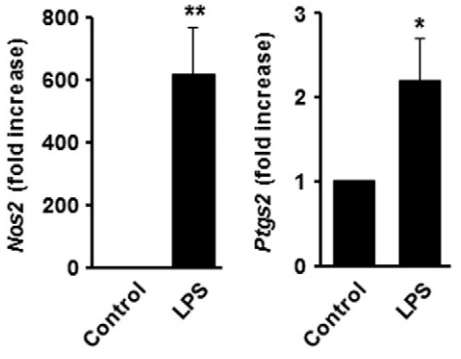



**Fig. 4.** LPS induces the expression of *nitric oxide synthase 2* (*nos2*) and *prostaglandin-endoperoxide synthase 2* (*ptgs2*) in primary cultures of microglia. Primary microglia were isolated from mesencephalon tissue of embryonic 14 day brain and cultured, as described in the Materials and methods. At DIV 18, microglia were treated with LPS (10 μM, 8 h), as indicated, and were subjected to qRT-PCR. mRNA levels of *nitric oxide synthase 2* (*nos2*) and *prostaglandin-endoperoxide synthase 2* (*ptgs2*) were examined and normalized against the level of β-actin. LPS induced mRNA levels of *nos2* and *ptgs2*. $^{*}p < 0.05$, $^{**}p < 0.01$; Student's *t*-test *versus* vehicle control.

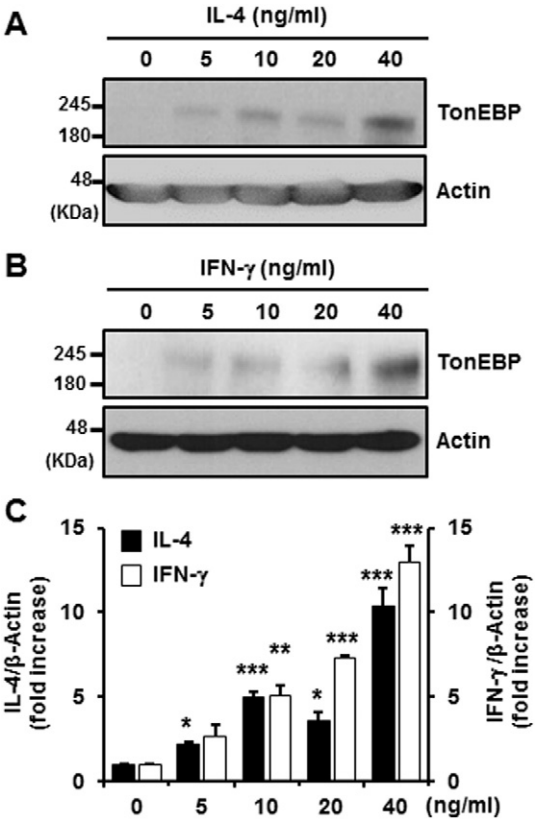
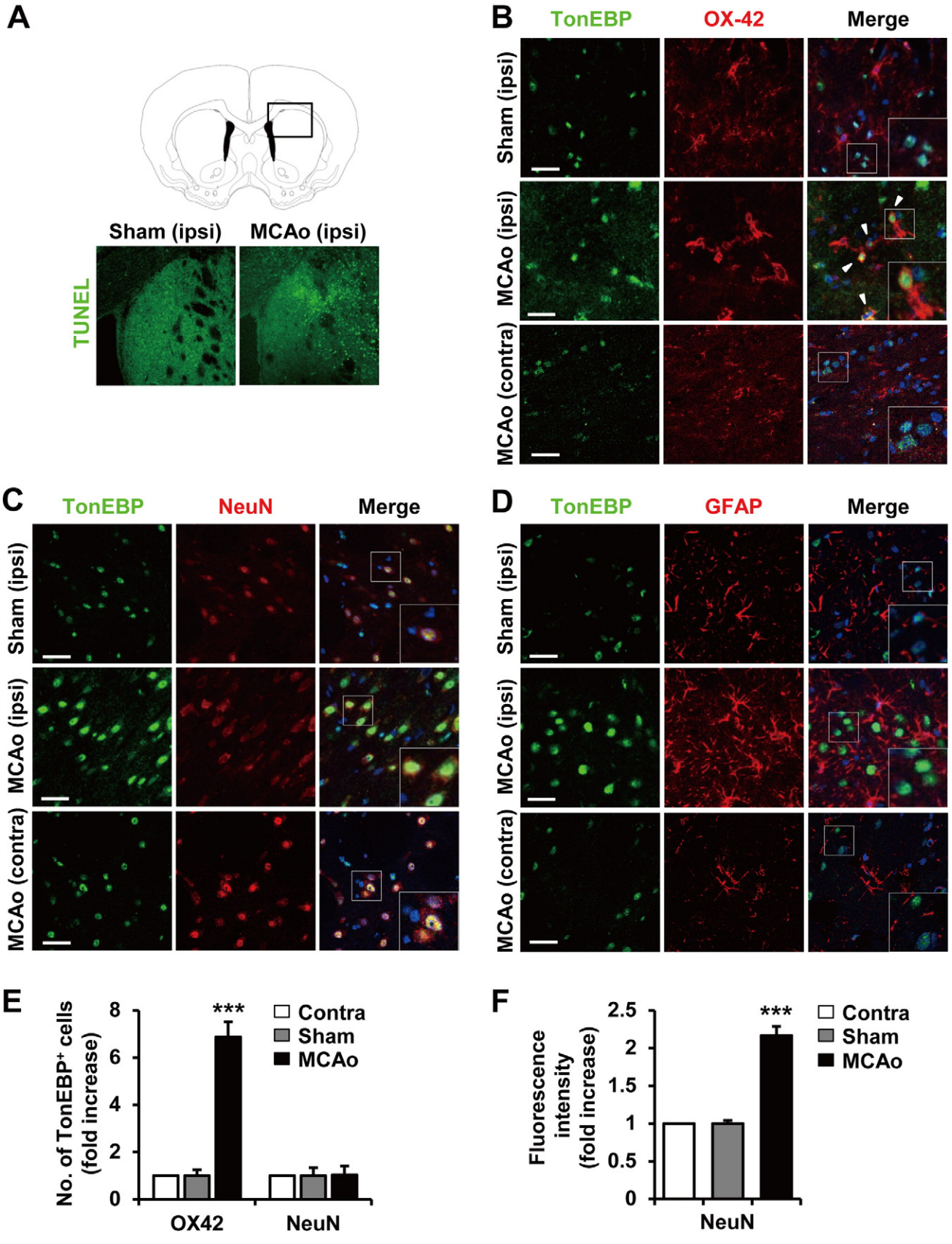



**Fig. 5.** TonEBP expression is increased in response to interleukin-4 and interferon-γ treatment in primary cultures of microglia. Primary microglia were isolated from mesencephalon tissue of embryonic 14 day brain and cultured, as described in the Materials and methods. At DIV 18, various concentrations of recombinant rat interleukin-4 (IL-4) and interferon-γ (IFN-γ) were treated, as indicated, and cell lysates were subjected to Western blot analysis using anti-TonEBP and β-actin antibodies. Note that IL-4 and IFN-γ induced the expression of TonEBP in microglia. Representative immunoblots (A, B) and quantification (C) from three independent experiments are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA, followed by Newman–Keuls post-hoc test *versus* vehicle control.

included in the analysis. The level of fluorescence in a given region (cytosol or nucleus, as indicated) was measured by using ImageJ software.

### 2.9. RNA extraction and real-time quantitative PCR

Primary microglia were plated into 60 mm culture dish pre-coated with poly-D-lysine at a density of $5 \times 10^5$ cells/dish. After treatment



with LPS (10 μM for 8 h) or NaCl (75 mM for 4 h), cells were prepared by homogenization using TRIzol reagent (Invitrogen), phenol-chloroform extraction (Junsei) and isopropanol precipitation (Merck millipore). The RNA was reverse-transcribed to yield first-strand cDNA using reverse transcriptase (Promega). The cDNA was mixed with pairs of primers. The sequences for the forward and reverse primers were as follows: rat *interleukin-6* (*il-6*; 5′-TCCTACCCCAACTTCCAATGCTC-3′ and 5′-TTGGATGGTCTTGGTCCTTAGCC-3′) and *tumor necrosis factor-α* (*tnf-α*; 5′-AAATGGGCTCCCTCTCATCAGTTC-3′ and 5′-TCTGCTTGGTGGTTTGCT ACGAC-3′) and *betaine-γ-amino-butyric acid transporter* (*bgt-1*; 5′-CCTCCATGGCCTGTGTACCGC-3′ and 5′-GAGTTCTTGCTTGACTGGAGAG-3′), *sodium dependent myo-inositol cotransporter* (*smit*; 5′-TGAACACT TCATTGGGCTGGTA-3′ and 5′-GAGCGGATGTAAATAGGGATGAAA-3′), *nitric oxide synthase2* (*nos2*; 5′-ACACAGTGTCGCTGGTTTGA-3′ and 5′-AACTCTGCTGTTCTCCGTGG-3′), and *prostaglandin-endoperoxide synthase2* (*ptgs2*; 5′-TCACCCGAGGACTGGGCCAT-3′ and 5′-CAGCGAACCG CAGGTGCTCA-3′). Step One Plus Real-Time PCR system (Life Technologies) was used for the quantification.

### 2.10. Statistical analysis

Student's *t*-test or one-way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc test was used. Data represents mean ± S.D. $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. TonEBP expression is increased in microglia in a rodent model of neuroinflammation induced by LPS

On the basis of previous studies reporting the expression of TonEBP in macrophages and other immune cells in peripheral tissues (Berga-Bolanos et al., 2010; Buxade et al., 2012; Halterman et al., 2012a), we have investigated whether TonEBP is expressed in resting and activated microglia. For this purpose, we performed stereotaxic surgery and injected either LPS (5 μg in 3 μl of phosphate-buffered saline (PBS)) or PBS (3 μl) into rat brain. LPS or PBS was stereotaxically delivered unilaterally into the SN, a region which was previously shown to express a relatively high level of TonEBP (Maallem et al., 2006). Three days after the injection, brains were removed, sectioned, and processed for immunostaining. Microglial activation was confirmed by the increase in the number of OX-42-positive cells and morphological changes of microglia (Fig. 1). Microglia in the LPS-injected ipsilateral side transformed from a ramified to an activated form, whereas microglia in the contralateral side of LPS injection or ipsilateral side of PBS injection exhibited ramified morphologies, characteristic of resting microglia (Fig. 1A). We observed that the expression of TonEBP was markedly enhanced in OX-42-positive cells mainly in the nucleus specifically in the ipsilateral side of LPS injection (Fig. 1A, E). We confirmed that neither the contralateral side of the LPS-injected brain nor the ipsilateral side of the PBS-injected control brain showed TonEBP expression in OX-42-positive cells (Fig. 1A, E). No signal was detected by immunostaining with control immunoglobulin (Fig. 1B). Expression of TonEBP in neurons and astrocytes has been reported in normal and kainic acid-induced lesioned brain (Maallem et al., 2006; Shin et al., 2014). Therefore, we examined if LPS induced any changes in the level or localization of TonEBP in neurons and astrocytes by double-labeling with either anti-NeuN or anti-GFAP antibodies and anti-TonEBP antibodies (Fig. 1C–F). Consistent with previous reports, TonEBP was detected in NeuN-positive cells in PBS-injected brain sections. In contrast with $OX\text{-}42^+$ cells, we found that LPS treatment did not alter the number of $TonEBP^+/NeuN^+$ double-positive cells (Fig. 1C, E). However, LPS injection markedly increased the fluorescence intensity of TonEBP signals in NeuN-positive cells (Fig. 1C, F). In GFAP-positive cells, TonEBP was barely detected in both PBS- and LPS-injected brain sections (Fig. 1D). Together, these results suggest that LPS injection induces the expression of TonEBP in microglia and neurons in rat brain, implying a role during neuroinflammation.

### 3.2. LPS induces TonEBP expression in rat primary microglia

Peripheral macrophages are known to infiltrate into the CNS in the condition of neuroinflammation. Histologically, CNS-resident microglia and macrophages that infiltrated into the brain from peripheral tissues cannot be distinguished, as both of these myeloid-origin cell types express common classic macrophage markers (Iba-1, OX-42, F4/80, and Mac-1) (Carson et al., 2006). Thus, the OX-42-positive cells detected in the LPS-injected brain can represent heterogeneous cell population composed of both activated microglia and infiltrated macrophages. To confirm the expression of TonEBP in microglia, we isolated primary microglia from mesencephalon tissue of embryonic 14 day brain and confirmed that more than 95% of cells were OX-42-positive (Fig. 2A). In primary cultures of microglia, LPS treatment increased the expression of inducible nitric oxide synthase (iNOS) in a concentration-dependent manner, as expected. Importantly, we found that TonEBP expression was also induced by LPS treatment in a dose-dependent fashion (Fig. 2B, C). When the cellular localization of TonEBP was examined in primary microglia, faint expression of TonEBP was detected in the nucleus of cells treated with vehicle control. In response to LPS treatment, TonEBP expression markedly increased in both the cytosol and the nucleus (Fig. 2D, E). In the cytosol and nucleus, LPS induced TonEBP expression within 1 h, which returned to basal level when observed at 18 h after treatment. Taken together, results from both *in vivo* and *in vitro* clearly show that LPS induces TonEBP expression in microglia.

In the kidney, where osmolality rapidly fluctuates, activation of TonEBP induces the transcription of osmoprotective genes, such as *sodium/chloride/betaine cotransporter* (*bgt-1*) (Miyakawa et al., 1998) and *sodium/myoinositol cotransporter* (*smit*) (Miyakawa et al., 1999; Rim et al., 1998), which play a role in increasing the concentration of intracellular osmolytes and restoring osmotic balance. We thus examined if LPS stimulation of microglia resulted in the induction of such hyperosmotically stimulated genes (Fig. 3A–D). Results from quantitative real time polymerase chain reaction (qRT-PCR) showed that in striking contrast to the marked induction of *smit* by NaCl, treatment of microglia with LPS failed to induce the expression of *smit*. Similarly, LPS only marginally increased *bgt-1* expression, in contrast to the dramatic induction triggered by NaCl. By contrast, LPS markedly induced the expression of *interleukin-6* (*il-6*) and *tumor necrosis factor-alpha* (*tnf-α*), genes which were not induced by NaCl in microglia.

Previously, in macrophages, TonEBP has been shown to regulate the expression of multiple toll-like receptor (TLR)-induced genes independently of osmotic stress (Buxade et al., 2012). In particular, expression of *Nos2*, *Il6* and *Ptgs2* mRNAs in response to LPS was severely impaired in macrophages lacking TonEBP. Consistent with the induction of TLR-induced genes in macrophages (Buxade et al., 2012), LPS markedly induced the expression of *Il-6* (Fig. 3A), *Nos2* and *Ptgs2* mRNAs in microglia (Fig. 4A–C).

**Fig. 6.** Ischemic brain injury induced by middle cerebral artery occlusion (MCAo) results in TonEBP expression in microglia. Focal cerebral ischemia was induced by transient MCAo, as described in the Materials and methods. Rats were divided into two groups (sham and MCAo) and each group contained at least four animals. (A) Brains were dissected at 24 h after ischemia and brain sections including caudoputamen were used for the analysis (top box). TUNEL was examined at 24 h after MCAo (bottom). Brain sections were co-immunostained with anti-OX-42 and anti-TonEBP antibodies (B), anti-NeuN and anti-TonEBP antibodies (C), or anti-GFAP and anti-TonEBP antibodies (D). Arrowheads show the expression of TonEBP specifically in the nucleus of OX-42-positive cells. (E) Quantification of $OX\text{-}42^+$, $NeuN^+$, or $GFAP^+$ cells expressing TonEBP in sham and MCAo sections, as indicated. Number of TonEBP-expressing cells in each population ($OX\text{-}42^+$, $NeuN^+$, or $GFAP^+$) was counted and normalized against the number in SN sections of the contralateral side. (F) Quantification of fluorescence intensity of TonEBP signal in $NeuN^+$ cells. ***$p < 0.001$; Student's *t*-test *versus* contralateral side of sham control.

### 3.3. Ischemic brain injury induces TonEBP expression in microglia

Our results suggest that conditions that induce neuroinflammation, such as pathogen infection, trauma, stroke and brain injury, might regulate TonEBP expression in microglia. To investigate if inflammatory cytokines control the expression of TonEBP, primary microglia were treated with IFN-γ or IL-4 and the level of TonEBP was examined by Western blot analysis. Similar to LPS treatment, both cytokines, IFN-γ and IL-4 induced TonEBP expression in a dose-dependent fashion (Fig. 5A–C). To investigate if TonEBP expression is controlled by inflammatory signals *in vivo*, we utilized the transient middle cerebral artery occlusion (MCAo) model to induce ischemic brain injury. MCAo was performed through 2 h of occlusion, followed by 24 h of reperfusion. Before examining the induction of TonEBP after MCAo, brain injury in infarcted areas was monitored through TUNEL staining to detect cell death. As previously reported (Lee et al., 2012), damaged areas were located in caudoputamen (Fig. 6A). We observed that MCAo markedly induced the expression of TonEBP in OX-42-positive cells mainly in the nucleus, specifically in the ipsilateral side (Fig. 6B). We confirmed that TonEBP expression was not detected in OX-42-positive cells the ipsilateral side of the sham control brain (Fig. 6B). In sham control brain, TonEBP was mostly, if not all, detected in $NeuN^+$ cells, the level of which was highly increased by MCAo (Fig. 6C). In $GFAP^+$ cells, TonEBP was undetected both in control and MCAo-lesioned brain (Fig. 6D). Together, these results suggest that TonEBP is expressed primarily in neurons, and inflammation induced by either LPS and MCAo triggers TonEBP both in microglia and neurons.

## 4. Discussion

Increasing lines of evidence show that TonEBP is expressed in several isotonic tissues and suggest that tonicity-independent mechanisms exist to regulate TonEBP expression and function (Halterman et al., 2012b). In the brain, although the expression of TonEBP appears widespread (predominantly detected in the nuclei of neurons and in a minor population of astrocytes), expression in microglia has not been reported to date (Loyher et al., 2004; Maallem et al., 2006). To the best of our knowledge, the present study provides the first evidence that TonEBP expression is induced by several inflammatory stimuli in microglia, the major cellular components which play crucial roles in the regulation of innate immunity and inflammation in the CNS.

It is interesting to note that in striking contrast to the marked increase of TonEBP in the brain in response to LPS and ischemic brain injury as we report here, previous studies showed that hypertonicity failed to induce TonEBP expression in microglia (Loyher et al., 2004; Maallem et al., 2006). These results suggest that in microglia, changes in tonicity are not a major regulator of TonEBP expression and TonEBP might be regulated primarily by other pathways. Given that (i) LPS induced the expression of TonEBP with little or subtle changes in the levels of hyperosmotically stimulated genes (such as *smit* and *bgt-1*) (Fig. 3) and that (ii) TonEBP expression was elevated by many other proinflammatory signals both *in vitro* (Figs. 2 and 5) and *in vivo* (Figs. 1 and 6), we favor the hypothesis that TonEBP expression in microglia is controlled by inflammatory signals in a tonicity-independent fashion.

A recent study reported a tonicity-independent regulation of TonEBP in the brain in a rodent model of seizure induced by kainic acid (Shin et al., 2014). Notably, TonEBP expression was specifically elevated in the nuclei of neurons, but not in microglia. Since kainic acid is well known to induce neuroinflammation, it will be interesting to investigate how TonEBP expression can be differentially regulated in specific cell types by distinct inflammatory signals.

LPS is a well-known trigger for inflammation, and as such, LPS treatment leads to the production of various kinds of pro-inflammatory cytokines, reactive oxygen species (ROS), and nitric oxide in microglia. However, the mechanism by which LPS controls the production of such immunomodulators is not fully understood. In peripheral macrophages, it has been shown that LPS induces the expression of TonEBP and that TonEBP, as a transcription factor, plays an essential part in the induction of multiple immunomodulatory genes, such as *tnf*, *il6*, and *nos2* (iNOS) (Buxade et al., 2012). Importantly, such function of TonEBP has been suggested to be independent of its osmomodulatory role (Buxade et al., 2012; Kaltschmidt and Kaltschmidt, 2009; Kim et al., 2013). In fibroblast-like synoviocytes, proinflammatory cytokines, TNF-α and IL-1β could induce the expression and nuclear localization of TonEBP, processes which were also suggested to occur independent of changes in tonicity (Yoon et al., 2011). Here we show that TonEBP is induced by IL-4 and interferon-γ in microglia, suggesting that TonEBP might be involved in M1 and M2 responses. More experiments will be needed to examine if TonEBP is selectively or preferentially induced by certain stimuli and how different cytokine environments control TonEBP function. Thus, the expression and function of TonEBP appear to be closely associated with inflammation both in the periphery (Halterman et al., 2012a; Yoon et al., 2011) and in the brain (current study), suggesting that inflammatory signals might represent important physiological and pathophysiological stimuli that control TonEBP.

In summary, the present study demonstrates that in microglia, inflammatory signals induce the expression of TonEBP both in the brain and in culture. Future research will be necessary to investigate the mechanism by which TonEBP expression is controlled in microglia in pathological neuroinflammatory conditions, such as neurodegenerative diseases, and more importantly, what role TonEBP plays during such conditions.

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