FULL-LENGTH ORIGINAL RESEARCH

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Intraperitoneal injection of lipopolysaccharide prevents seizure-induced respiratory arrest in a DBA/1 mouse model of SUDEP

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Abstract

Objective: Sudden unexpected death in epilepsy (SUDEP) is the cause of premature death of 50% patients with chronic refractory epilepsy. Respiratory failure during seizures is regarded as an important mechanism of SUDEP. Previous studies have shown that abnormal serotonergic neurotransmission is involved in the pathogenesis of seizure-induced respiratory failure, while enhancing serotonergic neurotransmission in the brainstem suppresses it. Because peripheral inflammation is known to enhance serotonergic neuron activation and 5-HT synthesis and release, we investigated the effect of intraperitoneal lipopolysaccharide (LPS)-induced inflammation on the S-IRA susceptibility during audiogenic seizures in DBA/1 mice.

Methods: After DBA/1 mice were primed by exposing to sound stimulation for three consecutive days, they were tested for seizure severity and seizure-induced respiratory arrest (S-IRA) induced by sound stimulation under different conditions. We determined the dose and time course of the effects of intraperitoneal administration of LPS on audiogenic seizures and S-IRA. The effects of blocking TLR4 or RAGE receptors and blocking 5-HT receptors on the LPS-induced effect on S-IRA were investigated. Statistical significance was evaluated using the Kruskal-Wallis test.

Results: Intraperitoneal injection of LPS significantly had dose-dependent effects in reducing the incidence of S-IRA as well as seizure severity in DBA/1 mice. The protective effect of LPS on S-IRA peaked at 8-12 hours after LPS injection and was related to both reducing seizure severity and enhancing autoresuscitation. Blocking TLR4 or RAGE receptor with TAK-242 or FPS-ZM1, respectively, prior to LPS injection attenuated its effects on S-IRA and seizure severity. Injection of a nonselective 5-HT receptor antagonist, cyproheptadine, or a 5-HT$_3$ receptor antagonist, ondansetron, was effective in blocking LPS-induced effect on S-IRA. Immunostaining results showed a significant increase in c-Fos-positive serotonergic neurons in the dorsal raphe.

Significance: This is the first study that demonstrates the effect of intraperitoneal LPS injection-induced inflammation on reducing S-IRA susceptibility and provides additional evidence supporting the serotonin hypothesis on SUDEP. Our study

suggests that inflammation may enhance brainstem 5-HT neurotransmission to promote autoresuscitation during seizure and prevent SUDEP.

KEYWORDS

audiogenic seizure, inflammation, seizure-induced respiratory arrest, serotonin, SUDEP

1 | INTRODUCTION

Epilepsy patients are at a 24- to 28-fold higher risk of sudden unexpected death compared to the general population.[1] This kind of unanticipated death of a relatively healthy person with epilepsy where no structural or toxicological cause of death can be identified after postmortem analysis is called sudden unexpected death in epilepsy patients (SUDEP). SUDEP is the cause of premature death of 17% of people with epilepsy and 50% of people with chronic refractory epilepsy.[2,3] In terms of potential years of life lost, SUDEP ranks second only to stroke among all neurologic disorders in the USA.[1] Growing clinical and animal research suggests impaired respiratory function during a seizure as an important mechanism of SUDEP.[4–7] Understanding the mechanisms underlying the impairment of respiration during seizure will advance the development of therapeutic strategies against SUDEP.

Respiratory dysfunction during generalized seizure leads to an increase in CO_2 level in epilepsy patients.[8] Serotonin/5-hydroxytryptamine (5-HT) is an important neurotransmitter that enhances respiration through regulating the medullary respiratory center and arousal network in response to an elevated level of CO_2.[9–11] Accumulating clinical evidence suggests that abnormal 5-HT neurotransmission is implicated in the pathogenesis of respiratory dysfunction during seizure.[12,13] Animal data also support the involvement of impaired 5-HT neurotransmission in SUDEP.[14,15] DBA/1 mice, being a model of SUDEP, exhibit generalized seizure in response to loud sound stimulation, which develops into tonic seizure leading to S-IRA.[4] They have abnormal 5-HT neurotransmission in the brainstem, making them susceptible to S-IRA.[11] Enhancing 5-HT neurotransmission in the brainstem by exogenous injection of 5-HT precursor (5-hydroxytryptophan)[16] or selective serotonin reuptake inhibitors (SSRIs)[17,18] or by optogenetic stimulation of the 5-HT neurons in the dorsal raphe nucleus[19] suppresses seizure-induced respiratory arrest (S-IRA) in these mice.

There is an overarching consensus on the bidirectional interaction between 5-HT signaling and immune response both in brain and in periphery.[20] Peripheral inflammation modulates 5-HT neuron activation, 5-HT synthesis, and 5-HT release. Peripheral LPS injection enhances c-Fos expression of the 5-HT neurons in the dorsal raphe nucleus[21] and increases the concentrations of tryptophan and 5-HT catabolite 5-hydroxyindoleacetic acid (5-HIAA) in mouse brain and 5-HT

Key Points

- Intraperitoneal LPS-induced inflammation shows dose- and time-dependent protection against seizure-induced respiratory arrest (S-IRA) in DBA/1 mice
- Injection of 10 mg/kg of LPS was most effective and peaked in 8-12 hours after injection
- Intraperitoneal LPS-induced inflammation also reduces seizure severity, which only partially contributes to its protective effect on S-IRA
- Intraperitoneal LPS treatment increased the number of c-Fos-positive serotonergic neurons in the dorsal raphe in DBA/1 mice
- This protective effect of LPS may be mediated through activating TLR4 and RAGE signaling and enhancing brain 5-HT transmission

levels in the hippocampus.[20,22] These findings support a hypothesis that LPS-induced peripheral inflammation would be protective against S-IRA.

To test this hypothesis, we induced inflammation by intraperitoneal injection of LPS and investigated its effect on S-IRA susceptibility in DBA/1 mice. Our data showed that LPS-based inflammation is protective against S-IRA. Using TAK-242 or FPS-ZM1 to block TLR4 or RAGE, respectively, we demonstrate that the S-IRA protective effect of LPS involved the activation of TLR4 and RAGE signaling. Using 5-HT receptor antagonists, we demonstrate that the S-IRA protective effect of LPS is specifically mediated by enhanced 5-HT neurotransmission. To the best of our knowledge, this is the first study that demonstrates the relationship between peripheral inflammation and S-IRA prevention.

2 | MATERIALS AND METHODS

2.1 | Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine, which are in accordance with National Institutes of Health guidelines for

the care and use of laboratory animals. DBA/1 mice were obtained from ENVIGO (Indianapolis, IN, USA). The mice were housed and bred in the Laboratory Animal Resource Center at the Stark Neurosciences Research Institute with water and food pellets ad libitum. On postnatal days (PND) 21, 22, and 23, the DBA/1 mice were primed by exposing them to 3 short sound stimuli of 10 seconds each with an interval of 10 seconds daily until the mice exhibited susceptibility to seizure-induced respiratory arrest (S-IRA). With this priming procedure, about 95 to 100% mice showed S-IRA on PND 23. Every attempt was made to reduce animal use and to minimize pain and discomfort to the animals.

2.2 | Drugs

Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Cat #L2880), cyproheptadine hydrochloride (Cat #C6022), and ondansetron hydrochloride (Cat #03639) were obtained from Sigma-Aldrich. TAK-242 (Cat #A3850) was obtained from APEX Bio, and FPS-ZM1 (Cat #11909) was obtained from Cayman Chemical Co. All drugs were dissolved in saline. The final volume of each injection was less than 0.1 mL.

2.3 | Seizure induction and resuscitation

Seizures and S-IRA were evoked in the DBA/1 mice by exposing them to audiogenic stimulation.[4] Primed DBA/1 mice were placed individually in an acrylic box (1 ft × 1 ft × 1 ft) within a sound-attenuated chamber. Mice were subjected to a broadband sound stimulation generated by an electric bell (Morris #78140) at an intensity of 106 dB sound pressure level (SPL). Audiogenic stimulation was given for a maximum duration of 60 seconds or until the animal exhibited tonic hind limb extension. When an audiogenic seizure susceptible mouse was exposed to a sound stimulation, it burst into a wild frenzied run which progressed to a generalized seizure. The generalized seizure developed into a tonic extension phase which was followed by S-IRA, which was characterized by loss of righting reflex, relaxation of pinnae, and loss of muscle tone.[23] Animals that displayed S-IRA during the priming process were resuscitated within 5 seconds after relaxation of pinna and general loss of muscle tone.[4,14,23] The animal was placed in a supine position and connected to a small animal ventilator (RWD life sciences) pumping 1 mL of 95% oxygen at 180 strokes/min. This process was effective in resuscitating > 95% mice. Susceptibility to S-IRA in the primed DBA/1 mice was always confirmed 24 hours before the experiment. All behavior patterns were video recorded and visually analyzed offline. The severity of the response was scored as no response = 0, wild run = 1, generalized tonic clonic seizure = 2, and tonic extension = 3.[24]

2.4 | Immunohistochemistry

For immunohistochemistry experiments, mice were deeply anaesthetized with sodium pentobarbital (100 mg/kg, ip) and transcardially perfused with 20 mL PBS (pH 7.4) followed by 10 mL of 4% PFA. The brain was fixed overnight at 4°C in 4% PFA and dehydrated in 30% sucrose for 7 days. A brain region containing the dorsal raphe nucleus (approx. AP-4.47 mm, ML 0 mm, V −3.5 mm)[19] was sectioned into 30-μm-thick coronal sections with a cryostat. Every third section containing the dorsal raphe was washed in 1X PBS + 0.3% (v/v) Triton X-100 (PBST) for 3 × 20 minutes, blocked with 5% normal goat serum (Southern Biotech, Cat # 0060-01) in PBST for 2 hours at room temperature, and incubated in a cocktail of primary antibodies against serotonin (5-HT Rabbit polyclonal antibody, catalog #20080, ImmunoStar, Hudson, USA 1:20 000 dilution) and c-Fos (mouse polyclonal antibody against c-Fos (Abcam, ab208942, 1:1000)) for 20 hours at 4°C. After incubation with primary antibodies, the brain sections were washed in PBST (3 × 20 minutes) and then incubated with a cocktail of goat anti-rabbit antibody conjugated with Alexa Fluor™ 488 (A1134, Invitrogen Thermo Fisher, 1:500) and goat anti-mouse antibody conjugated with cy3 (A10521, Invitrogen, Thermo Fisher, 1:500) for 2 hours. The sections were stained with 4',6-diamidino-2-phenylindole (DAPI) (1:1000) for 10 minutes to counterstain cell chromatin and washed in PBST (3 × 20 minutes). The brain sections were finally mounted on slides with Fluormount-G® (SouthernBiotech Cat #0100-01) and imaged under a ZEISS Axio Imager 2 fluorescent microscope with Neurolucida® system (MBF bioscience).

2.5 | Statistical analysis

Data are reported as mean ± SEM and 95% confidence interval. The incidence of S-IRA and seizure severity was compared using Kruskal-Wallis test using IBM SPSS Statistics for Windows. Bonferroni correction was performed as a post hoc test for pairwise comparison among multiple groups. Graphs were created using OriginPro 9. 1. Statistical significance was inferred if $P < .05$.

3 | RESULTS

3.1 | Intraperitoneal injection of LPS prevents S-IRA in a dose-dependent manner and reduces seizure severity in DBA/1 mice

The effects of different doses of LPS on the incidence of S-IRA and seizure severity evoked by audiogenic stimulation in DBA/1 mice were examined. Primed PND 24 mice

were intraperitoneally injected with saline (N = 15) or 1 mg/kg (N = 11), 2.5 mg/kg (N = 12), 5 mg/kg (N = 15), or 10 mg/kg (N = 20) of LPS. Audiogenic testing was performed after 12 hours of LPS injection. Animals treated with 1, 2.5, 5, and 10 mg/kg of LPS showed 75%, 58.3%, 40%, and 15% of S-IRA, respectively (Figure 1A). There were significantly lower incidences of S-IRA in the 5 and 10 mg/kg groups than the control group (*P* < .05 and *P* < .001, respectively, Kruskal-Wallis test). We further analyzed the anticonvulsant effects of different doses of LPS after 12 hours of injection. All saline-treated control animals exhibited tonic extension (overall seizure severity = 3.0 ± 0.0). LPS-treated animals exhibited a reduced incidence of tonic extension or generalized seizures. Animals treated with 1, 2.5, 5, and 10 mg/kg of LPS showed an overall seizure severity of 2.6 ± 0.2, 2.8 ± 0.1, 2.1 ± 0.3, and 2.3 ± 0.2, respectively (mean ± SEM) (Figure 1B). Statistically significant differences in severity of response were observed in 5 mg/kg and 10 mg/kg groups (*P* < .05 for both, Kruskal-Wallis test). The results suggest that LPS (ip)-induced inflammation reduced the incidence of S-IRA in a dose-dependent manner, with concomitant reduction in seizure severity.

3.2 | The effect of LPS on S-IRA peaks in 8-12 hours after injection

Since 10 mg/kg of LPS (ip) was most effective in reducing S-IRA incidence, we further examined the effects of 10 mg/kg LPS at different time points. Because seizures cause significant release of 5-HT,[25] we did not give multiple audiogenic stimulations to an animal after injecting LPS or saline. Animals tested at 1, 4, 8, 12, 18, 24, and 72 hours after LPS injection showed 57.1%, 66.7%, 14.3%, 15.00%, 28.6%, 85.7%, and 100% incidence of S-IRA, respectively (Figure 2A). Reductions in the incidence of S-IRA started

as early as 1 hour after LPS injection, but statistically significant reductions were detected at 8 (*P* < .01) and 12 (*P* < .001) hours after LPS injection. Therefore, a 12-hour time protocol was employed for subsequent experiments. Animals tested at 0, 1, 4, 8, 12, 18, 24, and 72 hours after injection of 10 mg/kg of LPS exhibited an overall seizure severity score of 3.0 ± 0.0, 2.9 ± 0.1, 2.5 ± 0.3, 2.1 ± 0.4, 2.3 ± 0.2, 2.3 ± 0.4, 2.9 ± 0.1, and 3.0 ± 0.0, respectively (Figure 2B). Significant reduction in seizure severity was observed 12 hours after LPS injection (*P* < .05) (Kruskal-Wallis test).

3.3 | LPS has a stronger effect on S-IRA than on seizure severity

To determine whether the effect of LPS on reducing seizure severity is sufficient to account for its protective effect on S-IRA, we further analyzed the effect of 10 mg/kg LPS on S-IRA and seizure at 12 hours after injection in all LPS-treated animals (N = 58) used in this study and compared them with their saline-treated littermate controls (N = 36). LPS reduced the incidence of audiogenic seizures, with 100% and 82.76% of mice having audiogenic seizures in the control and LPS groups, respectively (Figure 3. *P* < .01, Kruskal-Wallis test). LPS reduced the incidence of tonic seizures (100% versus 56.90% in control and LPS groups, respectively, *P* < .001). We also observed an overall reduction in S-IRA (100% versus 32.76% in control and LPS groups, respectively, *P* < .001, Kruskal-Wallis test). Furthermore, 12 of 58 LPS-treated mice that displayed tonic seizure auto-resuscitated and did not die (20.7%). In contrast, none of the 36 control mice autoresuscitated (Figure 3, *P* < .01, Kruskal-Wallis test). The results suggest that reduction in S-IRA by LPS is not only because of a reduction in incidence of tonic seizures but also due to an increase in autoresuscitation after tonic seizures.
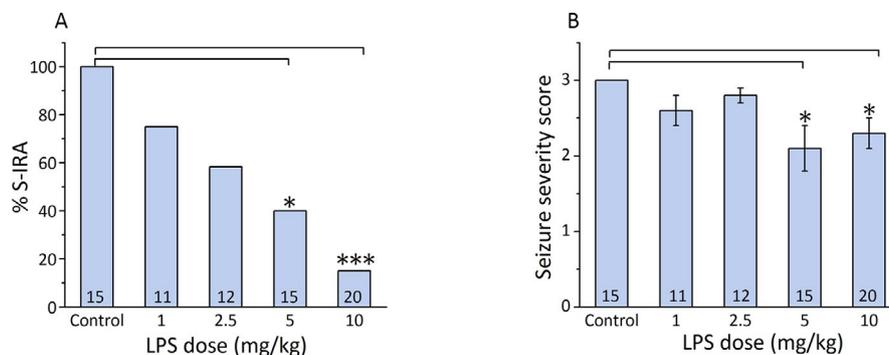

FIGURE 1 Effects of different doses of LPS on reducing the incidence of S-IRA and seizure severity in DBA/1 mice in response to audiogenic stimulation. Primed DBA/1 mice were intraperitoneally injected with saline or 1, 2.5, 5, or 10 mg/kg of lipopolysaccharide (LPS), and audiogenic testing was performed 12 hours after injection. A, Injection of 5 and 10 mg/kg LPS significantly reduced the incidence of S-IRA compared to saline-treated control (Kruskal-Wallis test followed by Bonferroni correction) B, LPS reduced the severity of audiogenic seizures in DBA/1 mice at 5 and 10 mg/kg doses (Kruskal-Wallis test followed by Bonferroni correction) *P* < .05; ***P* < .001. Error bars = SEM. The number inside each vertical bar indicates the number of mice included in that particular group
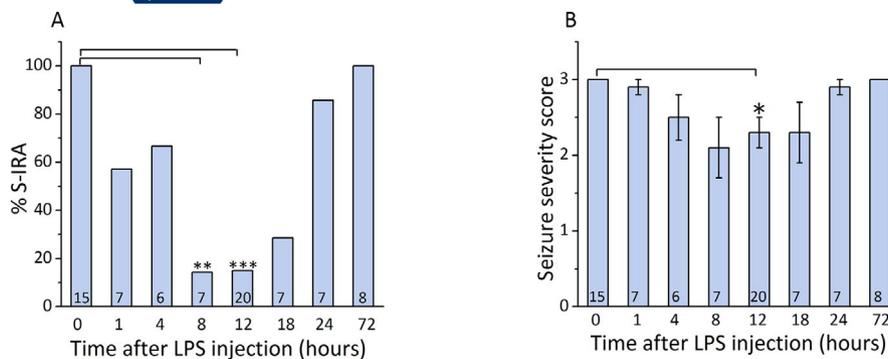
FIGURE 2 The time course of LPS on reducing S-IRA and seizure severity in DBA/1 mice in response to audiogenic stimulation. Primed DBA/1 mice were injected with 10 mg/kg of lipopolysaccharide (LPS), and audiogenic testing was performed after 1, 4, 8, 12, 18, 24, and 72 hours after LPS injection. A, Significant reduction in the incidence of seizure-induced respiratory arrest (S-IRA) occurred in 8-12 hours after LPS injection (Kruskal-Wallis test followed by Bonferroni correction). B, LPS reduced the severity of audiogenic seizures after 12 hours. (Kruskal-Wallis test followed by Bonferroni correction) *$P < .05$; **$P < .01$, ***$P < .001$. Error bars = SEM. The number inside each vertical bar indicates the number of mice included in that particular group
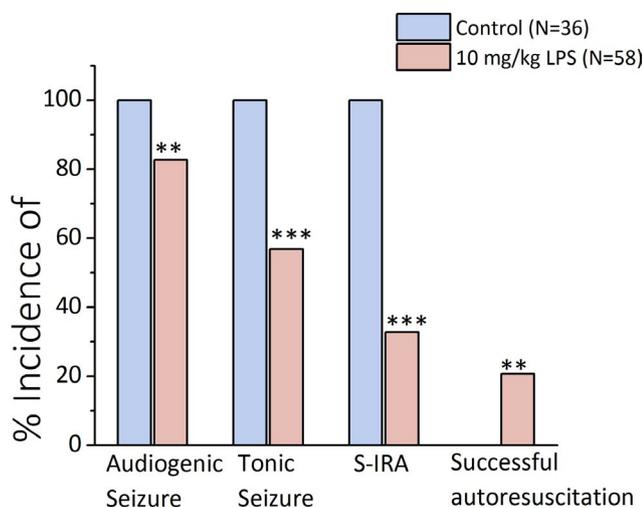

FIGURE 3 Effect of LPS on the incidence of audiogenic seizures, tonic seizure, and S-IRA in DBA/1 mice in response to audiogenic stimulation. Primed DBA/1 mice were injected with LPS (10 mg/kg, ip), and audiogenic testing was performed after 12 hours of LPS injection. LPS resulted in significant reductions in incidences of audiogenic seizure, tonic seizure, and seizure-induced respiratory arrest (S-IRA) compared to saline-treated mice (Kruskal-Wallis test). LPS also resulted in successful autoresuscitation in 20.7% of mice after tonic seizure, which was 0% in control group *$P < .05$; **$P < .01$, ***$P < .001$ when comparisons are made between the control and LPS groups

3.4 | Blocking TLR4 or RAGE receptors prevents the effect of LPS on S-IRA and seizures

LPS is a natural ligand for Toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE). TLR4 and RAGE signal via a common downstream messenger MyD88 and trigger inflammation. To investigate whether LPS exerted its effect through activating TLR4 and RAGE signaling, we used TAK-242 (3 mg/kg), a selective inhibitor of TLR4 that effectively attenuates inflammation in endotoxin shock,[26] or FPS-ZM1 (1 mg/kg), a RAGE antagonist that inhibits RAGE receptors and signaling induced by LPS/ ischemia.[27,28] Primed DBA/1 mice were injected with TAK-242 (3 mg/kg, ip) or FPS-ZM1 (1.5 mg/kg, ip) 35 minutes before injection of 10 mg/kg of LPS. Audiogenic testing was performed 12 hours after LPS injection. Similar to earlier results, the LPS-treated group showed significant reduction in incidence of S-IRA (N = 19, 47.4%) compared with the control group (N = 13, 100%, Kruskal-Wallis test, $P < .01$) (Figure 4A). However, treatment with TAK-242 + LPS or FPS-ZM1 + LPS resulted in increased incidences of S-IRA in both groups (N = 15, 73.3% and N = 14, 78.6%, respectively), both of which were not significantly different from the control group (Figure 4A). There were also no significant differences in the incidences of S-IRA between the control group and the TAK-242 only group (N = 6, 100%) or the FPS-ZM1 only group (N = 6, 100%).

LPS-treated animals exhibited a reduction in seizure severity (N = 19, 2.3 ± 0.3) compared with control group (N = 13, 3.0 ± 0.0) (Kruskal-Wallis test, $P < .05$) (Figure 4B). However, the seizure severity scores of the TAK-242 + LPS (N = 15, 2.8 ± 0.1) and FPS-ZM1 + LPS groups (N = 14, 2.9 ± 0.1) were not significantly different from the control group (N = 8, 3.0 ± 0.0). The seizure severity scores of the TAK-242 group (N = 6, 3.0 ± 0.0) or the FPS-ZM1 group (N = 6, 3.0 ± 0.0) were not significantly different from the control group (N = 13, 3.0 ± 0.0). Our results suggest that TLR4 or RAGE receptors may be involved in mediating LPS-induced protective effects on S-IRA and on seizure severity.
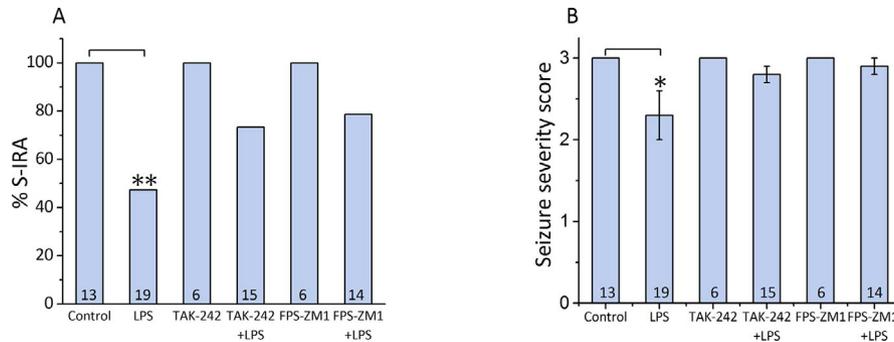
FIGURE 4 Blocking TLR4 and RAGE receptors before LPS injection prevents the protective effects of LPS on S-IRA and seizure severity in DBA/1 mice. Primed DBA/1 mice were injected with TAK-242 (a TLR4 antagonist, 3 mg/kg, ip) or FPS-ZM1 (a RAGE antagonist, 1.5 mg/kg, ip) 35 minutes before injection of 10 mg/kg of lipopolysaccharide (LPS). Audiogenic testing was performed 12 hours after LPS injection. A, LPS reduced incidence of S-IRA compared to the control group (Kruskal-Wallis test followed by Bonferroni correction, $P < .01$). Blocking TLR4 or RAGE receptors reduced S-IRA protection by LPS. TLR4 or RAGE antagonist alone did not affect the incidence of S-IRA. B, LPS reduced the overall seizure severity (Kruskal-Wallis test followed by Bonferroni correction, $P < .05$) compared to the control group. TLR4 or RAGE antagonist alone did not affect the severity of audiogenic seizures. However, blocking TLR4 or RAGE receptors reversed the anticonvulsive effects of LPS. $*P < .05$; $**P < .01$; Error bars = SEM. The number inside each vertical bar indicates the number of mice included in that particular group

3.5 | 5-HT antagonists reduce the suppressive effect of LPS on S-IRA

We hypothesized that the preventive effects of LPS on S-IRA and seizure severity are mediated by enhanced 5-HT neurotransmission in the brain. Based on a previous study showing that injection of nonselective 5-HT receptor antagonist—cyproheptadine (2 mg/kg, ip) 30 minutes prior to audiogenic stimulation increased the incidence of S-IRA and tonic seizures in DBA/1 mice,[29] we tested whether a similar dose of cyproheptadine would block the suppressive effect of LPS on S-IRA. Twelve hours after primed DBA/1 mice were injected with saline or 10 mg/kg of LPS, cyproheptadine (2 mg/kg, ip) was administered, which was followed by audiogenic testing in 35 minutes. Consistent with the above results, the LPS-treated group showed a significant reduction in the incidence of S-IRA (N = 9, 33.3%) (Kruskal-Wallis test, $P < .05$) when compared with the control group (N = 8, 100%) (Figure 5A). However, treatment with LPS + cyproheptadine resulted in a higher incidence of S-IRA (N = 9, 44.4%), which was not significantly different from the control group. There was also no significant difference in the incidence of S-IRA between the control group (N = 8, 100%) and the cyproheptadine only group (N = 10, 90.0%). No significant differences were seen in the seizure severity scores among all 4 groups (Kruskal-Wallis test, $P > .05$) (Figure 5B).

Similarly based on previous studies showing that pretreatment with a 5-HT$_3$ receptor antagonist—ondansetron (1-2 mg/kg, ip) blocked the protective effect of optogenetic stimulation of the 5-HT neurons in the dorsal raphe on S-IRA[19] and reduced the suppressing effect of fluoxetine on S-IRA,[18] we further investigated whether a similar dose of ondansetron would reverse the S-IRA suppressive effect of LPS. Mice were injected with saline or 10 mg/kg of LPS, and ondansetron (1 mg/kg, ip) was administered 12 hours after LPS injection. Audiogenic testing was performed 35 minutes after ondansetron treatment. Consistent with previous results, LPS-treated group showed significant reduction in the incidence of S-IRA (N = 10, 40.0%) (Kruskal-Wallis test, $P < .05$) compared with the control group (N = 8, 100%) (Figure 5C). However, treatment with LPS + ondansetron resulted in a higher incidence of S-IRA (N = 10, 80.0%), which was not significantly different from the control group. There was also no significant difference in the incidence of S-IRA between the control group (N = 8, 100%) and the ondansetron only treated group (N = 6, 100%). No significant differences were seen in the seizure severity scores among all 4 treatment groups (Kruskal-Wallis test, $P > .05$) (Figure 5D).

3.6 | Effect of LPS on S-IRA is mediated by activation of 5-HT neurons in dorsal raphe

Using c-Fos as a marker for neuronal activation, we applied immunohistochemistry to investigate the effect of intraperitoneal LPS administration on serotonergic neuron activity in the dorsal raphe. Primed PND 25 DBA/1 mice were intraperitoneally injected with 10 mg/kg of LPS (N = 9) or saline (N = 8), and brains were harvested 12 hours after injection. The total numbers of 5-HT-positive cells and 5-HT and c-Fos double-positive cells were quantified using NIH ImageJ software. LPS-treated animals showed $6.14 \pm 0.73\%$ of c-Fos-positive 5-HT neurons out of total 5-HT-positive neurons compared to the saline-treated animals 3.99 ± 0.60 (two-tailed Student's t test, $P < .05$) (Figure 6). The results suggest that intraperitoneal LPS injection increases the number of active 5-HT neurons in the dorsal raphe nucleus in DBA/1 mice.
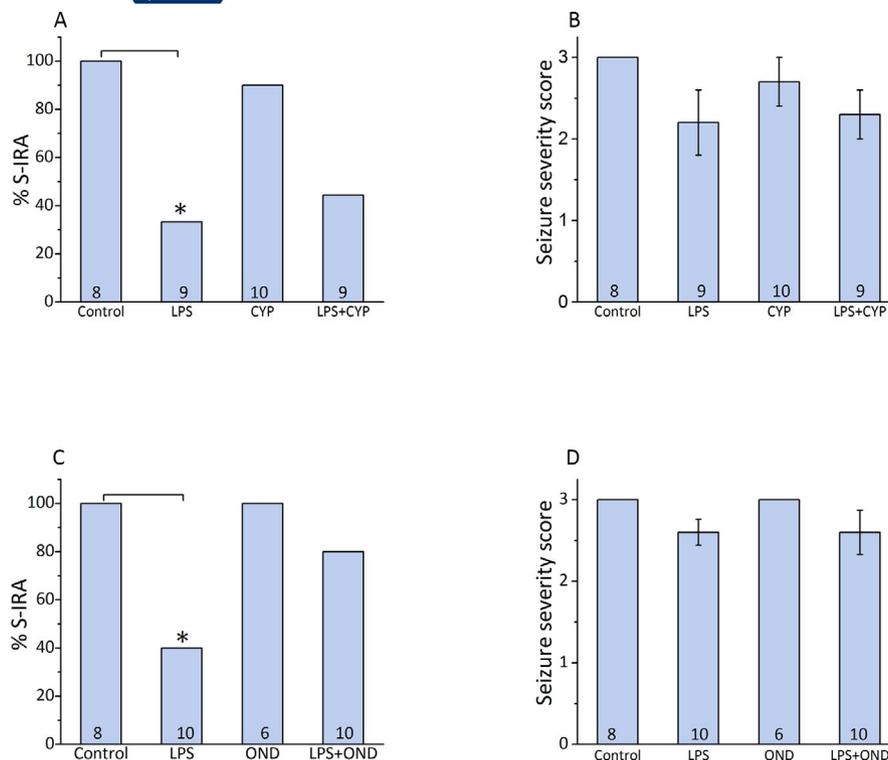
FIGURE 5 5-HT antagonists reduce the suppressive effect of LPS on S-IRA. Primed DBA/1 mice were injected with LPS (10 mg/kg, ip) and then a nonspecific 5-HT receptor antagonist—cyproheptadine (CYP) (2 mg/kg ip) or a 5-HT$_3$ receptor antagonist—ondansetron (OND) (1 mg/kg, ip) 12 h later. Audiogenic testing was performed after 35 min of 5-HT antagonist treatment. A, LPS-treated group showed significantly reduced incidence of S-IRA, while cyproheptadine reduced the protective effects of LPS on S-IRA ($P < .05$, Kruskal-Wallis test followed by Bonferroni correction). B, No significant differences were observed in the seizure severity scores among all 4 treatment groups ($P > .05$, Kruskal-Wallis test followed by Bonferroni correction). C, LPS-treated group showed significantly reduced incidence of S-IRA ($P < .05$). Ondansetron greatly reduced the protective effects of LPS on S-IRA (Kruskal-Wallis test followed by Bonferroni correction). D, No significant differences were observed in the seizure severity scores among all 4 groups ($P > .05$, Kruskal-Wallis test). *$P < .05$ when compared with the control group. Error bars = SEM. The number inside each vertical bar indicates the number of mice included in that particular group

Since LPS is known to disrupt blood-brain barrier (BBB),[30] it would be important to consider whether BBB disruption may lead to passage of LPS into brain to exert the observed protective effects. Our results with Evans blue extravasation assay[31] showed that there is no significant increase in the BBB permeability after 12 hours of LPS treatment (Figure S1) ($P > .05$, one-way ANOVA), suggesting that disruption of BBB may not be a significant factor in mediating the observed protection on S-IRA.

4 | DISCUSSION

In this study, we induced inflammation in DBA/1 mice by intraperitoneal injection of lipopolysaccharide (LPS) and determined its effect on S-IRA susceptibility using an audiogenic seizure model. We found that peripheral LPS administration was protective against S-IRA, with the maximum effect at 10 mg/kg and in 8-12 hours after its injection. This effect could be attenuated by blocking TLR4 or RAGE receptors prior to LPS injection or by

injection 5-HT receptor antagonists, cyproheptadine and ondansetron. Immunostaining to 5-HT and c-Fos showed significantly higher number of active serotonergic neurons in the dorsal raphe nucleus in the LPS group than the control group. Our results suggest that peripheral LPS-induced inflammation is effective in preventing S-IRA by activating TLR4/ RAGE signaling and enhancing 5-HT neurotransmission.

LPS is found on the outer membrane of most Gram-negative bacteria and activates the innate immune response via TLR4, which activates MAPKs, JNK, ERK, p38, and NF-kB pathways leading to the production of proinflammatory cytokines.[32] Peripheral administration of LPS[20,33] or cytokines like interleukin-6 (IL-6) has been shown to increase extracellular concentrations of 5-HT in different brain regions.[34–36] These findings suggest that peripheral inflammation can increase the 5-HT levels in the brain stem. Based on these reports, we propose that LPS-induced inflammation is protective against S-IRA. Our observation that intraperitoneal LPS administration prevents S-IRA in DBA/1 mice is in good agreement with previous studies showing that inflammation
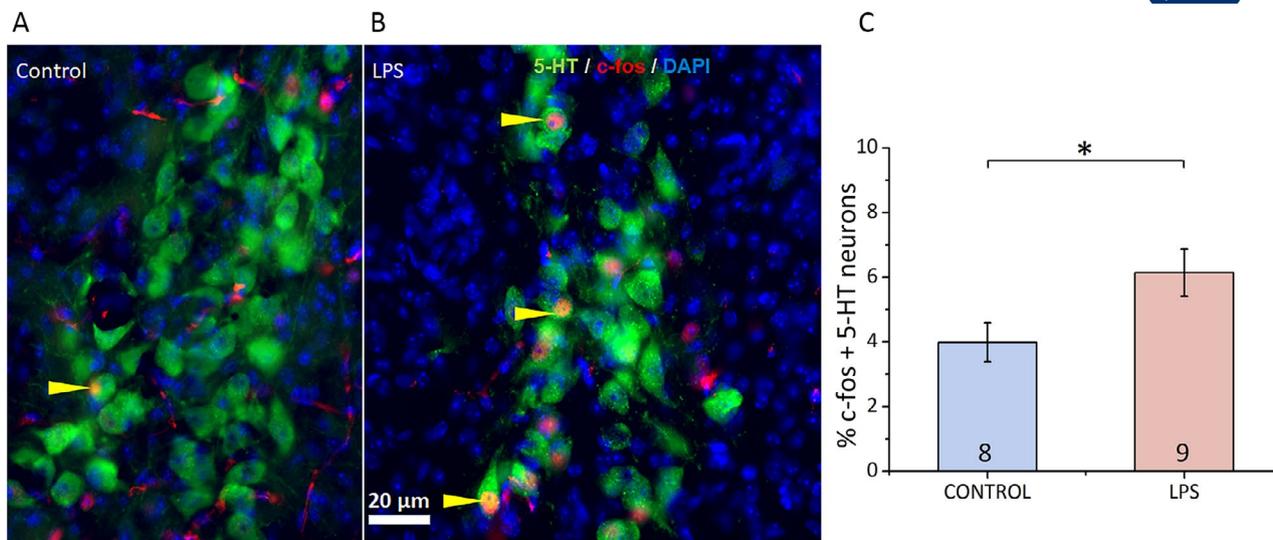
FIGURE 6 Intraperitoneal LPS injection increases the number of c-Fos-positive 5-HT neurons in the dorsal raphe. Photomicrographs illustrating immunostaining for 5-HT neurons (green) colabelled with c-Fos (red) and cell chromatin (blue) in control (A) and LPS-treated (B) animals. c-Fos-positive 5-HT neurons are depicted with yellow triangles. LPS-treated animals (9 animals, 30 slices) show more c-Fos-positive 5-HT cells compared to saline-treated control animals (8 animals, 23 slices). C, Percentage of c-Fos-positive 5-HT neurons out of total 5-HT neurons in LPS-treated mice was significantly higher than that of saline-treated control mice (6.14 ± 0.73% in LPS group vs. 3.99 ± 0.60 in saline group), (*$P < .05$ two-tailed Student's t test)

increases 5-HT levels in brain[22] and that increased 5-HT levels in brain stem protects against S-IRA.[29]

We observed an inverse relationship between LPS dose and S-IRA susceptibility: Increasing dosage of LPS injection is correlated with decreasing percentage of S-IRA. The S-IRA protection by LPS was observed as early as 1 hour, which peaked around 8-12 hours and lasted for up to 24 hours with complete reversal by 72 hours. Our earlier experiments (Figures 1 and 2) showed better effect of LPS in reducing the percentages of S-IRA than later experiments (Figure 4). Since this change in efficacy occurred after a new batch of LPS was ordered from the same company, it could possibly be due to batch variations of the LPS purchased at different times. Reduction in the incidence of S-IRA occurred concomitant with a reduction in severity of seizure which could be due to the anticonvulsive effects of the increased 5-HT. This observation is in line with previous studies where anticonvulsive effects of 5-HT have been reported.[37,38] However, this anticonvulsing effect of LPS leads to a question whether its protective effect on S-IRA is due to reducing the severity of seizure, particularly the tonic seizure that almost always leads to S-IRA and death in this mouse model. Our further analysis of incidences of audiogenic seizure, tonic seizure, S-IRA, and autoresuscitation after tonic seizure indicates that about twenty percent of LPS-treated animals that had tonic seizure did not develop S-IRA and survived (Figure 3), suggesting that LPS has a direct effect on enhancing respiratory function and resuscitation in addition to reducing seizure severity. The data provide convincing evidence that intraperitoneal injection of LPS promoted autoresuscitation during seizure.

Our data show both at behavioral and cellular level that the effect of LPS on reducing S-IRA could be attributed to enhancing serotonergic neurotransmission in the brain. Firstly, we demonstrate that cyproheptadine—a nonselective 5-HT receptor antagonist and ondansetron—a 5-HT₃ receptor antagonist reversed the S-IRA preventive effect of LPS. This observation is in line with previous studies where these agents have been shown to reverse the S-IRA preventive effects induced by enhancing 5-HT neurotransmission.[14,18,19,29] Secondly, we show that the number of c-Fos (neuronal activation marker)- and 5-HT-positive neurons was significantly higher in the dorsal raphe nucleus of LPS-treated animals compared to the saline-treated animals. Our study is in good agreement with Hollis et al (2006)[21] where they have shown that peripheral LPS administration increases Fos expression in 5-HT neurons of the dorsal raphe nucleus. Our data suggest that activation of 5-HT neurons in dorsal raphe may be a possible mechanism for enhanced 5-HT neurotransmission in LPS-treated animals. Increased activity (c-Fos expression) of the 5-HT neurons suggests an increased release of serotonin to the respiratory center, thereby stimulating the respiratory circuit.[39] However, additional studies are needed to understand how intraperitoneal injection of LPS activates the 5-HT neurons in the brainstem or if other mechanisms are involved in this effect.

Inflammatory mediators increase 5-HT release in brain. Intraperitoneal injection of LPS has been shown to increase c-Fos expression in the dorsal raphe nucleus 5-HT neurons[21] and increases 5-HT levels in the hippocampus.[20,33] IL-1β has been shown to elevate extracellular brain 5-HT levels.[20,36]

Interleukin-6 (IL-6) administration has been shown to increase extracellular concentrations of 5-HT and evoked release of 5-HT in the rat striatum.[34] These findings are in line with our observations that intraperitoneal LPS injection is protective against S-IRA, and this effect can be reversed by 5-HT antagonists. However, other studies have shown that peripheral inflammation reduces 5-HT levels in brain. Cytokines like IL-1β and TNF-α also activate the p38 MAPK pathway that increases the expression and function of serotonin transporters (SERT) that increase clearance of extracellular 5-HT.[40–43] Elevated IL-1β level has also been shown to upregulate 5-HT1A autoreceptors in raphe nucleus which provides feedback inhibition to the 5-HT neurons.[44,45] These studies refute the reports of other groups that have shown that inflammation increases 5-HT levels in brain. Further studies are necessary to identify the signaling pathways by different inflammatory mediators and their relative contributions in altering the brain 5-HT levels to explain the S-IRA protective effect of LPS.

Previous studies have shown that neuroinflammation promotes epileptogenesis[46] and TLR4 activation by HMGB1[47] and that LPS[48] decreases seizure threshold and is associated with a chronic increase in hippocampal neuronal network excitability.[47–49] These reports contradict with our observation that acute inflammation by relatively high doses of LPS is anticonvulsive. One possible explanation could be that the anticonvulsive effect of LPS in the DBA/1 model is mediated by increasing brain serotonin levels, which has been shown to be anticonvulsive,[37] whereas the proconvulsive effects of chronic inflammation reported by other groups are potentially mediated by transcriptional activation of genes contributing to cellular and molecular plasticity.

LPS is known to disrupt blood-brain barrier (BBB).[30] Banks et al, 2015, showed significant BBB disruption after 24 hours, but not after 4 hours, of injection of 3 mg/kg of LPS.[30] In contrast, the dose-response relationship of LPS effect in our study does not support an essential role of BBB disruption in the effect of LPS on S-IRA. Furthermore, our results from Evans blue extravasation assay showed no significant changes in BBB permeability after 12 hours after injection of up to 10 mg/kg LPS. Considering the much larger molecular weight of LPS (50-100 kDa), it is not supported that direct passage of LPS into brain could make a significant contribution to the S-IRA protection effect by LPS in our experiment condition.

Taken together, this study demonstrated the effect of intraperitoneal injection of LPS on preventing respiratory arrest in a mouse model of SUDEP and its possible mechanism by enhancing serotonin neurotransmission in the midbrain dorsal raphe nucleus. Our study shows the possibility that certain inflammatory mediators may augment 5-HT levels in brain stem. Identifying the signaling pathway involved in this mechanism will help developing pharmacological agents that can mimic the effect of LPS seen in this study and provide a new approach to prevent SUDEP.

CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.