# Recombinant rabies virus expressing chimeric Omp31/sodC or AHCY gene from Brucella melitensis elicits high level of antibodies and secretary cytokines in mice

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**Research article**

**Keywords:** Rabies virus; Brucella; Omp31/SodC gene; AHCY gene; Cellular and humoral immunity.

**Posted Date:** October 18th, 2019

**DOI:** https://doi.org/10.21203/rs.2.16210/v1

# Abstract

Background: The rabies virus (RV) vector LBNSE expressing foreign antigens has shown considerable promise as vaccines against viral and bacteria diseases, which is effective and safe. We produced a new RV-based vaccine vehicle expressing Brucella melitensis Omp31/sodC or AHCY gene by reverse genetics technology. The aim of this study was to investigate the cellular and humoral immunity of recombinant viruses. Results: The recombinant virus rLBNSE-SOD and rLBNSE-AHC retained growth properties similar to those of vector rLBNSE both in BSR and mNA cell culture. The Omp31/SODC and AHCY gene was expressed and detected by immunostaining. To compare the immunogenicity of LBNSE-SOD and LBNSE-AHC, mice were immunized with each of recombinant virus by intramuscular (i.m.). Then mice were bled at days 7, 14 after the immunization for the measurement of cytokines and virus neutralizing antibody (VNAs). The parent virus (LBNSE) without expression of any foreign molecules was included for comparison. It was found that mice inoculated with LBNSE-SOD and LBNSE-AHC showed no any signs of disease and exhibited seroconversion against RV. Our findings showed that mice were immunized with each of these recombinant RVs by intramuscular (i.m.) developed efficacy cellular and humoral immunity. The mRNA level of cytokines (IFN-γ and IL-2; IL-4 and IL-10) and VNA level against rabies virus in the blood of mice were increasing after immunization with recombinant RVs. There were no obvious histopathological changes in the brain samples of all mice. Conclusions: The studies suggested that recombinant RVs expressing Omp31/SodC or AHCY gene would elicit high level of antibodies and secretary cytokines and provided a promising vaccine candidate in mice.


# Background

Brucellosis, caused by *Brucella spp*., is a worldwide spread zoonotic disease transmitted from domestic animals to humans. *Brucella* causes abortions and sterility in infected domestic animals and various insidious syndromes in humans. The disease is a major cause of direct economic losses and is an impediment to trade and export [1]. Identifying novel *Brucella* antigens that can elicit a protective immune response, without the drawbacks of live attenuated vaccines, could enable development of next-generation vaccines. Previous study showed S-adenosyl-L-homocysteine hydrolase (AdoHcyase, AHCY) was identified and expressed in *Escherichia coli*. The recombinant AHCY induced a strong antibody response and a high level of protection against *B. melitensis16M* challenge, which indicated that rAdoHcyase could be a useful candidate for the development of subunit vaccines against *B. Melitensis* [2]. In previous study, intramuscular injection with a plasmid DNA carrying Omp31/SodC gene (pcDNA-Omp31/SodC) was able to induce a protective immune response [3]. And intraspleen immunization with pcDNA- Omp31/SodC vaccine efficiently induced Omp31/SodC -specific CD4+ and CD8+ T cells [4]. Recently, an improved recombinant *Brucella abortus* vaccine strain RB51SOD by over-expressing Cu-Zn superoxide dismutase (SOD), has been shown to confer better protection than parental strain RB51 in a mouse model, which indicated that SOD could be a new immunogenic candidate gene for the development of subunit vaccines against *B. Melitensis* [5]. Oral immunization of mice with recombinant

Lactococcus lactis expressing SOD gene of *Brucella abortus* triggers protective immunity against *B. abortus* infections [6].

The reverse genetics system has developed rapidly in recent years, which is helpful to create genetically engineered vaccine [7, 8]. Recombinant rabies virus vaccine expressing proteins of other pathogens stably show powerful protection against other infectious diseases as vaccine vector[9-11], while the safety concerns of recombinant rabies virus vaccine have previously been stated in our study [12]. In previous reports, it was indicated that recombinant RV expressing chemokines/cytokines including granulocyte-macrophage colonystimulating factor (GM-CSF), macrophage-derived chemokine (MDC), and macrophage inflammatory protein (MIP-1a), can recruit and/or activate more DCs to enhance the immunogenicity[12, 13].

In present study, we generated a recombinant RV attenuated vaccine strain expressing *Brucella abortus* Omp31/SodC (LBNSE-SOD) or AHCY gene (LBNSE-AHC) which is immunogenic candidate gene and plays an important role in inducing the protective immunity as well. In order to further investigate the immunological characteristics of the recombinant RV, forty 5-week-old female mice were experimentally immunized with LBNSE-SOD and LBNSE-AHC. And hematocytes analysis, pathological changes, humoral and cell-mediated immune responses were evaluated in this study.

# Results

### Construction and rescue of infectious chimeric clone and detection of rRABV expressing *Brucella* Omp31/SodC or AHCY gene

To construct recombinant rabies virus (RV) expressing *Brucella* Omp31/SodC or AHCY gene, a recombinant virus was developed using the RV infectious cDNA clone pLBNSE as the backbone as described in Methods. Infectious clone was shown in Figure 1A, which *Brucella* Omp31/SodC or AHCY gene was inserted into pLBNSE between the G and L genes by *Bsi*wI and *Nhe*I. The rescued chimeric clone was named as LBNSE-SOD and LBNSE-AHC confirmed by IFA and DNA sequencing. Several nucleotide changes including foreign genes inserted restriction endonuclease (RE) sites were observed throughout the genome.

To detect the rescued infectious clone from the cell culture supernatant, the transfected cells were fixed and examined by an indirect fluorescence assay (IFA). It was indicated that the BSR cells transfected with LBNSE (Fig. 2B), LBNSE-SOD (Fig. 2C) and LBNSE-AHC (Fig. 2D) showed green fluorescence, respectively. However, control cells remained negative (Fig.2A). Full-genome sequencing results showed that recovered virus LBNSE-SOD and LBNSE-AHC maintained the nucleotide sequence of the original plasmids pLBNSE-Omp31/SodC and pLBNSE-AHCY.

### Growth curve of recombinant RVs

One concern about a recombinant RV expressing *Brucella* Omp31/SodC or AHCY gene was whether this will affect the viral replication cycle, resulting in a recombinant RV that grows slowly or at low titer. To study the replication kinetics of LBNSE-SOD and LBNSE-AHC, a multicycle growth curve was performed by infecting BSR cells at an MOI of 0.01. From the Figure 1, we can get the information of growth kinetics of the reconstituted viruses and the parental virus. The skeleton virus LBNSE and the chimeric virus LBNSE-SOD and LBNSE-AHC showed the similar growth kinetics in BSR cells (Figure 1B) and mNA cells (Figure 1C). The recombinant RV grew very similarly and reached approximately the same titer at similar time points, indicating there were no differences in viral spread. Therefore, the insertion of Omp31/SodC and AHCY genes did not have any major impact on the viral life cycle. The cloned virus was further amplified by subsequent passages in BSR cells. And the titer of passage-3 rescued virus was determined.

### Cells count in the heparinized blood of mice

To determine the effect of recombinant rabies viruses expressing *Brucella* genes on hematocyte differentiation, mice were immunized with each of rRV virus and heparinized blood were harvested and hematocytes were auto-counted by VetScan HM5 (Gurnee, IL). As showed in Fig. 3A and 3B, there are no significant different change of total white blood cells and red blood cells at different time-points between each group. Fig. 3C showed that the number of platelets started to increase at 7 dpi and significantly higher than control group in mice immunized with LBNSE, LBNSE-SOD and LBNSE-AHC ($p < 0.05$). In Fig. 3D, the number of lymphocytes also started to significantly increase at 7 dpi than control group in mice immunized with LBNSE and LBNSE-AHC, while significantly higher than control group in LBNSE-SOD and LBNSE-AHC groups by 14 dpi ($p < 0.05$), which was going down at 14 dpi in LBNSE group. As showed in Fig. 3E, ONLY LBNSE group showed significantly more number of monocytes than other groups ($p < 0.05$), and there are no significant different change in other groups through the experiment course. Finally, in Fig. 3F, ONLY LBNSE-AHC group showed significantly more number of neutrophils than other groups ($p < 0.05$), and there are no significant different change in other groups through the experiment course.

### The mRNA level of cytokines in the heparinized blood of mice

To address the cellular and humoral immunity after immunization with recombinant rabies viruses expressing *Brucella* genes, mice were immunized with each of rRV virus and heparinized blood were harvested and mRNA level of cytokines (IFN-γ and IL-2; IL-4 and IL-10) in the blood were measured by real-time PCR. Fig. 4A and 4B showed mRNA level of IL-2 and IFN-γ increased significantly at 7 dpi and reached the highest levels by 14 dpi in the LBNSE-SOD and LBNSE-AHC groups, compared to control group ($p < 0.05$). And LBNSE group showed significantly more mRNA level of IL-2 and IFN-γ at 7 dpi. As shown in Fig. 4C and 4D, mRNA level of IL-4 started to increase while IL-10 peaked at 7 dpi ($p < 0.01$). However IL-10 started to decline by 14 dpi in all immunized groups, while there is still significantly more mRNA level than control group. Of interest, immunization with LBNSE-SOD and LBNSE-AHC were able to express relatively more IL-10 levels in the sera than with parental virus. On the other hand, delivery with LBNSE-SOD could develop relatively more IL-4 levels in the blood than with LBNSE-AHC and parental virus.

### The ELISA level of cytokines in the sera of mice

To confirm the expression of Th1 and Th2 cells in the sera after immunization with recombinant rabies viruses expressing *Brucella* genes, ELISA level of cytokines (IFN-γ and IL-10) in the sera of mice were detected by mouse quantitative ELISA Kit according to the manufacturer's instructions. Fig. 5A showed ELISA level of IFN-γ increased significantly at 7 dpi and reached the highest levels by 14 dpi in all the vaccinated groups, compared to control group ($p < 0.05$). As shown in Fig. 5B, ELISA level of IL-10 peaked at 7 dpi and started to decline by 14 dpi in all immunized groups ($p < 0.05$), while there is no significant different change through the experiment in control group. Of note, immunization with LBNSE-SOD and LBNSE-AHC can express relatively more IFN-γ and IL-10 levels in the sera than with parental virus.

### The VNA level against rabies virus in the sera of mice

In order to address the VNAs production against rabies virus after recombinant RV immunization，blood samples were collected at 7, 14 dpi, and sera VNAs were determined by RFFIT. As shown in Fig. 6, VNA titres in sera increased significantly at 7 dpi and reached the highest levels by 14 dpi in the LBNSE, LBNSE-SOD and LBNSE-AHC groups, compared to control group. While there were no significant different VNA titres between in mice immunized with recombinant RVs and the parent virus ($p > 0.05$).

### Gross pathological and histopathological changes

To investigate the effects of recombinant rabies viruses expressing *Brucella* Omp31/SodC or AHCY gene on tissues pathological and inflammatory cell infiltration in brains. Mice were immunized with each of recombinant rabies virus and brains were collected and stained with H.E. No obvious pathological lesions were found in all organs of control mice treated with Medium and LBNSE group at necropsy, while 1 out of 10 mice in LBNSE-SOD and LBNSE-AHC group showed mild hemorrhage and swelling in lymph nodes (data not shown). Under microscopic examination from Fig.7, no perivascular-cuffing and neuronal degeneration showed in all brain samples of control and immunized groups. There is no inflammatory cell infiltration in the brains of control and LBNSE groups. While only one out of ten mice immunized with LBNSE-SOD and LBNSE-AHC showed mild inflammatory cell infiltration in brain.  vascular cuff vascular cuff

## Discussion

Our previous studies have shown that recombinant RABV expressing dendritic cell-activating molecules such as GM-CSF, MDC, MIP-1α and bacterial flagellin could enhance the innate and adaptive immune response to vaccination and led to better protection than with the parent virus when challenged with virulent RABV in mice[12, 13, 22, 23]. It is confirmed that recombinant rabies virus vaccine strain-based vectors expressing foreign genes or proteins provided the promising safe and effective vaccine candidates in mouse model. In this study, *Brucella* Omp31/SodC or AHCY gene was cloned into the pLBNSE genome respectively and recombinant virus LBNSE-SOD and LBNSE-AHC were rescued. After incorporation of Omp31/SodC and AHCY gene, the recombinant virus still grew to a high final titer of

about $10^{7.0}$ FFU/mL. From the multicycle growth curve, it showed that inset of Omp31/SodC and AHCY gene did not affect the growth and replication of recombinant rabies virus. Safety is a major concern for the use of live viral vector. However, this RV based vectors expressing Omp31/SodC and AHCY gene were completely safe in mice and apathogenic after intramuscular infection. For gross pathological, there was no obvious pathological lesion in all organs from almost all mice of LBNSE-SOD and LBNSE-AHC groups at necropsy (data not shown).

From hematocytes analysis results, there were the similar change tendency for different blood cells between LBNSE-SOD and LBNSE-AHC and parental virus. Except that ONLY LBNSE group showed significantly more number of monocytes than other groups and ONLY LBNSE-AHC group showed significantly more number of neutrophils than other groups. In previous study, T-cell proliferative responses and increased production of gamma interferon were also observed upon splenocyte restimulation with recombinant SOD and the pcDNA-SOD induced improved protection against challenge with the virulent strain B. abortus in BALB/c mice[24]. A liposome based DNA vaccine co-expressing SOD gene along with interleukin-18 (IL-18) was evaluated in experimental murine brucellosis. Higher lymphoproliferative response and IFN-gamma production were developed after treatment with recombinant DNA vaccine [25]. A recombinant DNA vaccine encoding SOD confered high protection against *Brucella abortus* 2308 by inducing specific CTL responses in mouse model[26]. In our study, the results of mRNA level of cytokines (IFN-γ and IL-2; IL-4 and IL-10) in the blood indicated that all cytokines except IL-10 showed continuous increasing by 14 dpi. The change trend of ELISA level of cytokines (IFN-γ and IL-10) in the sera showed in Figure 5 was consistent with the results of mRNA level in blood showed in Figure 4. High VNA titers were detected in mice immunized with LBNSE-SOD and LBNSE-AHC and parental virus, there were no significant difference between recombinant RVs and parental virus. These data predict that treatment with LBNSE-SOD and LBNSE-AHC also induced high adaptive immunity as parental virus. Further studies are needed to address if recombinant RVs is effective to complete protect mice against rabies virus challenge.

In conclusion, the results presented here suggest that recombinant virus delivering Omp31/SodC and AHCY gene induced good immune response against rabies virus. The large insert genes from *Brucella melitensis* can be efficiently expressed by RV-based backbone, which further indicates their potential function as efficacious antiviral vaccines. Our results of immune response following rLBNSE which expresses Omp31/SodC or AHCY gene from *Brucella melitensis* imply further researches on subunit vaccine immunizations in mice, especially in order to reduce the risks of the virulence reversion of attenuated strain in the field application.

# Conclusions

A new recombinant rabies virus expressing Brucella melitensis Omp31/sodC or AHCY gene was constructed in this study. The LBNSE- SOD and LBNSE-AHC showed the similar viral growth kinetics to parental virus both in BSR and mNA cell culture. Likewise, our findings show the expression of Omp31/sodC or AHCY gene in RABV results in increased cytokines secretary and VNA production. While

there were no obvious histopathological changes and inflammatory cell infiltration in brains of mice after immunized with recombinant rabies virus. It is suggested that recombinant RVs expressing Omp31/sodC or AHCY gene are more immunogenic and provide a promising vaccine candidate in mice. However, the immune response of expressed Omp31/sodC or AHCY gene is warranted to address in the further research.

# Methods

### Cells and viruses

Mouse neuroblastoma cells (NA) were maintained in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY). BSR cells, a cloned cell line derived from BHK-21 cells, were maintained in Dulbecco's modified Eagle's medium (Mediatech) containing 10% FBS. Recombinant RV strains were propagated in BSR cells. Fluorescein isothiocyanate (FITC)-conjugated antibody against the RV N protein was purchased from FujiRab (Melvin, PA). Challenge virus standard 11 (CVS-11) was propagated in NA cells.

### Plasmid construction

The recombinant rabies virus plasmids, pLBNSE, were constructed as described previously [12]. Briefly, the skeleton plasmid pLBNSE was developed from an SAD L16 cDNA clone in pcDNA3.1 (+) (Invitrogen, Carlsbad, CA). And the transcription unit with the *Bws*I and *Nhe*I restriction sites was designed between the G and L genes by removing the pseudogenes. To produce a new rabies virus vector expressing *Brucella* Omp31/SodC and AHCY gene, the genes were amplified from by using Pfu polymerase (Biolabs, Inc.). And the primers used for the construction of these genes were designed by using Primer5.0. The PCR product was digested with *Bsi*WI-*Nhe*I and linked to the *Bsi*WI-*Nhe*I-digested plasmid pLBNSE. This plasmid was named as pLBNSE-SOD and pLBNSE-AHC (Fig. 1a) and the insert gene was confirmed by restriction analysis and DNA sequencing.

### Recombinant viruses rescue and insert genes expressing detection

The full-length recombinant cDNA clones were purified and were co-transfected with the plasmids encoding the RV N, P, G, and L proteins into BSR cells which stably expressing T7 RNA polymerase [14] by standard methods [10], and infection was examined in 2 from 6 wells of co-transfected cells. The recombinant virus was rescued as described previously [15,16]. Briefly, BSR cells were transfected with 2.0 μg of the full-length infectious clone, 0.5 μg of N-, 0.25 μg of P-, 0.1 μg of L-, and 0.15 μg of G-expressing plasmids using SuperFect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacture's instruction. After 4 days, the culture medium was discarded and another fresh medium was added. And the culture supernatant was harvested after another 3 days, then the rescued recombinant viruses were examined by an indirect fluorescence assay using FITC-conjugated antibody against the RV N protein (Fujirebio Diagnostic, Inc. DA, USA). Rescued recombinant viruses were designated as LBNSE-SOD and LBNSE-AHC.

To verify the insert genes, the *Brucella* Omp31/SodC gene was amplified by using primers 5'-CC*ATCGAT*ATGCTGGTTGTTTCTGAACC-3' (forward) and 5'-CCG*CTCGAG*TTATTCGATC ACGCCGCAGGC-3' (reverse), while the *Brucella* AHCY gene was amplified by using primers 5'-CC*ATCGAT*ATGCAATTTTGCCTTCAG-3'(forward) and 5'-CCG*CTCGAG*TTAATACCTGTAG TGTTCCG-3'(reverse). PCR products were purified using QIAquick gel extraction kit (Qiagen, Germantown, MD) and cloned into the pCR-Blunt II vector (Invitrogen). The amplified Omp31/SodC and AHCY genes were inserted between the *Cla* I and *Xho* I sites in the plasmid pCAGGS and were designated as pCA-Omp31/SodC and pCA-AHCY, respectively. Then BSR cells were transfected with pCA-Omp31/SodC and pCA-AHCY using lipofectamine$^{TM}$ 2000 (Invitrogen) according to the manufacture's protocol. The transfected cells were fixed and examined by an indirect fluorescence assay (IFA), which using sera from mice against LBNSE-SOD or LBNSE-AHC as first antibody and Dylight 594-conjugated second antibody against the mouse IgG.

### The growth curve of recombinant virus

BSR cells were plated in 60-mm-diameter dishes. After 16 h incubation, the cells were infected at an MOI of 0.01 (multicycle growth) with LBNSE, LBNSE-SOD and LBNSE-AHC. After incubation at 37°C for 1 h, inocula were removed, and cells were washed three times with PBS to remove any unabsorbed virus. Three milliliters of complete medium was added back, and 100 μl of tissue culture supernatants was removed at the indicated time points after infection. Titers of virus aliquots were determined in duplicate on BSR cells.

### Vaccination experiments in mice

Forty 5-week-old female mice, whose serology is negative to *Brucella melitensis* and rabies virus, were purchased from Changchun Institute of Biological Products Co., Ltd. and raised by isolated cages in the Animal Facility, Division of Zoonosis, Institute of Special Economic Animal and Plant Sciences. Mice were randomly divided into 4 groups, and 10 mice in each group were used to verify the vaccination efficacy. Three groups of mice were vaccinated by i.m.route (biceps femoris muscle of right hindlimb) at 6 week of age with $10^6$ PFU of LBNSE, LBNSE-SOD, LBNSE-AHC with 100 μl per dose, respectively. One group of mice inoculated i.m. with DMEM as a sham-treated control during the experiment. At days 0, 7, 14 after immunization, heparinized blood was harvested and sera were collected from anesthetized mice for real-time PCR and serology test. At the end of experiment, all the mice were anesthetized and euthanized in a CO chamber.

The animal experiments herein described have been reviewed and approved by the local Animal Care & Use Committee of the Institute of Special Wild Economic Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Jilin, China animal (welfare assurance number: SEAPS-IACUC-2015-004).

### Hematocytes counts in the blood

Whole blood from each group were collected with EDTA for hematocyte count at day 0, and at day 7, 14 after immunization, including WBC (white blood cell), RBC (red blood cell), platelet, lymphocyte, monocyte and neutrophils. Blood cells were counted using an automated cell counter VetScan HM5 (Gurnee, IL) according to the protocol.

### Quantitative real-time PCR (qRT-PCR)

In order to measure the expression of cytokines in the blood, a quantitative real-time PCR assay was conducted in a BIO-RAD T100™ Thermal Cycler (Hercules, CA) as described previously [17-19]. Briefly, total RNA was extracted from blood samples according to the Qiagen RNeasy kit (Qiagen, Redwood, CA) and treated with DNase (Qiagen). The reverse transcriptase and DNA polymerase were used from a TransScript$^{TM}$ Green One-Step qRT-PCR SuperMix kit. Each PCR reaction ran in duplicate with 100 ng DNase-treated RNA and 5 nM each primer pairs. Amplification was operated at 50℃ for 2 min and 95℃ or 10 min, followed by 40 cycles in two steps: 95℃ for 15 s and 60℃ for 1 min. The copy numbers of cytokines□IL-2□IL-10□IL-4□IFN-γ□were normalized to those of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Levels of cytokines expression in samples of mice vaccinated with LBNSE and LBNSE-SOD, LBNSE-AHC are presented as the fold change over that detected in control group.

### Total IgG ELISA assay

In order to confirm the level of cellular and humoral immunity, ELISA levels of IL-10 and IFN-γ in the sera were detected by Mouse Quantitative ELISA Kit (Cat:M1000B and Cat:MIF00, R&D Systems, Inc. Minneapolis, MN) according to the kit's protocol. The optical density was examined at 450 nm using a spectrophotometer (BioTek Instruments, VT). Calculation of IL-10 and IFN-γ concentration was developed by linear regression analysis using software KC4 Signature Ver. 3.4 (Bio-Tek Instruments). A standard curve was made using known concentrations of mouse IL-10 and IFN-γ provided in the ELISA Kit (R&D Systems). The mouse IL-10 and IFN-γ concentrations of samples from vaccinated and control groups were determined according to the standard curve using a 4-parameter logistic regression equation of the KC4 program. The results were presented as pg/L for each sample.

### Rapid fluorescent focus inhibition test (RFFIT)

RV neutralizing antibodies (VNAs) were detected using the rapid fluorescent focus inhibition test (RFFIT) as described previously [20], and titers of VNAs were normalized to international units (IU/ml). Briefly, 50μl of serial five-fold dilutions of serum were added in Lab-Tek Chamber slides (Nalge Nunc International, Rochester, NY). 50% Fluorescing Foci dose (Fifty FFD50) of challenge virus standard (CVS-11) was added to each chamber and incubated for 90 min at 37°C. NA cells ($10^5$cells) were added into each chamber and the slides were incubated at 37°C for 20 hours. Then the cells were fixed and stained with FITC-conjugated anti-RABV N antibodies. After washing with PBS, twenty fields in each chamber were observed under a fluorescent microscope. Then the 50% endpoint titers were determined following the Reed–Muench formula. Then the titers of VNAs were normalized to international units (IU/ml).

### Histopathology observation

At necropsy, the tissue samples from brains were harvested and fixed in 10% neutral-buffered formalin for histopathology observation following hematoxylin and eosin (H&E) staining as described previously [21].

### Statistical analysis

The statistical significance of the differences between various groups was analyzed by Graphpad Prism 5 v. 5.01 (Graphpad Software Inc., La Jolla, CA, USA). Student's t-test or one-way ANOVA was used to evaluate the significant difference. Statistical significance was set at $p< 0.05$ and extremely significance at $p< 0.01$.

# Abbreviations

RV: Rabies virus; i.m: intramuscular; VNA: virus neutralizing antibody; rLBNSE: recombinant virus LBNSE; rRABV: recombinant rabies viruses; AHCY: S-adenosyl-L-homocysteine hydrolase; SOD: superoxide dismutase; NA: Mouse neuroblastoma cells; FBS: fetal bovine serum; CVS-11: Challenge virus standard 11; WBC: white blood cell; RBC: red blood cell; H&E: hematoxylin and eosin; RE: restriction endonuclease.

# Declarations

Ethics approval and Consent to Participate

The animal experiments herein described have been reviewed and approved by the local Animal Care & Use Committee of the Institute of Special Wild Economic Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Jilin, China animal (welfare assurance number: SEAPS-IACUC-2015-004). And we have followed the 3 Rs guiding principles during the course of animal experiment.

Availability of data and materials

The data sets during and/or analyzed during the current study available from the corresponding author on reasonable request.

### Competing interests

The authors have agreed that there is no conflict of interest between them.

Consent for publication

Not applicable.

### Funding

The study was financially supported by the grant of National Natural Science Foundation of China (NSFC)'Molecular mechanism for synaptic recycling dysfunction induced by G protein of street rabies virus in primary mouse neural cells'(31572505) , the National Program for Key Research Projects of China (2016YFD0500900 and 2017YFD0500900) and "311" program of Taizhou city. The funder Yongjun Wen and Zhenguang Li designed the study and wrote the article.

### Authors' contributions

ZL and HZ conceived and designed the experiments. YY, FW, MW and NS performed the experiments. ZL and SC analyzed the data. ZL, HZ, ZF, and YW wrote the article. All authors read and approved the final manuscript.

### Acknowledgements

We thank all of the technicians who were involved in the animal care and sample collection. And we

are grateful of Dr.Wenzhi Xue for many helpful suggestions and his critical reading of the manuscript.

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# Figures

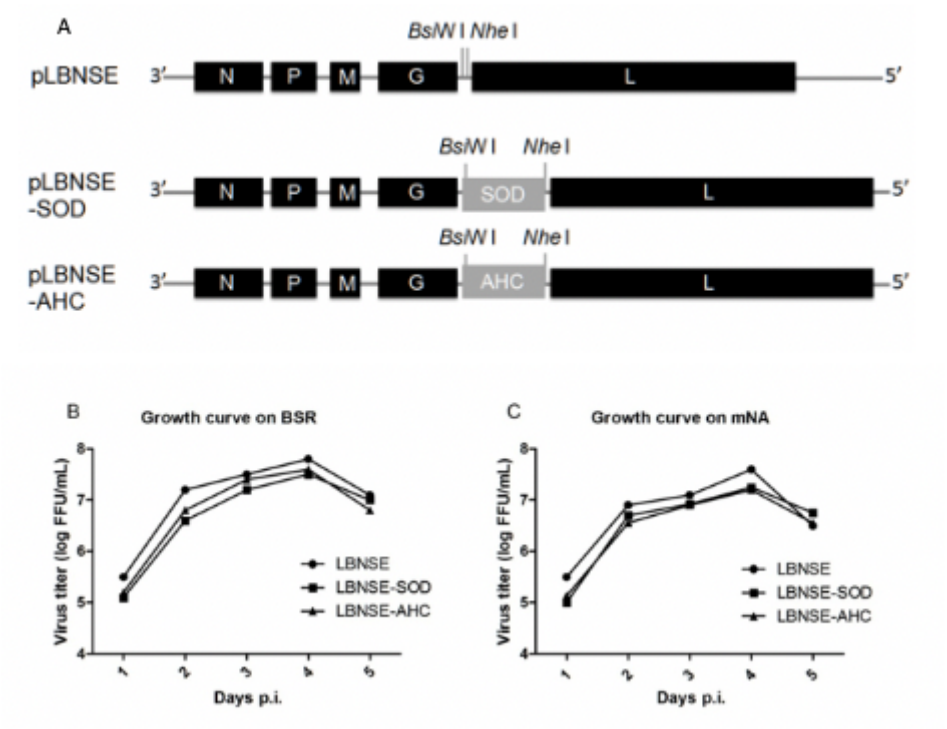
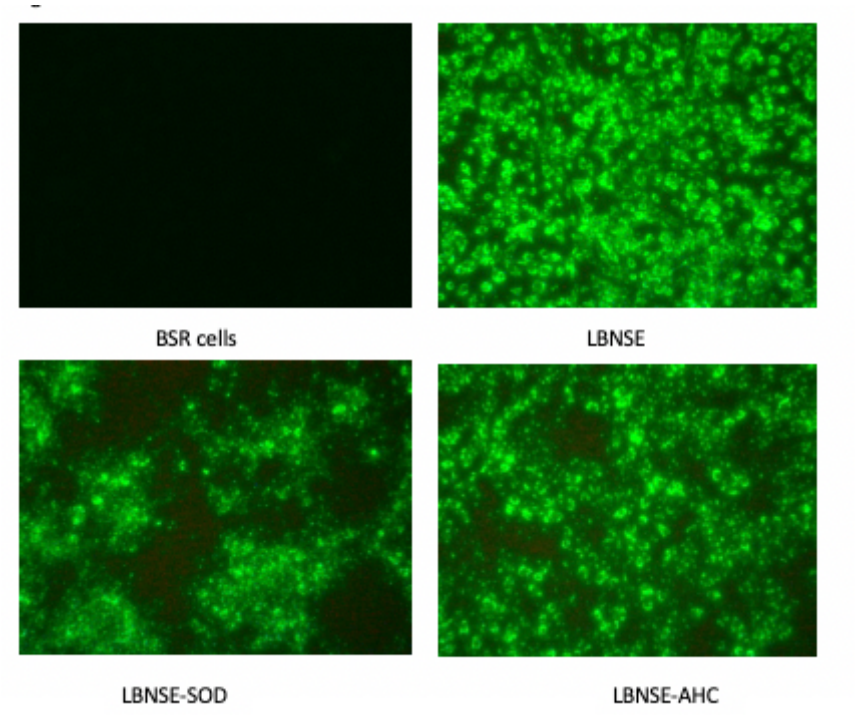


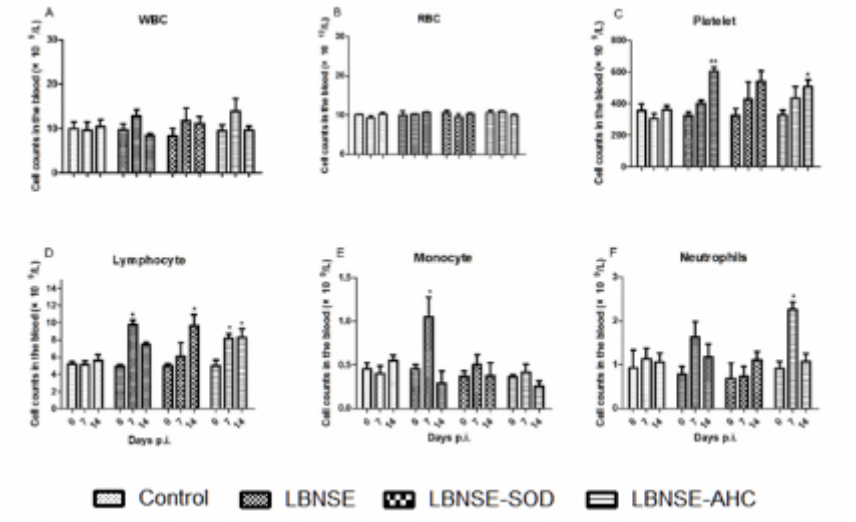

**Figure 1**

Construction strategy and the growth curve of recombinant virus. A. Schematic diagrams of two full-length cDNA clones used to rescue recombinant virus. The construction of the individual plasmid, based on pLBNSE, is displayed. BsiwI and NheI were introduced ahead of L gene in pLBNSE. The Brucella OMP or AHC gene was inserted by BsiwI and NheI. B, C. The growth curve of recombinant vaccine vectors grow in cells. BSR cells (B) or mNA cells (C) were infected with LBNSE, LBNSE-SOD and LBNSE-AHC at a MOI of 0.01 (multicycle growth) or 5 (one-step growth curve). Aliquots of tissue culture supernatants were collected and viral titers were determined in duplicate as indicated.




## Figure 2

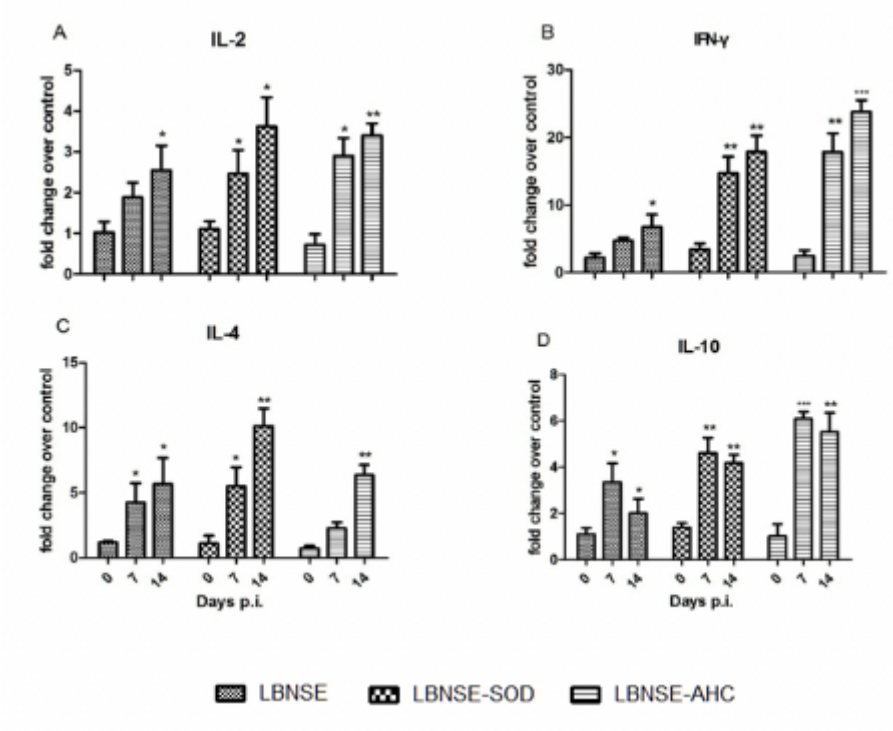
Rescue of recombinant rabies viruses. rLBNSE-SOD and rLBNSE-AHC were rescued as described previously. Briefly, the full-length infectious clone, and helper plasmids of N, P, L, G were co-transfected into BSR cells using Lipofectamine 2000 transfection reagent (Grand Island, NY) according to the manufacturer's protocol. After 4 days incubation at 34℃, the culture medium was discarded and fresh medium replenished for further incubation (3 more days). Cell culture medium was harvested and tested for rescued virus after reaction with FITC-conjugated anti-RABV N antibody.




## Figure 3

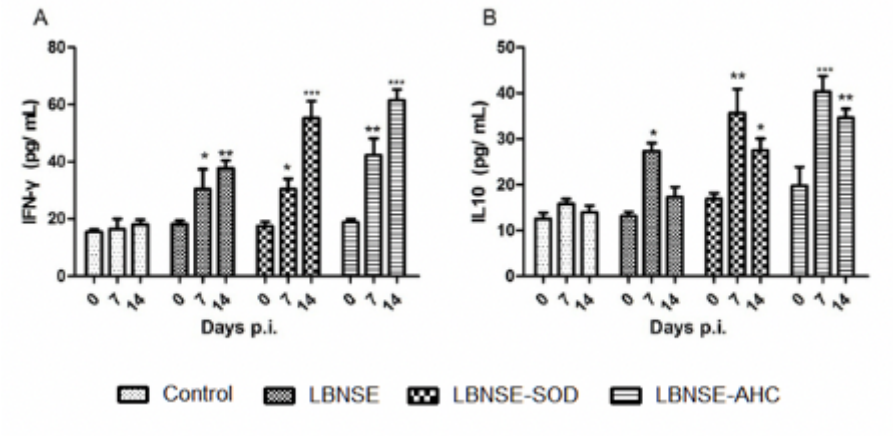
Cells count in the blood of mice. Kunming mice (groups of 9) at the age of 4–6 weeks were treated with Medium and immunized (i.m.) with LBNSE, LBNSE-SOD and LBNSE-AHC at dose of 106 FFU/mL

(100μl/mouse). Mice were euthanized at day 0, and at day 7, 14 after immunization. Whole blood from each group was collected for hematocyte count: WBC (white blood cell) (A), RBC (red blood cell), (B) Platelet (C), Lymphocyte(D), Monocyte (E), Neutrophils(F). Values represent the average from three animals. Data are expressed as mean values ± SEM. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$) between the corresponding time points of four groups.



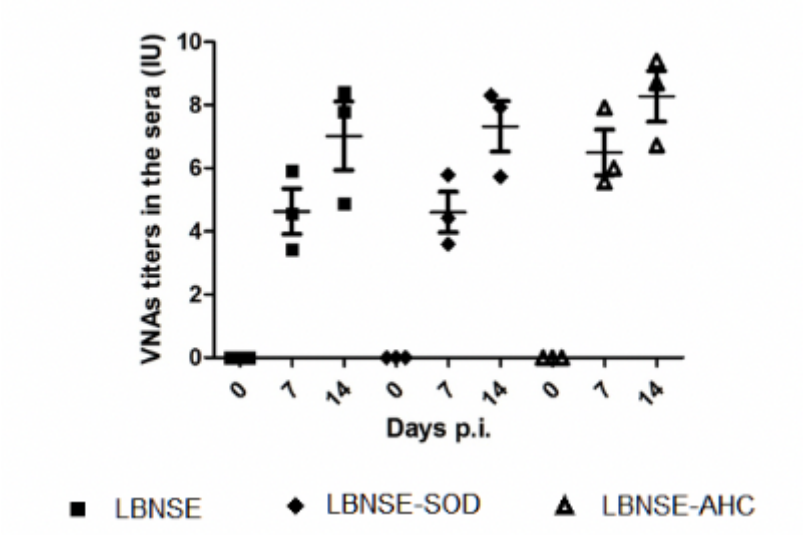

## Figure 4

The mRNA level of cytokines detected in the blood of mice. Kunming mice (groups of 9) at the age of 4–6 weeks were treated with Medium and immunized (i.m.) with LBNSE, LBNSE-SOD and LBNSE-AHC at dose of 106 FFU/mL (100μl/mouse). Mice were euthanized at day 0, and at day 7, 14 after immunization. Whole blood from each group was collected for the copy number of mRNA level of cytokines measure by real-time PCR: IL-2 (A), IFN-γ (B), IL-4(C), IL-10(D). Values represent the average from three animals. Data are expressed as mean values ± SEM. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between the corresponding time points of four groups.



## Figure 5

The ELISA level of cytokines measured in the sera of mice. Kunming mice (groups of 9) at the age of 4–6 weeks were treated with Medium and immunized (i.m.) with LBNSE, LBNSE-SOD and LBNSE-AHC at dose of 106 FFU/mL (100μl/mouse). Mice were euthanized at day 0, and at day 7, 14 after immunization. Sera from each group were collected for the ELISA level of cytokines detection by mouse Quantitative ELISA Kit according to the manufacturer's instructions: IFN-γ (A), IL-10 (B). Values represent the average from three animals. Data are expressed as mean values ± SEM. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between the corresponding time points of four groups.




## Figure 6

The VNA level against rabies virus in the sera of mice. Kunming mice (groups of 9) at the age of 4–6 weeks were treated with Medium and immunized (i.m.) with LBNSE, LBNSE-SOD and LBNSE-AHC at dose of 106 FFU/mL (100μl/mouse). Mice were euthanized at day 0, and at day 7, 14 after immunization. Sera from each group were collected for the VNA level against rabies virus detection by FAVN assay. Values represent the average from three animals. Data are expressed as mean values ± SEM. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between the corresponding time points of experimental groups.

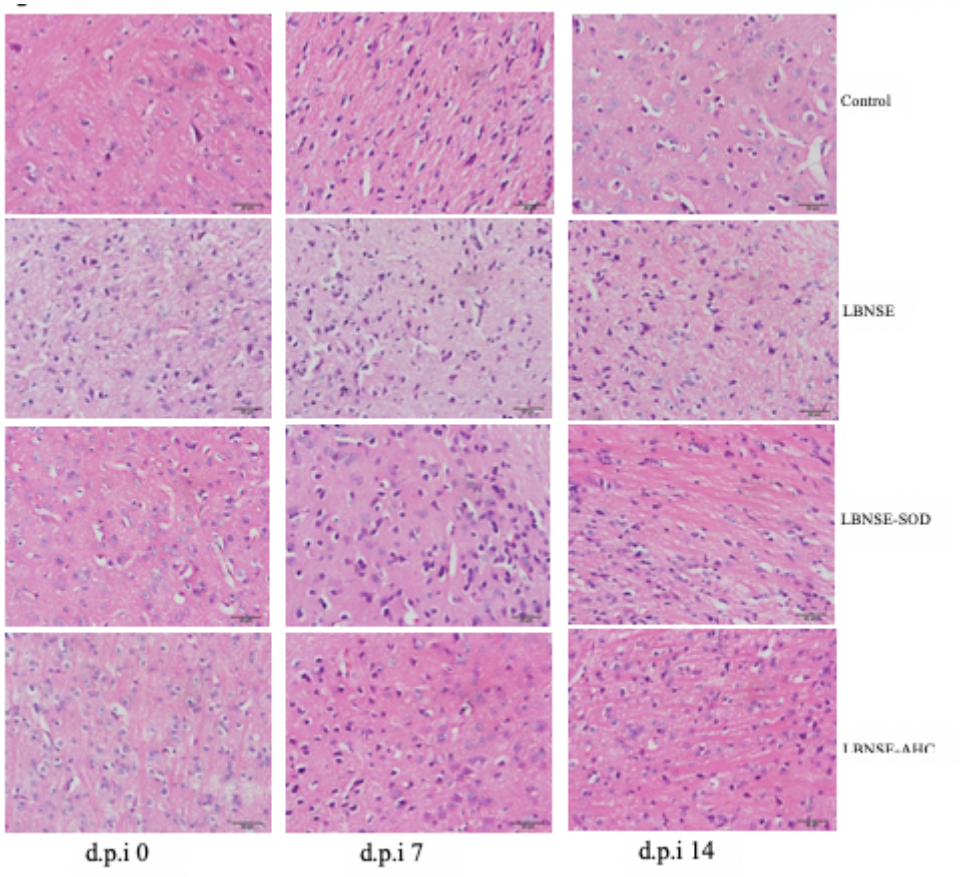



**Figure 7**

Histopathology observation of the brains in all groups. Kunming mice (groups of 9) at the age of 4–6 weeks were treated with Medium and immunized (i.m.) with LBNSE, LBNSE-SOD and LBNSE-AHC at dose of 106 FFU/mL (100μl/mouse). Mice were euthanized at day 0, and at day 7, 14 after immunization. Brain samples from each group were collected for histopathology observation by HE staining.

# Supplementary Files

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