Astrocyte EV-Induced lincRNA-Cox2 Regulates Microglial Phagocytosis: Implications for Morphine-Mediated Neurodegeneration

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INTRODUCTION

Impairment of microglial functions, such as phagocytosis and/or dysregulation of immune responses, has been implicated as an underlying factor involved in the pathogenesis of various neurodegenerative disorders. Our previous studies have demonstrated that long intergenic noncoding RNA (lincRNA)-Cox2 expression is influenced by nuclear factor κB (NF-κB) signaling and serves as a coactivator of transregulatory factors to regulate the expression of a vast array of immune-related genes in microglia. Extracellular vesicles (EVs) have been recognized as primary facilitators of cell-to-cell communication and cellular regulation. Herein, we show that EVs derived from astrocytes exposed to morphine can be taken up by microglial endosomes, leading, in turn, to activation of Toll-like receptor 7 (TLR7) with a subsequent upregulation of lincRNA-Cox2 expression, ultimately resulting in impaired microglial phagocytosis. This was further validated in vivo, wherein inhibition of microglial phagocytic activity was also observed in brain slices isolated from morphine-administered mice compared with control mice. Additionally, we also showed that intranasal delivery of EVs containing lincRNA-Cox2 siRNA (small interfering RNA) was able to restore microglial phagocytosis. This was further validated in vivo, wherein inhibition of microglial phagocytic activity was also observed in brain slices isolated from morphine-administered mice compared with control mice. Additionally, we also showed that intranasal delivery of EVs containing lincRNA-Cox2 siRNA (small interfering RNA) was able to restore microglial phagocytic activity in mice administered morphine. These findings have ramifications for the development of EV-loaded RNA-based therapeutics for the treatment of various disorders involving functional impairment of microglia.

Extracellular vesicles (EVs) comprising microvesicles, exosomes, and apoptotic bodies play important roles as cargo-carrying vesicles mediating communication among diverse cells types and tissues, including the CNS.15–18 Studies by others15 and us16 have demonstrated that EVs released from astrocytes, the most abundant cell type in the CNS, can modulate immune responses. Phagocytic cells via their cell surface receptors can recognize pathogenic microorganisms and cellular debris that, in turn, trigger the internal cellular machinery resulting in the ingestion of the particles into the phagosomes, which can fuse with the lysosomes to be ultimately degraded in the phagolysosomes.1,2 It has been well documented that the “find-me” and “eat-me” signals released by apoptotic cells serve to attract the phagocytes, resulting in the engulfment and clearance of apoptotic cells to maintain tissue homeostasis.9,10 In the CNS, impairment of microglial phagocytosis renders the microglia unable to engulf and eliminate toxic pathogens and cellular debris, ultimately culminating in a toxic inflammatory milieu, a common feature underlying various neurodegenerative disorders.11,12 Microglia express a wide array of phagocytic receptors that are involved in receptor-mediated phagocytosis in the CNS.13 Dysregulated expression of microglial phagocytic receptors has been shown to result in impaired microglial phagocytosis.14 The current study was undertaken to understand how morphine exposure impairs microglial phagocytosis involving the astrocyte-microglial crosstalk.

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CNS, can be conduits for delivering the cargo of macromolecules to neighboring as well as distant cells, culminating in a wide spectrum of functional changes in the recipient cells. Exploiting this cargo delivery function of EVs has been critical in understanding how EVs can also be developed as vehicles for targeted delivery of therapeutic drugs to tissues. For example, an exciting study by Zhuang et al. has demonstrated that intranasal delivery of exosomes loaded with curcumin can target the microglia, resulting in reduction of lipopolysaccharide (LPS)-induced neuroinflammation.

Long intergenic noncoding RNAs (lincRNAs) are regulatory RNA molecules and play pivotal roles in the regulation of many cellular processes. Our previous studies have demonstrated that the transcription of lincRNA-Cox2 is regulated by the nuclear factor κB (NF-κB) signaling pathway and is required for the transcription of late primary inflammatory-response genes. In the current study, we demonstrate that morphine-stimulated astrocyte-derived EVs (morphine-ADEVs) can be taken up by microglial cells, leading, in turn, to impaired microglial phagocytosis via the Toll-like receptor 7 (TLR7)-NF-κB-lincRNA-Cox2 axis. Additionally, we also demonstrate that intranasal delivery of lincRNA-Cox2 small interfering RNA (siRNA) can restore microglial phagocytic activity in morphine-administered mice.

RESULTS
Morphine Induces Release of EVs from Astrocytes
Astrocytes perform essential functions to sustain homeostasis within the CNS, including proper maintenance of water and ion balance, removal of extracellular glutamate, and release of neurotrophic factors. In addition, astrocytes have developed multiple avenues of intercellular communication and transfer of various factors including RNA. It is well known that astrocytes can communicate via direct cell-cell interactions and gap junctions; however, over the last few years, it is increasingly being appreciated that astrocytes also communicate with many, if not all, cells within the CNS, through the release and uptake of EVs. Herein we first sought to isolate and characterize EVs from conditioned media of human astrocyte A172 cells, as well as mouse and human primary astrocytes, using a differential ultracentrifugation procedure (Figure 1A). Purified EVs were characterized by western blot for signature exosomal markers, as well as by transmission electron microscopy (TEM) and atomic force microscopy (AFM). As shown in Figure 1, immunoblotting of the EV lysates revealed the presence of exosomal markers TSG101, CD63, and Alix. Additionally, immunoblotting using calnexin was also done to demonstrate that isolated ADEVs were free of contamination with cell debris (Figure 1B). Further characterization of EVs using TEM and AFM demonstrated EVs ranging from 40 to 100 nm in diameter (Figures 1C and 1D). Given that astrocytes express high levels of mu opioid receptors and have been shown to respond to morphine, we next sought to examine the effect of morphine on the number and size of ADEVs using nanoparticle tracking analysis (NTA). As shown in Figures 1E and 1F, exposure of astrocytes to morphine (10 μM, 24 hr) resulted in significantly increased numbers of released EVs compared with the control cells.

There was, however, no effect on the size distribution of EVs in the presence of morphine.

EVs Are Taken up by Microglia and Reach the Endosomes
Because microglia are the primary phagocytic immune cells within the CNS, we next asked whether ADEVs could be taken up by microglia and, if so, could they reach the endosomes, the site for RNA-sensing TLRs. For this, we cultured mouse primary microglia with EVs isolated from mouse primary astrocytes that were transfected with a plasmid encoding the exosomal marker TSG101 fused with mCherry. As observed in Figure 2A, mouse primary microglia cultured with RFP-tagged EVs (30 min) demonstrated a fine granular fluorescent pattern within the cytoplasm, suggesting thereby that EVs could reach and release their contents into the microglial cells. We next sought to assess whether ADEVs taken up by microglia could reach the endosomes; mouse primary microglia were exposed to mouse primary astrocyte-derived RFP-tagged EVs for 30 min, followed by harvesting of cells for localization of EVs at the endosomes.

Endosomal receptors TLR7 and TLR8 are known to bind AU- or GU-rich single-stranded RNA ligands, leading to activation of the downstream NF-κB signaling pathway. For example, Forsbach et al. have identified 15 AU- or GU-containing 4-mers that function as TLR7/8 RNA agonists. Interestingly, EV microRNAs (miRNAs) containing the TLR7/8 binding motifs have also been shown to function as TLR7/8 agonists. Based on our findings that ADEVs taken up by the microglia could reach the endosomes, we next sought to investigate whether ADEV-miRNAs could also serve as TLR7/8 RNA agonists. For this, we performed an TLR7/8 binding elements search within the known mouse mature miRNA sequence database (miRBase, version 21). Moreover, the extracellular mouse miRNAs annotation was also obtained from the EV miRNA databases, ExoCarta and EVpedia. We found that almost 1,079 out of 2,049 mouse miRNAs contained at least one AU- or GU-rich 4-mers, as reported by Forsbach et al., with 38 of them reported to be present in EVs (Figure 2C, Table S1). Based on these 38 reported EV-miRNAs, which are potential TLR7/8 RNA agonists, we next conducted motif prediction using MDS2, and three possible motifs were predicted (Figure 2D) covering 66.0% to 79.0% of the 38 EV-miRNAs as potential TLR7/8 RNA agonist. Next, we sought to examine whether morphine-ADEVs also contained the TLR7/8 motifs. For this, RNA was purified from control- or morphine-ADEVs isolated from human primary astrocytes and subjected to RNA-sequencing (RNA-seq) analyses. As shown in Figure 2E, 15 of the 4-mer containing miRNAs were significantly upregulated and, 9 of the 4-mer containing miRNAs were downregulated in morphine-ADEVs compared with control-ADEVs. Selective differentially expressed miRNAs identified from the RNA-seq analysis were further validated using qPCR (Figure 2F). Interestingly, the total reads from the RNA-seq data for miRNAs in
morphine-ADEVs were significantly increased compared with that in control-ADEVs, indicating thereby that morphine-ADEV-miRNAs could serve as TLR7/8 RNA agonists in the recipient microglia.

**Morphine-ADEV-Induced Nuclear Translocation of Endogenous NF-κB p65 Involves Endosomal TLR7 Signaling in Microglia**

Having determined that EVs can reach the endosomes and deliver AU- and GU-rich miRNAs, which could activate TLR7/8, the next logical step was to investigate the downstream signaling of the TLR7/8 pathway, in particular, nuclear translocation of NF-κB and microglial activation. To this end, we thus sought to examine whether morphine-induced EVs released from astrocytes could activate NF-κB in microglia. Mouse primary microglia were stimulated with either control- or morphine-ADEVs isolated from mouse primary astrocytes for 30 min followed by immunocytochemistry for translocation of p65. As shown in Figure 3A, microglial cells stimulated with morphine-ADEVs demonstrated significantly increased translocation of NF-κB p65 from the cytoplasm into the nucleus compared with cells exposed to control-ADEVs (Figure 3A). Intriguingly, and as expected, morphine-ADEVs failed to promote nuclear translocation of p65 in TLR7 knockout (KO) microglial cells (Figure 3B). These findings were further confirmed by immunoblotting. To further investigate whether morphine-ADEV-mediated translocation of NF-κB could be attributed to the miRNA cargo in the EVs, we transfected mouse primary astrocytes with either control or Dicer-siRNA for 24 hr, and subsequently exposed them to morphine for an additional 24 hr. EVs were then isolated from the conditioned media and exposed to either wild-type (WT) or TLR7 KO mouse microglial cells for 30 min, followed by detection of nuclear translocation of the NF-κB p65 subunit by western blotting and fluorescent immunostaining. As shown in Figures 3C–3E...
and Figure S1, EVs isolated from morphine-stimulated, Dicer KO astrocytes failed to induce nuclear translocation of NF-κB p65 in WT microglia. These data thus underpin the pivotal role of miRNA cargo of the morphine-ADEVs in activating the TLR7-NF-κB signaling pathway.

Figure 2. EVs Are Internalized by Microglial Cells and Localize to Late Endosomes

(A and B) Mouse primary microglial cells were incubated with EVs purified from pEF6.mCherry-TSG101-transfected mouse primary astrocytes for 20 min. Paraformaldehyde fixed microglial cells were permeabilized and stained for (A) Iba1 or (B) EEA1, followed by FITC-conjugated secondary antibody and visualized by fluorescence microscopy. Scale bars, 10 μm. (C) Barplot showing the number of miRNAs (light blue) and EV-miRNAs (red) that contain various numbers of AU- or GU-rich motifs. (D) Sequence logos AU- and GU-rich motifs of EV miRNAs. (E) Heatmap of expression profile of AU- and GU-rich miRNAs in EVs from control and morphine-stimulated human primary astrocytes. (F) qPCR validation of representative differentially expressed miRNAs identified from the RNA-seq. All experiments were done at least three independent times. *p < 0.05 versus control.

Morphine-ADEVs Induce lincRNA-Cox2 Expression in Microglia via the TLR7/NF-κB Signaling Pathway

Studies from others and us have demonstrated that lincRNA-Cox2 is an early-primary gene regulated by NF-κB signaling in myeloid cells such as macrophages and microglia. Herein we sought
to examine whether morphine-ADEVs that are taken up by microglial cells could, via the TLR7-NF-κB axis, lead to upregulation of lincRNA-Cox2 expression. To address this, we pretreated mouse primary microglia with either SC-514 (NF-κB inhibitor) or chloroquine (TLR7 inhibitor) followed by stimulation with either control- or morphine-ADEVs isolated from mouse primary astrocytes for 4 hr. Expression of lincRNA-Cox2 was analyzed by real-time PCR. As presented in Figure 4A, expression of lincRNA-Cox2 was significantly increased in microglial cells stimulated with morphine-ADEVs compared with its expression in microglia exposed to control-ADEVs. In microglia pretreated with either SC-514 or chloroquine, morphine-ADEVs failed to induce expression of lincRNA-Cox2 (Figure 4A). To further validate our results, we also assessed lincRNA-Cox2 expression in mouse primary microglia isolated from mouse primary astrocytes for 4 hr. Expression of lincRNA-Cox2 was analyzed by real-time PCR. As presented in Figure 4A, expression of lincRNA-Cox2 was significantly increased in microglial cells stimulated with morphine-ADEVs compared with its expression in microglia exposed to control-ADEVs. In microglia pretreated with either SC-514 or chloroquine, morphine-ADEVs failed to induce expression of lincRNA-Cox2 (Figure 4A). To further validate our results, we also assessed lincRNA-Cox2 expression in mouse primary microglia isolated from either C57BL/6N WT or TLR7 KO mice that were stimulated with either control- or morphine-ADEVs isolated from mouse primary astrocytes for 4 hr. Expression of lincRNA-Cox2 was analyzed by real-time PCR. As presented in Figure 4A, expression of lincRNA-Cox2 was significantly increased in microglial cells stimulated with morphine-ADEVs compared with its expression in microglia exposed to control-ADEVs. In microglia pretreated with either SC-514 or chloroquine, morphine-ADEVs failed to induce expression of lincRNA-Cox2 (Figure 4A). To further validate our results, we also assessed lincRNA-Cox2 expression in mouse primary microglia isolated from either C57BL/6N WT or TLR7 KO mice that were stimulated with either control- or morphine-ADEVs isolated from mouse primary astrocytes. As shown in Figure 4B, exposure of WT microglia to morphine-ADEVs resulted in an upregulation of lincRNA-Cox2 expression, and in contrast and as expected, morphine-ADEVs failed to upregulate lincRNA-Cox2 expression in TLR7 KO microglial cells. Next, to determine the site of action of lincRNA-Cox2, we performed RNA fluorescent in situ hybridization (FISH) for lincRNA-Cox2 in microglia. As shown in Figure 4C, FISH analysis revealed nuclear, perinuclear, as well as cytoplasmic expression of lincRNA-Cox2 in microglial cells. Further validation of these findings was done by assessing the expression of lincRNA-Cox2 in subcellular fractions of microglia by real-time PCR. As shown in Figure 4D, lincRNA-Cox2 expression was predominantly localized to the nucleus in the microglia (Figure 4D), and as expected, GAPDH mRNA was localized to the cytosol, and U1 small nuclear RNA (snRNA) was confined to the nucleus. Consistent with previous studies, these results indicate that lincRNA-Cox2 could serve as a pivotal mediator regulating the expression of other genes at the level of transcription in microglia.

**Morphine-ADEVs Inhibited Microglial Phagocytosis**

To determine the functional effects of induced lincRNA-Cox2 expression in microglia, we first designed lincRNA-Cox2 siRNAs, and the knockdown efficiency of our siRNAs was evaluated by real-time PCR in siRNA-transfected BV-2 cells. As shown in Figure 5A, both lincRNA-Cox2 siRNAs significantly downregulated the expression of lincRNA-Cox2 in microglia. We next performed microarray analysis to compare the gene expression profiles in both control and lincRNA-Cox2 knockdown BV-2 microglia. We have previously reported that lincRNA-Cox2 is a coregulator of transcription factor(s) controlling inflammatory responses in microglia and macrophages. The interrelationship between lincRNA-Cox2 and phagocytosis
in microglia, however, remains largely unknown. Based on the microarray analysis, in lincRNA-Cox2 knocked-down microglia, we identified several phagocytic genes, including Lrp1, Syk, and Pld2, that were negatively regulated by lincRNA-Cox2 (Figure 5B). These findings were also validated in mouse primary microglia. As shown in Figure 5C, in mouse primary microglia exposed to morphine-ADEVs there was decreased expression of Lrp1, Pld2, and Syk compared with cells exposed to control-ADEVs. Interestingly, knocking down lincRNA-Cox2 significantly increased the expression of Lrp1, Pld2, and Syk in microglia exposed to either control- or morphine-ADEVs compared with scrambled siRNA-transfected microglia (Figures S2A and S2B). Next, we sought to validate the role of morphine-ADEVs on microglial phagocytosis. For this, mouse primary microglial cells were transfected with either control- or lincRNA-Cox2 siRNA for 24 hr followed by exposure of cells to PKH26-labeled ADEVs (isolated from mouse primary astrocytes) for an additional 24 hr and subsequent incubation with fluorescent beads for 2 hr. As shown in Figure 2C, there was decreased internalization of the beads (green) in morphine-ADEV (red)-stimulated microglia compared with cells stimulated with control-ADEVs (red). In microglial cells transfected with lincRNA-Cox2 siRNA, however, there was increased internalization of beads (green) in morphine-ADEV (red)-stimulated microglia compared with cells transfected with control-siRNA and exposed to morphine-ADEVs (red). These findings thus underpinned the roles of morphine-ADEVs and lincRNA-Cox2 in microglial phagocytosis. We next sought to examine whether the TLR7-NF-κB signaling pathway was involved in phagocytosis. As presented in Figures 5D and 5E, in the presence of inhibitors of either TLR7 or NF-κB, morphine-ADEVs failed to abrogate microglial phagocytosis. These findings were further confirmed in TLR7 KO microglia. As shown in Figure 5F, morphine-ADEVs failed to inhibit phagocytosis in TLR7 KO microglia. Together, these findings demonstrated that morphine-ADEVs could be taken up by microglia, leading to activation of the TLR7-NF-κB signaling axis. This activation pathway, in turn, resulted in upregulation of lincRNA-Cox2 expression, culminating in impaired microglial phagocytosis.

Knockdown of lincRNA-Cox2 Restored Morphine-Impaired Phagocytic Activity In Vivo
Having determined that knockdown of lincRNA-Cox2 in microglia was able to restore ADEV-mediated morphine-induced phagocytic
impairment, we next sought to investigate whether knockdown of lincRNA-Cox2 could also restore microglial phagocytic activity in morphine-administered mice. Targeting therapeutic drugs to the CNS is a major challenge in the field owing to the tight blood-brain barrier. Intranasal administration is a non-invasive route for drug delivery, which is widely used for the treatment of rhinitis and nasal polyps.36,37 In recent times, EVs have not only been recognized for their role as communication vesicles delivering cargo to neighboring or distant cells, but have also been implicated as conduits for efficient delivery of RNA drugs into the brain.20,38 Before proceeding to test the efficacy of siRNA-loaded EVs in our system, it was first essential to assess the biodistribution of intranasally administered EVs. For this, WT mice were given intraperitoneal injections of either saline or morphine (10 mg/kg) twice a day for 5 consecutive days. Simultaneously, we also initiated intranasal delivery of ADEVs (20 μg each time) loaded with either control or lincRNA-Cox2 siRNA to

Figure 5. Knockdown of lincRNA-Cox2 Restores EV-Mediated Impairment of Microglial Phagocytosis

(A) Real-time PCR for lincRNA-Cox2 in microglia transfected with lincRNA-Cox2 siRNA. (B) Heatmap of differentially expressed phagocytosis-associated genes in lincRNA-Cox2 knockdown BV-2 cells compared with control cells. (C) Mouse primary microglial cells were transfected with lincRNA-Cox2 siRNA for 24 hr, followed by exposure of cells to astrocyte EVs for 4 hr, and subsequently assessed for the expression of Lrp, Pld2, and Syk by qPCR. (D and E) Mouse primary microglial cells were pre-treated with TLR7 inhibitor (chloroquine 25 μM) or NF-κB inhibitor (SC-514, 5 μM) for 1 hr, followed by exposure of cells to astrocyte EVs and subsequent exposure to fluorescent beads for 2 hr. Phagocytosis was measured using (D) fluorescence microscopy (scale bar, 20 μm; high-magnification images are shown in the bottom right corners) and (E) fluorometric plate reader. (F) TLR7 KO microglial cells were exposed to astrocyte-derived EVs and assessed for phagocytosis as described above. All data are presented as mean ± SD. *p < 0.05 versus control; #p < 0.05 versus treatment using Student’s t test.
the two groups of mice 1 hr prior to morphine or saline injections. One hour after the last injection on day 5, mice were sacrificed followed by microglial isolation and assessment of lincRNA-Cox2 expression by real-time PCR. As shown in Figure 6C, the expression of lincRNA-Cox2 was significantly upregulated in microglia isolated from morphine-administrated mice compared with the saline-injected controls. Furthermore, intranasal delivery of lincRNA-Cox2 siRNA-loaded ADEVs resulted in a significant knockdown of lincRNA-Cox2 in microglia isolated from morphine-administered mice. The next step was to perform phagocytosis assay on the brain slices from the two groups of mice that were intranasally delivered loaded EVs. As shown in Figures 6D and 6E, there were fewer fluorescent beads phagocytosed in morphine-administered microglia compared with saline-injected controls, suggesting thereby that morphine administration impaired microglial phagocytosis in the adult brain (Figures 6D and 6E). Intriguingly, intranasal delivery of lincRNA-Cox2 siRNA-loaded EVs restored microglial phagocytic activity in morphine-administered mice compared with the morphine-administered mice that were treated with control siRNA-loaded EVs.

**DISCUSSION**

EVs play an important role in cell-cell communication among diverse cells types and tissues, including the CNS. The primary function of these vesicles is the delivery of their cargo comprising cellular miRNAs, proteins, and DNA to neighboring as well as distant cells, resulting in impairment of cellular functions. For example, in our previous study, we have shown that both morphine and HIV Trans-Activator of Transcription (Tat) protein upregulated miR-29b in ADEVs, which, in turn, resulted in neuronal death. Along these lines, studies by Yelamanchili et al. also demonstrated that HIV infection resulting in significant upregulation of miR-21 in blood macrophage-derived EVs in turn directly activated the TLR7-dependent downstream necroptosis pathway. Our miRNA-seq profiling of morphine-ADEVs demonstrating enrichment of miRNAs with an AU- or GU-rich motif critical for direct activation of TLR7 is in keeping with published reports on the role of EV miRNAs in this process. The process by which EV-miRNA-mediated activation of TLR7 leads to upregulation of lincRNAs that regulate cellular function(s), however, remains less understood. In the current study, we demonstrate that exposure of mouse
Phagocytes play a vital role in the maintenance of tissue homeostasis by promoting rapid clearance of apoptotic cells, pathogens, and other insults, leading, in turn, to the elimination of immunogens and prevention or resolution of inflammation. In rodents, intranasal administration of exosomes is considered a noninvasive method for rapid delivery of exosome-encapsulated drug(s) to the brain with selective uptake by microglial cells. These findings have ramifications for the development of EV-loaded RNA drug target(s) as therapeutics for a multitude of neurodegenerative disorders including those associated with opiate abuse.

In conclusion, our findings suggest that exposure of microglial cells to morphine-ADEVs results in impaired phagocytic function via the TLR7- NF-κB-lincRNA-Cox2 axis. We also demonstrate that knockdown of lincRNA-Cox2 restores morphine-impaired phagocytic activity in microglia in vivo. These findings have ramifications for the development of EV-loaded RNA drug target(s) as therapeutics for a multitude of neurodegenerative disorders including those associated with opiate abuse.
EVs (20 μg/100 μL) in saline were administered intranasally as drops with a small pipette every 2 min into alternating tissue sides of the nasal cavity for a total of 10 min. To determine tissue distribution of EVs in vivo, we harvested various tissues and monitored for efficiency of delivery and biodistribution using a Xenogen IVIS 200 imager.

**Cell Culture and Cell Lines**

The human astrocytic cell line A172 (no. CRL-1620; American Type Culture Collection [ATCC]) was cultured as described previously and maintained in DMEM with high glucose containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/mL), and 100 μg/mL streptomycin. In this study, A172 cells were used within 30 passages. Human primary astrocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and were cultured in DMEM/F12 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% heat-inactivated FBS, 2 mM glutamine, sodium bicarbonate, gentamicin, non-essential amino acids, and vitamins. In this study, A172 cells were used within 30 passages. Human primary microglia cells were obtained from 1- to 3-day-old C57BL/6N mice and plated on poly-D-lysine pre-coated cell culture dishes. Mouse primary astrocytes were prepared from whole brains of post-natal (1- to 3-day-old) C57BL/6N mice and plated on poly-D-lysine pre-coated culture dishes. Development of the cultures was comprised of >95% glial cells.

Mouse primary microglia were obtained from whole brains of postnatal (1- to 3-day-old) C57BL/6N mice and plated on poly-D-lysine pre-coated culture dishes. Development of the cultures was comprised of >95% glial cells.

**EV Isolation**

EVs were prepared from the supernatant of primary astrocytes and A172 cells by differential centrifugations as previously described. In brief, conditioned media were harvested, centrifuged at 1,000 × g for 10 min to eliminate cells, and again spun at 10,000 × g for 30 min, followed by filtration through a 0.22-μm filter to remove cell debris. EVs were pelleted by ultracentrifugation (Beckman Ti70 rotor; Beckman Coulter, Brea, CA, USA) at 100,000 × g for 70 min. EVs were assessed for their protein content using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). TSG101 and CD63 were detected by western blot as exosome markers. EVs were further quantified by NTA using a NanoSight (model NS300), as previously reported.

**Cell and EV Transfection**

Plasmid and siRNA transfections were performed using Lipofectamine 2000 (catalog no. 11668027; Life Technologies) according to the manufacturer’s instructions. In brief, cells were transfected with plasmid (500 ng) or targeted siRNA (20 pM) mixed with 2 μL of Lipofectamine 2000 diluted in 100 μL of Opti-MEM (catalog no. 31985062; Life Technologies). The resulting siRNA-lipid complexes were added to the cells, incubated for 6 hr, and the medium changed into fresh DMEM. Next, the medium was changed to 10% FBS-containing medium for 20-hr incubation. The transfected cells were then ready for use in experiments. pEF6.mCherry-TSG101 was a gift from Quan Lu (Addgene plasmid 38318). CD63-pEGFP was a gift from Paul Luzio (Addgene plasmid 62964). Sequences of mouse Dicer1 siRNA oligonucleotides used in this study were: mouse Dicer1-siS1, 5'-GrUrGrUrCrArUrGrUrGrArUrGrUrArUrUr-3'; mouse Dicer1-si AS1, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUr-3'; mouse Dicer1-si AS2, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUr-3'; mouse Dicer1-si AS3, 5'-GrCrArGrArUrGrCrArUrGrArUrUr-3'; mouse Dicer1-si AS4, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUr-3'.

EVs were transfected with siRNA using Exo-Fect Exosome Transfection Reagent (SBI; System Biosciences) according to the manufacturer’s instructions. Sequences of mouse lincRNA-Cox2 siRNA oligonucleotides used in this study were: Control-(si)S1, 5'-GrUrGrUrCrArUrGrUrGrArUrGrUrArUrUr-3'; Control-(si) AS1, 5'-GrUrGrUrCrArUrGrUrGrArUrGrUrArUrUr-3'; lincRNA-Cox2-(si)S1, 5'-GrCrArGrArUrGrArUrGrArUrGrUr UrUr-3'; lincRNA-Cox2-(si) AS1, 5'-GrCrArGrArUrGrArUrGrArUrGrUr UrUr-3'; lincRNA-Cox2-(si) AS2, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUrUr-3'; lincRNA-Cox2-(si) AS3, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUrUr-3'; lincRNA-Cox2-(si) AS4, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUrUr-3'; lincRNA-Cox2-(si) AS5, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUrUr-3'; lincRNA-Cox2-(si) AS6, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUrUr-3'; lincRNA-Cox2-(si) AS7, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUrUr-3'; lincRNA-Cox2-(si) AS8, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUrUr-3'; lincRNA-Cox2-(si) AS9, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUrUr-3'; lincRNA-Cox2-(si) AS10, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUr Ur-3'; lincRNA-Cox2-(si) AS11, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUrUr-3'; lincRNA-Cox2-(si) AS12, 5'-UrArGrArArCrArGrArGrArGrArCrArGrUrUr Ur-3'.

**Western Blotting**

Treated cells or EVs were lysed using the Mammalian Cell Lysis kit (Sigma, St. Louis, MO, USA) and quantified using the micro BCA Protein Assay kit (Pierce, Rockford, IL, USA). Equal amounts of the corresponding proteins were electrophoresed in a 10% SDS-polyacrylamide gel (10%–12%) under reducing conditions followed by transfer to polyvinylidene fluoride (PVDF) membranes. The blots were blocked with 5% non-fat dry milk in PBS. Western blots were then probed with antibodies recognizing the CD63 antibody (1:1,000; ab216130; Abcam, Cambridge, MA, USA), TSG101 (1:1,000; ab125011; Abcam, Cambridge, MA, USA), NF-kB p65 (1:2,000; ab16502; Abcam, Cambridge, MA, USA), histone H3 (1:1,000; catalog no. 9715S; Cell Signaling Technology, Cambridge, MA, USA), and caspase-3 (1:1,000; catalog no. 9661S; Cell Signaling Technology, Cambridge, MA, USA).
Technology, Danvers, MA, USA), and β-actin (1:4,000; A5316; Sigma, St. Louis, MO, USA). The secondary antibodies were alkaline phosphatase-conjugated to goat anti-mouse/rabbit IgG (1:5,000). Signals were detected by chemiluminescence and imaged on the FLA-5100 (Fujifilm, Valhalla, NY, USA) digital image scanner; densitometry was performed utilizing ImageJ software (NIH).23

**Electron Microscopy**

EV pellets were prepared for negative staining employing a slightly modified procedure. Using wide-bore tips, 3 μL of EV pellet was gently placed on 200-mesh Formvar-coated copper grids, allowed to adsorb for 4–5 min, and processed for standard uranyl acetate staining. In the last step, the grid was washed with three changes of PBS and allowed to semi-dry at room temperature before observation in TEM (Hitachi H7500 TEM; Hitachi, Tokyo, Japan).

**Atomic Force Microscopy**

The 1-(3-aminopropyl) silatrane (APS) mica functionalization procedure was used, in which freshly cleaved mica is treated with APS, as previously described.24,25 Twenty microliters (8.3 × 10⁷ EVs/mL) of EV sample was deposited on APS mica for 20 min at room temperature. Then 200 μL of the PBS buffer was added to the sample. The sample was then subjected to AFM imaging. AFM imaging of EVs was carried out using the Asylum Research MFP3D (Santa Barbara, CA, USA) instrument. Imaging was performed in tapping mode at room temperature. An MSNL probe with cantilever "E" (Bruker Corporation) was employed for imaging. The nominal spring constant of the MSNL "E" cantilevers was ~0.1 N/m.

**RNA Isolation and Sequencing**

RNA was extracted from EVs, which were isolated from conditioned media of human primary astrocytes of three donors. RNA samples were then shipped on dry ice to LC Sciences (Houston, TX, USA) for miRNA sequencing.

**Real-Time PCR**

For quantitative analysis of mRNA expression, comparative real-time PCR was performed with the use of SYBR Green PCR Master Mix (Applied Biosystems). The sequences for the amplification of lincRNA-Cox2 were: 5′-AGTATGGGATAACCAGCTGAGGT-3′ (forward) and 5′-GAATGCTCAGAGTGAGAAATAG-3′ (reverse); the primer sequences for the amplification of GAPDH were as follows: 5′-CCATGATCACGAAGGTGGTTT-3′ (forward) and 5′-GAATGCTCAGAGTGAGAAATAG-3′ (reverse); the primer sequences for the amplification of U1 were as follows: 5′-CCATGATCACGAAGGTGGTTT-3′ (forward) and 5′-GAATGCTCAGAGTGAGAAATAG-3′ (reverse); the primer sequences for the amplification of GAPDH were as follows: 5′-GCCTAGAACCACGTGAA-3′ (forward) and 5′-GGATTTTTAATAGC-3′ (reverse); the primer sequences for the amplification of GAPDH were as follows: 5′-GCCTAGAACCACGTGAA-3′ (forward) and 5′-GGATTTTTAATAGC-3′ (reverse); the primer sequences for the amplification of GAPDH were as follows: 5′-GCCTAGAACCACGTGAA-3′ (forward) and 5′-GGATTTTTAATAGC-3′ (reverse); the primer sequences for the amplification of GAPDH were as follows: 5′-GCCTAGAACCACGTGAA-3′ (forward) and 5′-GGATTTTTAATAGC-3′ (reverse); the primer sequences for the amplification of GAPDH were as follows: 5′-GCCTAGAACCACGTGAA-3′ (forward) and 5′-GGATTTTTAATAGC-3′ (reverse); the primer sequences for the amplification of GAPDH were as follows: 5′-GCCTAGAACCACGTGAA-3′ (forward) and 5′-GGATTTTTAATAGC-3′ (reverse); the primer sequences for the amplification of GAPDH were as follows: 5′-GCCTAGAACCACGTGAA-3′ (forward) and 5′-GGATTTTTAATAGC-3′ (reverse). All reactions were run in triplicate.

**Immunofluorescence**

Cells grown on sterile coverslips were incubated with TSG101-mCherry/CD63-EGFP-labeled EVs at 40% confluency. After 20–30 min, cells were washed extensively with PBS and then fixed with 4% paraformaldehyde for 20 min at room temperature followed by permeabilization with 0.3% Triton X-100 in PBS for 30 min. The samples were incubated with a blocking buffer containing 10% goat serum (NGS) in PBS for 2 hr at room temperature followed by addition of rabbit anti-Iba1 antibody (1:1,000; Wako Laboratory Chemicals) or rabbit anti-NF-kB p65 antibody (1:1,000; Abcam) or rabbit anti-EEA1 antibody (1:200; Cell Signaling Technology) and incubated overnight at 4°C. Primary Abs were labeled with secondary anti-rabbit Abs conjugated to the fluorescent probes Alexa Fluor 488 or Alexa Fluor 594, and nuclei were labeled with DAPI.

**Candidate TLR7/8 RNA Agonists miRNA Search and Motif Prediction**

To identify the potential mouse miRNAs that are capable of functioning as the TLR7/8 RNA agonists, we performed an unbiased search as follows: first, all known mouse mature miRNAs were collected from miRBase30 (Version 21), and the mouse EV-miRNAs were also obtained from two EV databases: ExoCarta31 and EVpedia.24 Next, previously reported 15 AU- and GU-rich 4-mers from the Forsbach et al.27 study were scanned for all known mouse miRNAs. Finally, the EV-associated miRNAs that contained the reported AU- and GU-rich 4-mers were considered as TLR7/8 RNA agonists. Furthermore, sequence motif analysis was performed based on the selected candidates using the tool MDS2.33

**Cell Fractionation**

Cells were fractionated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit to generate cytosolic and nuclear pools as described by the manufacturer (Thermo Fisher).

**lincRNA In Situ Hybridization**

In situ hybridization for lincRNA was performed as described previously.37 Biotin-labeled antisense DNA probes (5′-catggttctcagagttcagc-3′, 5′-gaacagtttccagtctctctc-3′, 5′-ggaaactcagagtgtctgt-3′, 5′-gactacctgctgtagatt-3′, and 5′-tggtagagttctttc-3′) were designed using the online probe designer at http://www.singlemoleculefish.com and synthesized by Integrated DNA Technologies. The cells were fixed in 4% paraformaldehyde for 10 min, and washed and permeabilized with 0.25% Triton X-100 in PBS for 15 min. Next, the slides were washed in PBS three times, followed by incubation in hybridization buffer for 1 hr. Subsequently, the DNA probes in hybridization buffer were added and incubated in a humid chamber overnight at 35°C. The next day, the slides were washed in 2 × saline-sodium citrate (SSC) three times for 2 min each at 42°C and 0.2 × SSC three times for 2 min each at 42°C. Blocking was performed with blocking solution (3% NGS + 1% BSA in PBS) at room temperature for 2 hr and then incubated with horseradish peroxidase (HRP)-labeled reagent (SA-HRP) and cell-type marker at 4°C overnight in a humidified chamber. On the third day, slides were washed three times for 5 min each in TBS at room temperature with agitation. Slides were incubated in streptavidin-HRP for 30 min at room temperature, then washed three times for 5 min each in TBS buffer at room temperature. After that the slides were incubated with the appropriate labeled secondary antibody in 1% BSA, 1% NGS diluted in PBS at room temperature for 1 hr. Then the slides were washed in 0.1% Tween 20 in TBS.
three times for 5 min each at room temperature. The slides were then mounted using Prolong gold anti-fade reagent with DAPI (Invitrogen).

**Adult Microglia Isolation**

Microglia were isolated from whole-brain homogenates by Percoll gradient centrifugation according to previous reports with slight modifications. In brief, the brains were homogenized in PBS (pH 7.4) by passing through a 70-μm nylon cell strainer. Resulting homogenates were centrifuged at 600 × g for 6 min. Supernatants were removed and cell pellets were resuspended in 70% isotonic Percoll (GE Healthcare, Uppsala, Sweden) at room temperature. A discontinuous Percoll density gradient was layered as follows: 70%, 50%, 35%, and 0% isotonic Percoll. The gradient was centrifuged for 20 min at 2,000 g, and microglia were collected from the interphase between the 70% and 50% Percoll layers. Cells were washed and then resuspended in sterile PBS followed by flow cytometry analysis by gating the myeloid cells for the CD11b+/CD45dim population.

**Phagocytosis Assay**

The phagocytic activity of adult microglia of live brain slices was analyzed as described previously. Brains from 8-week-old mice were washed in carbogen-saturated (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 126; KCl 2.5; MgSO₄ 1.3; CaCl₂ 2.5; NaH₂PO₄ 1.25; NaHCO₃ 26; and D-glucose 10 (pH 7.4; all from Sigma). Coronal slices (130 μm) were prepared using a Vibratome (Microm, Walldorf, Germany) at 4°C and allowed to rest in ACSF buffer at room temperature for 1 hr before incubation with fluorescein isothiocyanate (FITC)-labeled rabbit IgG-coated latex beads 0.1 μm mean particle size (1:100; Cayman Chemical) for 60 min at 37°C. The slices were washed and fixed with 4% paraformaldehyde. To visualize microglia, slices were permeabilized (0.25% Triton X-100, 1% BSA, and 10% goat serum in PBS) and incubated with anti-Iba1 (1:750; Wako), followed by goat anti-rabbit Alexa Fluor 488 (1:250; Invitrogen) and Hoechst 33258 (1:10,000; Sigma). Fluorescent images were acquired at room temperature on a Zeiss Observer Z1 microscope (Carl Zeiss, Germany); images were processed using AxioVs 40.8.0.0 software (Carl Zeiss Microimaging). Photographs were acquired using an AxioCam MRm digital camera (Carl Zeiss, Germany).

To investigate the ability of microglia to internalize particles, microglial cells (2 × 10⁵ cells/mL) were plated on a 24-well plate and allowed to adhere overnight, and then incubated with FITC-labeled rabbit IgG-coated latex beads as described above, intensively washed, and finally visualized at ×20 magnification with a microscope.

**Statistical Analysis**

Statistical analysis was performed using Student’s t test or one-way ANOVA followed by Holm-Sidak test (SigmaPlot 11.0). The appropriate test was clarified in the figure legends. Results were judged statistically significant if p < 0.05 by ANOVA.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three figures and two tables and can be found with this article online at [https://doi.org/10.1016/j.omtn.2018.09.019](https://doi.org/10.1016/j.omtn.2018.09.019).

**AUTHOR CONTRIBUTIONS**

G.H. and S.B. designed the experiments and wrote the manuscript. K.L., F.N., L.Y., B.W.D., C.T., Z.S., and M.K. performed the experiments. J.S., J.C., and G.H. performed bioinformatics analysis. S.C., Y.L.L., X.-M.C., G.H., and S.B. analyzed data.

**CONFLICTS OF INTEREST**

The authors have no conflicts of interest.

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