ORIGINAL RESEARCH

# LACC1 Promoted Nerve Injury in an Anesthesia-Induced Cognitive Disorder Model via RIP2 Expression through ROS-NOD2 Induction

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## ABSTRACT

**Objective** • This study aimed to investigate the impact of laccase domain-containing 1 (LACC1) on the model of anesthesia-induced cognitive disorder and its underlying mechanisms.

**Methods** • We collected and analyzed data from 24 patients with postoperative cognitive dysfunction (POCD) through microarray analysis. In the animal model, mice were exposed to 1.3% isoflurane in a humidified atmosphere with 30% oxygen as the carrier gas. Additionally, PC12 cells were stimulated with 2% isoflurane.

**Results** • In mice subjected to anesthesia, both mRNA and protein expression levels of LACC1 were up-regulated. Moreover, LACC1 expression was increased in the anesthesia-induced cognitive disorder model. *In vitro*, LACC1 promoted the production of reactive oxygen species (ROS). Furthermore, LACC1 was found to exacerbate cognitive impairment in mice under anesthesia. The induction of RIP2 expression mediated this effect via the ROS-NOD2 pathway. The regulation of ROS-NOD2 played a critical role in controlling the impacts of LACC1 in both *in vivo* and *in vitro* models.

**Conclusions** • Our findings demonstrate that LACC1 contributes to nerve injury in the anesthesia-induced cognitive disorder model by inhibiting the ROS-NOD2/RIP2 signaling pathway. This discovery offers a novel perspective for understanding anesthesia-induced cognitive disorders. (*Altern Ther Health Med.* [E-pub ahead of print.])


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## INTRODUCTION

Postoperative cognitive dysfunction (POCD) refers to a condition characterized by cognitive impairments that can occur following surgery, often impacting memory, thinking, and overall mental function.[1-2] It is most commonly observed in older adults and remains a significant concern in the context of surgical interventions.[1] Previous studies have firmly established aging as a significant high-risk factor for POCD.[1-3]

The clinical presentations of POCD align with the categories of forgetfulness and dementia in traditional Chinese medicine (TCM).[2] POCD has the potential to result in both short-term and long-term postoperative health and economic consequences, including mental health issues, mortality, extended hospital stays, and increased healthcare-related expenses.[3] Research has demonstrated that various anesthesia methods may influence the incidence of POCD.[4] Several general anesthetics can impact neuronal processes, such as gene transcription, receptor effectiveness, synaptic vesicle dynamics, and intracellular calcium homeostasis.[5,6]

The elevated level of oxidative stress in POCD is linked to the activation of NADPH oxidase.[5] NADPH oxidase (Nox) serves as the primary enzyme responsible for generating reactive oxygen species (ROS) during ischemia-reperfusion injury (IRI), and it is particularly abundant in the kidney.[6] Notably, the kidney exhibits significant expression of Nox1, Nox2, and other Nox subunits, which serve as the primary source of ROS production in this organ.[7]

POCD has the capacity to activate Nox2 and Nox4, leading to a substantial increase in ROS production. The ROS produced by Nox enzymes are critical in mediating various cellular signaling pathways and play a vital role in regulating processes such as cell growth, division, proliferation, differentiation, apoptosis, and immune defense.[8]

RIP2 plays a key role in innate immunity, adaptive immunity, cell survival, and apoptosis, participating in numerous signaling pathways.[9] In the context of the apoptosis

signaling pathway, RIP2 exerts dual functions by both restraining and activating Caspase1 through its CARD domain (Caspase Activation and Recruitment Domain), leading to the up-regulation of the downstream pro-apoptotic factor, Bid (a pro-apoptotic protein), and the subsequent induction of apoptosis.[10] In the context of immune responses, RIP2 is responsible for triggering the production of inflammatory factors by mediating the signal transduction of receptors such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing proteins (NODs).[11]

Laccase domain-containing 1 (LACC1), previously known as C13orf31, is a genetic variant of laccase-containing domain 1, which includes the common coding single nucleotide polymorphism Ile254Val.[12] LACC1 has been associated with conditions such as leprosy, inflammatory bowel disease, and juvenile arthritis. However, only a limited number of studies have investigated the functional implications of LACC1 protein and its variants in mammals.[13-15] Therefore, this study aimed to explore the impact of LACC1 on the anesthesia-induced cognitive disorder model and explain its potential mechanisms.

## METHODS AND MATERIALS

### Study Design

The study design for this research involved male 20-month-old C57bl/6 mice, which were housed individually under controlled environmental conditions. These conditions maintained a temperature of 22 ± 1˚C and followed a 12-hour light/dark cycle. The mice were given unrestricted access to standard rat chow and sterile water. This study design allowed for examining the effects of LACC1 and anesthesia exposure on cognitive function in mice. Informed consent was obtained in accordance with the approved protocol by the Qiantang Campus Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University. Controls were recruited for peripheral blood draws. This study received approval from the Ethics Committee of Qiantang Campus Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University.

### Induction of Anesthesia

The study included three groups: (1) Model Group: mice in the model group were exposed to 1.3% isoflurane in a humidified atmosphere with 30% oxygen carrier gas for 4 hours; (2) Control Group: mice in the control group were exposed to 30% oxygen carrier gas for 4 hours; (3) LACC1 Group: mice in the LACC1 group received an injection of human LACC1 recombinant protein (1 μg/mice) for 2 days and were then exposed to 1.3% isoflurane in a humidified atmosphere with 30% oxygen carrier gas for 4 hours.

### Hematoxylin-Eosin (H&E) Staining

Upon sacrificing the mice, brain tissue was collected and fixed with 4% paraformaldehyde at room temperature for 24 hours. Lung tissue samples, also fixed with paraformaldehyde, were subsequently paraffin-embedded. The paraffin-embedded samples were then sectioned into 5 μm slices using a paraffin slicing machine and subjected to hematoxylin and eosin staining. The evaluation of lung tissues was performed using light microscopy (BH3-MJL; Olympus Corporation, Tokyo, Japan).

### Immunohistochemistry

Upon the sacrifice of the mice, brain tissue was collected and fixed with 4% paraformaldehyde at room temperature for 24 hours. The fixed brain tissue samples were subsequently paraffin-embedded. These samples were cut into 5 μm sections for further processing using a paraffin slicing machine. Triton X-100 was employed for staining. Immunofluorescence staining of brain tissue sections was carried out using an anti-LACC1 antibody (1:200, Cell Signaling Technology, Danvers, MA, USA) and conducted overnight at 4°C. Subsequently, fluorescence images were obtained using an Alex 555 conjugated secondary antibody (1:200, Thermo Fisher, MA, USA) after DAPI staining, and imaging was performed using the LSM 510 Meta microscope (Carl Zeiss).

### Immunofluorescence

Cell samples were fixed with 4% paraformaldehyde for 24 hours at room temperature. These fixed cell samples were then subjected to staining with Triton X-100 in PBS and subsequently blocked with 5% BSA in PBS for 1 hour at room temperature. Immunofluorescence staining of the cell samples was performed using an anti-LACC1 antibody (1:200, Cell Signaling Technology, Danvers, MA, USA) and conducted overnight at 4°C. Fluorescence images were acquired using an Alex 555 conjugated secondary antibody (1:200, Thermo Fisher, MA, USA) after DAPI staining. The LSM 510 Meta microscope (Carl Zeiss) did the imaging.

### Expression Plasmids, LACC1 Gene Silencing, Cell Culture, and Transfection

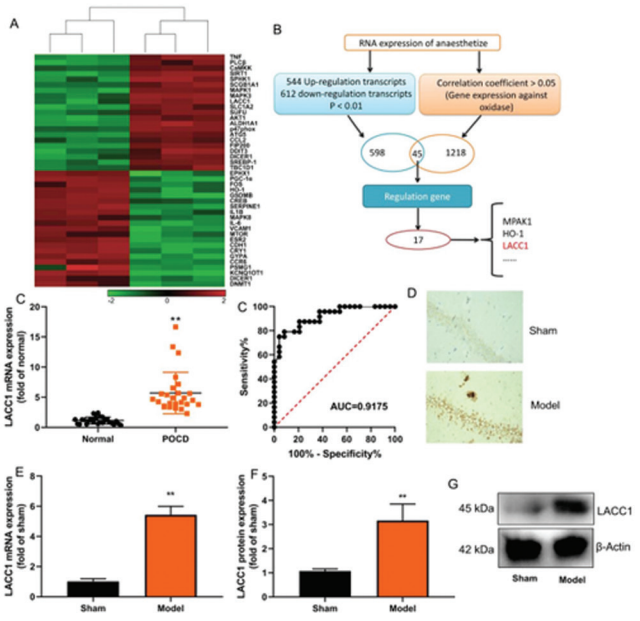
Human LACC1 was amplified through standard PCR using the sequence from GenBank, and the resulting fragment was subsequently inserted into the pGL3-Basic vector (Promega). Si-LACC1 mimics were procured from Santa Cruz Biotechnology. PC12 cells were cultured at 37°C in a 5% $CO_2$ environment using DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml).

Once PC12 cells reached 80% confluence, they were transfected with plasmids (LACC1, si-LACC1, negative control, and si-negative control) utilizing Lipofectamine 2000. Following transfection for 48 hours, PC12 cells were stimulated with a mixture of 2% isoflurane, 21% $O_2$, and 5% $CO_2$ for 6 hours, which was then added to the culture medium.

### Real-Time Quantitative RT-PCR

Total cellular RNA was extracted from lung tissue or cells using Trizol (Thermo Fisher Scientific). Subsequently, the extracted RNA was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase. MRNA expression was quantified using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and

**Figure 1.** LACC1 Expression in the Anaesthetize-Induced Cognitive Disorder Model




Note: Figure 1 illustrates the up-regulation of LACC1 expression in the anaesthetize-induced cognitive disorder model. The panel includes a microarray analysis and a schematic diagram of the results (A), serum mRNA levels of LACC1 expression (B), sensitivity analysis (C) conducted in patients with anaesthetize-induced cognitive disorder, LACC1 expression as demonstrated by immunohistochemical staining (D), and the expression of LACC1 mRNA and protein in anesthetize-induced mice with cognitive disorders (E, F, and G). The groups represented are Normal (normal volunteers group), POCD (Patients with the anaesthetize-induced cognitive disorder), Sham (sham control group), and Model (mice with the anesthetize-induced cognitive disorder). $^{**}P < .01$ indicates significant differences compared to the normal volunteers or sham control group.

SYBR® Premix Ex TaqTM II (Takara, Japan). Relative gene expression levels were normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH).

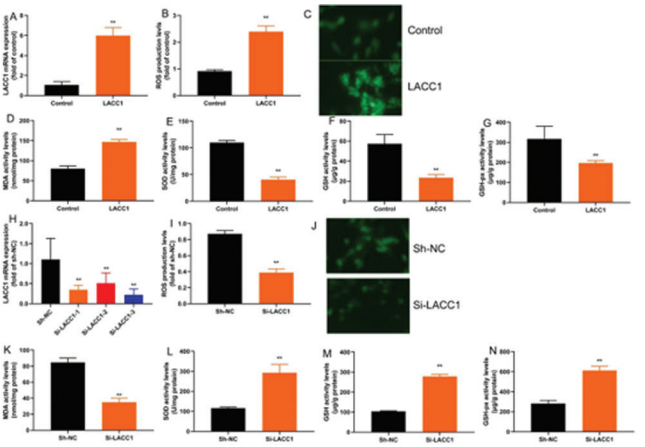
### ELISA Experiments

Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GSH-PX) and ROS levels in cultured supernatants were quantified using ELISA kits following the manufacturer's instructions. The ELISA plate reader used was from Bio-Rad Laboratories.

### Western Blot Analysis

Lung tissue or cells were collected and lysed in ice-cold lysis buffer. Protein concentrations were determined using a BCA protein quantitative kit. Total proteins were separated on 10% SDS-PAGE and transferred onto a PVDF membrane. The membranes were then blocked with 5% non-fat milk powder at room temperature for 2 hours and subsequently incubated with primary antibodies: LACC1 (ab24170, 1:1000, Abcam), NOD2 (ab31488, 1:1000, Abcam), RIP2 (ab75257, 1:1000, Abcam), and β-Actin (ab8226, 1:5000, Abcam) overnight at 4°C. The bound antibodies were detected using horseradish peroxidase-labeled secondary IgG (dilution 1:1000) and a Chemiluminescence Kit. Reactive bands were visualized using ECL western blotting detection reagent (GE Healthcare, USA).

**Figure 2.** LACC1-Mediated ROS Production in an *In Vitro* Model




Note: Figure 2 demonstrates the impact of LACC1 on ROS production in an in vitro model. It includes LACC1 mRNA expression (A), ROS production levels (B and C), as well as MDA, SOD, GSH, and GSH-px levels (D, E, F, and G) in the *in vitro* model following LACC1 over-expression. The figure also presents LACC1 mRNA expression (H), ROS production levels (I and J), and MDA, SOD, GSH, and GSH-px levels (K, L, M, and N) in the *in vitro* model by down-regulating LACC1. The represented groups are Control (control group), LACC1 (over-expression of LACC1), Sh-NC (Sh-control group), and Sh-LACC1 (down-regulation of LACC1). Data is presented as mean ± SD, with $^{**}P < .01$ signifying significant differences compared to the control group.

### Statistical Analysis

A two-tailed Student's *t*-test was utilized to compare different groups, providing a reliable measure of the differences observed. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Measurement data were presented as mean ± standard deviation ($\bar{x} \pm s$), allowing for a clear representation of the data's central tendency and variability. A significance level of $P < .05$ was applied to ensure the statistical significance.

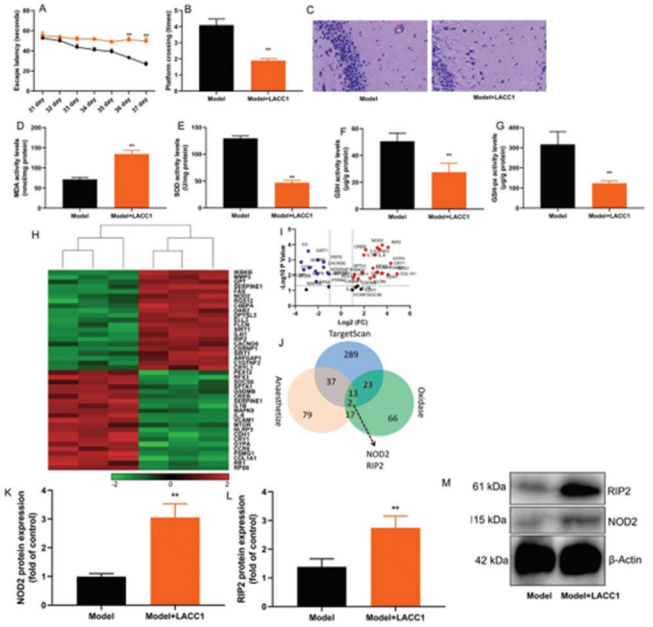
## RESULTS

### LACC1 Expression Up-Regulated in Anesthesia-Induced Cognitive Disorder Model

To investigate the expression and functional role of LACC1 in anesthesia-induced cognitive disorder, we collected and analyzed samples from 24 patients with POCD using microarray analysis, as shown in Figure 1A. Our analysis revealed that serum DCAF1 mRNA expression was significantly up-regulated in patients with POCD compared to the normal group, with an ROC value of 0.9175, as depicted in Figures 1B and 1C.

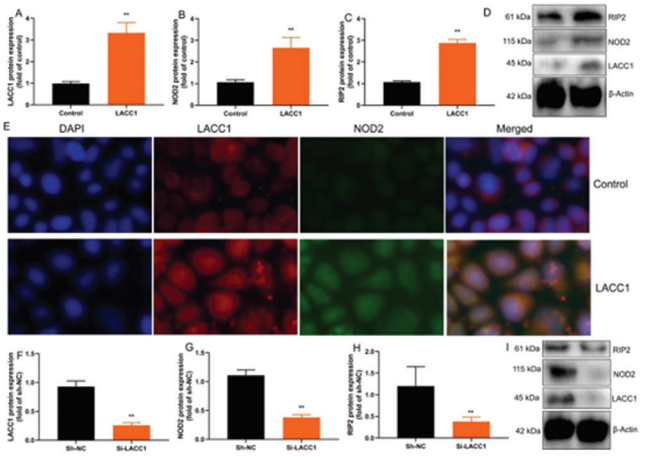
Additionally, immunohistochemical staining demonstrated an up-regulation of LACC1 expression in the brain tissue of mice subjected to anesthesia, as observed in Figure 1D. Furthermore, we observed increased LACC1 mRNA and protein expression in mice with anesthesia-induced cognitive disorder, as illustrated in Figure 1E-1G.

**Figure 3.** LACC1 Promotion of Cognitive Disorder in Mice Through Anesthetizing




Note: Figure 3 depicts the influence of LACC1 in promoting cognitive disorder in mice subjected to anesthesia. The figure includes data on escape latency (A), platform-crossing times (B), HE staining (C), as well as MDA levels (D), SOD, GSH, and GSH-PX levels (E, F, and G) in brain tissue. Additionally, it provides a microarray analysis and schematic diagram of results (H), a volcanic map (I), and another schematic diagram of results (J) highlighting the relationship between NOD2 and RIP2 protein expressions (K, L, and M). The groups represented are Model (mice with the anesthesia-induced cognitive disorder) and Model+LACC1 (mice with anesthesia-induced cognitive disorder treated with LACC1 protein). Data is presented as mean ± SD, with $^{**}P < .01$ signifying significant differences compared to the control group.

**Figure 4.** LACC1-Induced RIP2 Expression via ROS-NOD2 Induction




Note: Figure 4 demonstrates how LACC1 induces the expression of RIP2 through the induction of ROS-NOD2 in an *in vitro* model. The figure presents LACC1, NOD2, and RIP2 protein expressions (A, B, C, and D) following LACC1 over-expression. It also shows LACC1 and NOD2 expressions (E) in the *in vitro* model with LACC1 over-expression. Additionally, it includes LACC1, NOD2, and RIP2 protein expressions (F, G, H, and I) in the *in vitro* model with LACC1 down-regulation. The groups represented are Control (control group), LACC1 (over-expression of LACC1), Sh-NC (Sh-control group), and Sh-LACC1 (down-regulation of LACC1). Data is presented as mean ± SD, with $^{**}P < .01$ signifying significant differences compared to the control group.

### LACC1 Promotes ROS Production in an *In Vitro* Model

This study investigated the impact of LACC1 on ROS-induced nerve injury in an *in vitro* model of anesthesia. LACC1 expression was observed to be increased in the *in vitro* model using LACC1 plasmid, as depicted in Figure 2A. Notably, the over-expression of LACC1 led to elevated ROS production and MDA levels while simultaneously reducing SOD, GSH, and GSH-PX levels in the *in vitro* model of anesthesia, as illustrated in Figure 2B-2G.

Conversely, Si-LACC1 mimics effectively reduced LACC1 expression in the *in vitro* model, as shown in Figure 2H. Consequently, the down-regulation of LACC1 resulted in decreased ROS production and MDA levels, as well as an increase in SOD, GSH, and GSH-PX levels within the *in vitro* model of anesthesia, refer to Figure 2I-2N.

### LACC1 Promotes Cognitive Disorder in Mice through Anesthesia

To investigate the impact of LACC1 on cognitive disorders induced by anesthesia in mice, human LACC1 recombinant protein was administered to mice with anesthesia-induced cognitive disorders. Treatment with human LACC1 recombinant protein resulted in an increased time (escape latency) required for the mice to locate the platform in the Morris Water Maze (MWM) pool and a reduction in platform-crossing times for mice subjected to anesthesia, as demonstrated in Figure 3A-3G. Additionally, human LACC1 recombinant protein exacerbated nerve injury, reduced levels of SOD, GSH, and GSH-PX, and elevated MDA levels in brain tissue samples, as illustrated in Figure 3D-3G.

To investigate the mechanisms by which LACC1 affects mice under anesthesia, the experimental microarray analysis was employed to reveal the regulatory impact of LACC1 on cognitive disorders induced by anesthesia in mice, as depicted in Figure 3H. The resulting volcano plot highlighted NOD2 and RIP2 as potential target candidates influenced by LACC1 in the context of cognitive disorders in anesthesia-induced mice, illustrated in Figure 3I-3J. Furthermore, treatment with human LACC1 recombinant protein-induced changes in NOD2 and RIP2 protein expressions within the brain tissue of mice subjected to anesthesia is presented in Figure 3K-3M.

### LACC1 Induces RIP2 Expression through ROS-NOD2 Induction

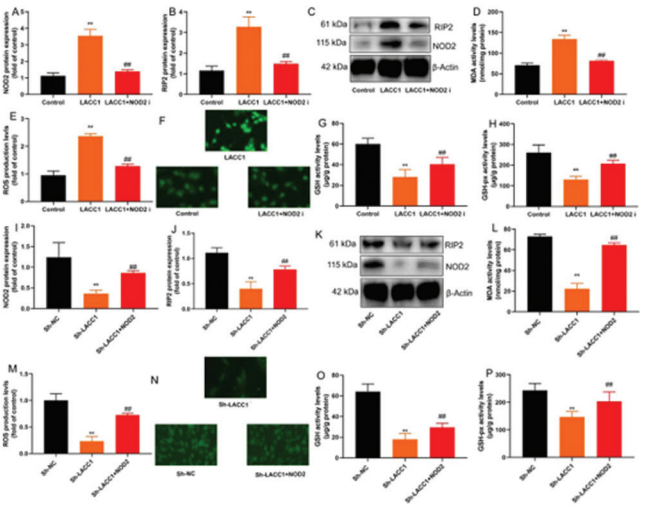
In an *in vitro* model of LPS-induced lung injury, the over-expression of LACC1 led to an upregulation of LACC1, NOD2, and RIP2 protein expressions, refer to Figure 4A-4D. Immunofluorescence analysis revealed that the over-expression of LACC1 promoted increased expressions of LACC1 and NOD2 within the *in vitro* model, as shown in Figure 4E. Conversely, the down-regulation of LACC1 resulted in the suppression of LACC1, NOD2, and RIP2 protein expressions in the *in vitro* model, refer to Figure 4F-4I.

### ROS-NOD2 Regulation Controls the Effects of LACC1 in the *In Vitro* Model

This study revealed the important role of ROS-NOD2 regulation in controlling the effects of LACC1 within the *in vitro* model. Notably, the use of an NOD2 inhibitor (GSK717, 50 nM) effectively suppressed the protein expression of NOD2 and RIP2 in the *in vitro* model with LACC1 over-expression, compared to LACC1 over-expression alone, as depicted in Figure 5A-5C. Furthermore, the NOD2 inhibitor led to a reduction in MDA and ROS production levels and an increase in GSH and GSH-PX levels in the *in vitro* model with LACC1 over-expression, as opposed to LACC1 over-expression alone, as shown in Figure 5D-5H.

Conversely, the introduction of the NOD2 plasmid induced enhanced protein expression of NOD2 and RIP2 in the *in vitro* model with LACC1 down-regulation, as compared to LACC1 down-regulation alone, as illustrated in Figure 5I-5K. This alteration resulted in increased MDA and ROS production levels and decreased GSH and GSH-PX levels in the *in vitro* model with LACC1 down-regulation, compared to LACC1 down-regulation alone (refer to Figure 5L-5P).

**Figure 5.** Regulation of LACC1 Effects in *In Vitro* Model by ROS-NOD2 Control




Note: Figure 5 illustrates how the regulation of ROS-NOD2 controls the effects of LACC1 in an *in vitro* model. It includes NOD2 and RIP2 protein expressions (A, B, and C), MDA levels (D), ROS production levels (E and F), as well as GSH and GSH-PX levels (G and H) in the *in vitro* model with NOD2 inhibitor. The figure also presents NOD2 and RIP2 protein expressions (I, J, and K), MDA levels (L), ROS production level (M and N), and GSH and GSH-PX levels (O and P) in the *in vitro* model with NOD2 plasmid. The groups depicted are Control (control group), LACC1 (over-expression of LACC1), LACC1+NOD2 i (over-expression of LACC1 and NOD2 inhibitor), Sh-NC (Sh-control group), Sh-LACC1 (down-regulation of LACC1), and Sh-LACC1+ NOD2 (down-regulation of LACC1 and over-expression of NOD2). Data is presented as mean ± SD, with $^{**}P < .01$ indicating significance compared to the control group and $^{\#\#}P < .01$ indicating significance compared to the down-regulation of the LACC1 group.

## DISCUSSION

POCD refers to central nervous system complications in patients after general anesthesia. Clinically, it is characterized by manifestations such as mental confusion, anxiety, personality changes, memory impairment, and alterations in cognitive function, independent abilities, and skills.[16] Past studies have established that surgery, despite its therapeutic purpose, can be a traumatic experience for patients.[16,17]

Surgical stress has the potential to trigger immune system activation, leading to the overproduction of inflammatory cytokines, which can subsequently impact the development of POCD.[17] Currently, the effects of acupuncture anesthesia have been clinically validated, particularly in terms of reduced anesthesia medication requirements, decreased complications, and enhanced overall body protection.[17-18]

Previous research has consistently demonstrated the protective effects of acupuncture in preventing the occurrence of POCD.[16,18] The findings revealed an up-regulation of LACC1 expression in the anesthesia-induced cognitive disorder model. Leturiondo et al.[19] have confirmed that LACC1 plays a significant role in nerve injury among Amazon ethnic admixed populations. These findings suggest a potential association between LACC1 and its neurotoxicity in the anesthesia-induced cognitive disorder model. Oxidative stress serves as an indicator of brain tissue damage in patients undergoing surgery.[20] SOD is an enzyme responsible for scavenging oxygen-free radicals, and it provides insight into the body's overall antioxidant capacity.[21]

MDA on the other hand, is a toxic byproduct generated by the body during the stress response, and it reflects the extent of free radical damage to brain tissue.[22] The current data demonstrates that LACC1 promotes cognitive disorders and increases ROS-induced oxidative stress in both *in vivo* and *in vitro* models through anesthesia. Zhang et al.[23] have provided evidence that LACC1 plays a role in regulating oxidative stress within a co-culture system. This finding suggests a potential connection between LACC1 and its oxidative effects in the context of anesthesia-induced cognitive disorders.

Nox-produced ROS is a key player in mediating multiple signaling pathways within cells, contributing significantly to signal transduction processes that regulate cell growth, division, proliferation, differentiation, apoptosis, and immune defense.[24,25] In the present study, LACC1 induced the expression of RIP2 by activating ROS-NOD2. Additionally, Lahiri et al.[12] have reported the critical role of LACC1 in optimal NOD2-induced signaling and mitochondrial ROS production. As a result, it is possible that LACC1 could potentially enhance the cognitive disorder function in anesthesia.

RIP2 is crucial in downstream activation within the innate immune pattern recognition receptor NOD1 and NOD2 signaling pathways.[26] When phagocytic cells present muramyl dipeptide (MDP) to bind intracellularly with NOD after bacterial phagocytosis, or when MDP is directly internalized into the cytoplasm and binds with NOD, it triggers the intermolecular polymerization of NOD.[27] The polymerized NOD molecule can subsequently recruit RIP2, which contains the CARD domain, through its CARD structure.[26-27]

The polymerized NOD molecule can recruit RIP2, which includes the CARD domain, through its CARD

structure.[27] The present findings demonstrate that the regulation of ROS-NOD2 controls the effects of LACC1 within the *in vitro* model by influencing the regulation of RIP2 expression. These findings also offer a potential avenue for preventing or treating potential anesthesia-induced cognitive deficits.

### Study Limitations

This study has a few limitations. While the information provided is valuable, a more comprehensive exploration is necessary to disclose the details of this underlying process. Secondly, the utilization of human tissue samples was limited in this research, potentially impacting the generalizability of the findings. Future investigations will address these limitations by investigating the mechanistic aspects and expanding the use of human organizational samples to enhance the robustness and applicability of the study's conclusions.

## CONCLUSION

In conclusion, our study provides evidence that LACC1 significantly promotes nerve injury and elevates ROS-induced oxidative stress in the anesthesia-induced cognitive disorder model by inducing RIP2 expression through the ROS-NOD2 pathway. These findings suggest that LACC1 is implicated in the disruption of brain energy metabolism induced by anesthesia, which in turn initiates synaptic dysfunction, ultimately resulting in cognitive impairment. Consequently, LACC1 emerges as a promising molecular target for the prevention and management of POCD and anesthesia-induced cognitive disorders.

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None

### CONFLICT OF INTERESTS
The authors report no conflict of interest.

### AVAILABILITY OF DATA AND MATERIALS
The data supporting the findings of this study are available from the corresponding author upon request, subject to reasonable conditions.

### FUNDING
No funding was received.

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