Research report

Up-regulation of SETD3 may contribute to post-stroke depression in rat through negatively regulating VEGF expression

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Abstract

Post-stroke depression (PSD) is one of the most familiar complications of stroke, which refers to stroke patients who have varying degrees of depression (lasts for -2 weeks). SET domain-containing 3 (SETD3) is a conserved histone H3 methyltransferase, and the role of SETD3 in some diseases is increasingly being explored. However, the effects of SETD3 in PSD remain unclear. In this study, the PSD rat model was firstly constructed by Endothelin-1 injection combined with chronic unpredictable mild stress, and we discovered that SETD3 expression was up-regulated in PSD rat model. Additionally, SETD3 knockdown relieved the depressive symptom of PSD. Moreover, SETD3 knockdown promoted proliferation and differentiation of neural stem cells (NSCs). Due to the critical role of vascular endothelial growth factor (VEGF) in antidepressant and SETD3 can negatively regulate VEGF, we speculated that SETD3 may regulate PSD progression through VEGF. Our results demonstrated that SETD3 knockdown up-regulated VEGF expression. Furthermore, SETD3 modulated the proliferation and differentiation of NSCs through regulating VEGF expression. In conclusion, our study indicated that up-regulation of SETD3 contributed to PSD progression in rats through negatively regulating VEGF expression. The findings of this work suggest that SETD3 may be a promising target for treating PSD in the future.

1. Introduction

Post-stroke depression (PSD) is the most prevalent complication of psychological and behavioral disorders after stroke, with an incidence of 25–79% [1,2]. The main manifestations of PSD are depression, loss of interest after stroke, delaying the recovery of neurological function, increasing mortality, and greatly reducing the quality of life of PSD patients [3]. As the incidence of PSD is increasing year by year, cognitive impairment after PSD has attracted more and more attention from researchers [3–5]. Current research on the molecular pathogenesis of PSD mainly focuses on three main areas: inflammation, neurotrophic proteins and glutamate [6–8]. PSD could be caused by various factors, including physiology, psychology and social factors [9], but its pathogenesis still needs further investigation. Therefore, it is of great significance to find effective molecular therapeutic targets for PSD.

SET domain-containing 3 (SETD3) is a conserved protein (actin histidine methyltransferase. As a member of the protein lysine methyltransferase (PKMT) family, it facilitates to add methyl group to lysine residues [10]. SETD3 can induce monomethylation and dimethylation of the 4th and 36th lysines of histone H3 [11]. SETD3 has two domains: an N-terminal SET domain and a C-terminal domain homologous to a Rubisco large subunit methyltransferase interaction domain [12]. Previous study has found that proteins containing the SET domain of catalytically lysine methyltransferase can regulate multiple biological

Keywords:
SETD3
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Abbreviations: bFGF, basic fibroblast growth factor; CUMS, chronic unpredictable mild stress; DG, dentate gyrus; EGF, epidermal growth factor; ET-1, endothelin-1; FBS, fetal bovine serum; FST, forced swim test; IF, immunofluorescence; LSMT, large subunit methyltransferase; NSCs, neural stem cells; PKMT, protein lysine methyltransferase; PSD, post-stroke depression; SCT, sucrose consumption test; SD, Sprague-Dawley; SETD3, SET domain-containing 3; VEGF, vascular endothelial growth factor.

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and pathological processes [13]. Previous studies have focused on the key role of SETD3 in cancers. For example, SETD3 affects the breast cancer cells viability and invasion [14]. SETD3 mediated by some miRNAs can modulate proliferation and metastasis of hepatocellular carcinoma cells [15]. In addition, SETD3 down-regulates KLC4 expression to relieve the radiotherapy sensitization in cervical cancer [16]. Moreover, cell cycle-dependent degradation of SETD3 weakens cell proliferation and tumorigenesis in liver cancer [17]. SETD3 also exerts an important regulatory function in other diseases. SETD3 relieves primary dystocia [12]. Mr-15b/mir-322 suppresses SETD3 expression to regulate muscle cell differentiation [18]. Furthermore, SETD3 inhibits vascular endothelial growth factor (VEGF) expression in hypoxic pulmonary hypertension [19]. However, there is no research on the investigation of SETD3 in the progression of PSD.

In this study, we investigated the effects of SETD3 on PSD progression. These findings from our work demonstrated that SETD3 was highly expressed in PSD rat model, and up-regulation of SETD3 contributed to PSD progression in rats through negatively regulating VEGF expression. This discovery suggested that SETD3 may be a pivotal regulator of PSD progression and a feasible target for future treatment.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley (SD) rats weighing 200–250 g were supplied by the Vital River Co. Ltd. (Beijing, China). The animal experiments were approved by the Medical Ethics Committee of Affiliated Hospital of Youjiang Medical University for Nationalities. These rats were placed in clean cages with a 12-hour light/dark schedule at 22 ± 2 °C, and were allowed free access to water and food.

Rats were divided into 6 groups (n = 6) as follows: Con, PSD, PSD + LV-shNC, PSD + LV-shSETD3, PSD + LV-shVEGF and PSD + LV-shVEGF + LV-shSETD3. Endothelin-1 (ET-1) induced middle cerebral artery occlusion was used to establish the rat model of stroke. In details, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (2%, 40 mg/kg). The anesthetized rats were mounted in a stereotaxic apparatus (RWD Life Science Co., Ltd., Shenzhen, China). The head of rats was shaved and disinfected. The skin was cut along the dorsal midline to expose the skull. An electric drill with a diameter of 2 mm was used to drill the skull and dura mater at the injection area to reveal the skull. Drilling was done on the skull at the suitable location. The lentivirus plasmids were injected into brain ventricles 48 h before the establishment of the stroke model. After being sterilizing with 75% alcohol, a 2.5 cm-long sagittal incision along the midline was made to reveal the skull. Drilling was done on the skull at the suitable location. We pierced cerebral dura mater with a needle and inserted the cannula into the left lateral ventricle of rats (4.8 mm posterior to the bregma, ± 2.5 mm lateral to the medial suture, 3.5 mm ventral to the skull surface). The 10 μL of 10^8 TU/mL LV-shNC (sense: TTTCGCGACGTGTCAAGTTCGTT; anti-sense: TTAGAAGCTGTCACAGTCA), LV-shSETD3 (sense: 5′-CACCCGGAACGGAAAGTTTCACATGAAATGAGGTAATCT-3′; anti-sense: 5′-AAGACACGGAGGATCGCCATGTT-3′), LV-shVEGF (sense: 5′-CACCCGACGTATGTGCCTGACACAGCTATGGCAGCACCAGTGC-3′; anti-sense: 5′-AAGACACGGAGGATCGCCATGTT-3′) plasmids were injected with 0.2 μL/min. Injectors were left intact for 5 min in place after completing the injection to ensure complete diffusion from the syringe tip.

2.2. Lentivirus plasmid injection into brain ventricles

The lentivirus plasmids were injected into brain ventricles 48 h before the establishment of the stroke model. After being sterilizing with 75% alcohol, a 2.5 cm-long sagittal incision along the midline was made to reveal the skull. Drilling was done on the skull at the suitable location. We pierced cerebral dura mater with a needle and inserted the cannula into the left lateral ventricle of rats (4.8 mm posterior to the bregma, ± 2.5 mm lateral to the medial suture, 3.5 mm ventral to the skull surface). The 10 μL of 10^8 TU/mL LV-shNC (sense: TTTCGCGACGTGTCAAGTTCGTT; anti-sense: TTAGAAGCTGTCACAGTCA), LV-shSETD3 (sense: 5′-CACCCGGAACGGAAAGTTTCACATGAAATGAGGTAATCT-3′; anti-sense: 5′-AAGACACGGAGGATCGCCATGTT-3′), LV-shVEGF (sense: 5′-CACCCGACGTATGTGCCTGACACAGCTATGGCAGCACCAGTGC-3′; anti-sense: 5′-AAGACACGGAGGATCGCCATGTT-3′) plasmids were injected with 0.2 μL/min. Injectors were left intact for 5 min in place after completing the injection to ensure complete diffusion from the syringe tip.

2.3. Sucrose consumption test

After the simulated stroke, the sucrose consumption test (SCT) was performed at 0, 1, 2, 3 and 4 weeks. After 24 h of water deprivation, two bottles (one containing 1% sucrose solution and the other containing standard drinking water) were placed in the cage for 1 h. At the end, the amount of liquid was examined. The sucrose preference score was expressed as a percentage of total fluid intake.

2.4. Forced swim test

After the simulated stroke, the forced swim test (FST) was performed at 0, 1, 2, 3 and 4 weeks. In brief, rats were forced to swim in a plastic bucket (60 cm high and 45 cm in diameter) filled with water (23–25 °C), the height of which prevents them from reaching the bottom. Then, the swimming time of rats was recorded, during which the rats were swimming along the wall or actively attempting to climb it. 5 min later, we removed the rats from the bucket, dried them with a towel, and warmed them under a lamp. No water or food was supplied for rats for 24 h after the test.

2.5. Open field test

Under quiet conditions, rats were put into an open-field box. The size of the open-field box was 100 cm × 100 cm, and divided into 5 × 5 small squares. The locomotor activity was assessed through the frequency of crossing the square. Four claws that enter the square scored 1 point. The frequency of rearing was applied to assess rearing activity. Two forelimbs that get off the ground scored 1 point. Each rat was detected 3 times, 3 min each time, and the average value was taken.

2.6. Isolation and culture of neural stem cells

Neural stem cells (NSCs) from the dentate gyrus (DG) of rats were dissected and cultured. Rats were euthanized by cervical dislocation.

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The dentate gyrus was separated from the brain under a dissecting microscope. The dentate gyrus was cut into 1 mm³ pieces, and then washed with D-Hank’s solution for 2 times. These pieces were treated with 0.25% trypsin-EDTA and DNase at 37 °C for 10 min, and then filtered through a 70 μm cell strainer. The cells were cultured in DMEM/F12 at 37 °C and 5% CO2. The medium was supplemented with 10% fetal bovine serum (FBS), 20 mg/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), 1% B27 and 1% penicillin/streptomycin. The medium was changed every 2 days. On the 7th day of culture, the neurospheres were formed by differential centrifugation and subcultured for further use.

2.7. The proliferation and differentiation of NSCs in vitro

Rats were intraperitoneally injected with 50 mg/kg of BrdU (10 mg/mL). The injections were started from the stroke modeling, twice a day for the first 3 days, then twice a week, and finally, once every 8 h for the last 2 days before the rats were sacrificed. The NSCs proliferation was measured through BrdU incorporation observation. The NSCs differentiation was assayed by immunofluorescence (IF) assay by using NeuN, TuJ1 and Nestin antibodies. DAPI-positive cells were used to show the total number of cells. The Image-Pro Plus software was utilized to analyze images.

2.8. Immunofluorescence staining

NSCs were immobilized using 4% formaldehyde and next washed with PBS, followed by permeabilization and blocking with 0.1% Triton X-100 in PBS containing 1% bovine serum albumin (5%). Then, they were incubated with primary antibodies against BrdU (ab8152, Abcam, Shanghai, China), NeuN (ab177487, Abcam, Shanghai, China), TuJ1 (ab18207, Abcam, Shanghai, China) and Nestin (ab105389, Abcam, Shanghai, China) overnight at 4 °C. Following rinsing by PBS, secondary antibodies were supplemented and incubated in darkness. Finally, DAPI staining was conducted. Cell samples were put under one inverted fluorescence microscope (IX-71; Olympus) for imaging.

2.9. RT-qPCR

Hippocampus tissues from rats were treated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and homogenized. Total RNA was extracted from the tissue homogenate. The PrimeScript® RT reagent Kit (Takara, Dalian, China) was used in the reverse transcription for RNAs to cDNA. The expression of SETD3 and VEGF was measured through the One-Step SYBR RT-PCR Kit (TaKaRa, Tokyo, Japan). GAPDH acted as an internal reference, and genes' expression was analyzed by the 2−ΔΔCT method. The primers were presented as follows: SETD3.

F 5'-CAACCTGGAAGATGCAGCGTGT-3'
R 5'-CAGCCTGGAATCAGAGCAGCGT-3'
VEGF.

F 5'-GGGAAATGATTGACCTG-3'
R 5'-CAGCCGAGAATTGAGACGGA-3'
GAPDH.

F 5'-CTCTCCCTGTGACAGTACGACG-3'
R 5'-CCCAATACGACAAATTTGGTT-3'

2.10. Western blot

Hippocampus tissues were lysed to isolate proteins. Protein extracts were separated by SDS-PAGE, and next moved onto PVDF membranes. Then, proteins were probed with primary antibodies against SETD3 (ab176582; 1:2000; Abcam), VEGF (ab32152; 1:1000; Abcam) and β-actin (ab179467; 1:1000; Abcam). After washing, membranes were mixed with secondary antibody. Bands were evaluated through the enhanced chemiluminescence (ECL) Kit (Pierce, Thermo Fisher Scientific, IL, USA).

2.11. IHC

The paraffin-embedded hippocampus samples were de-paraffinized, rehydrated by ethanol, and then subjected to microwave antigen retrieval with citrate buffer. Antibodies against VEGF (1:50, Santa Cruz) were applied as primary antibodies. After blocking, samples were mixed with primary antibodies and secondary antibodies. The samples were stained with dianminobenzidine (DAB) and then counterstained with hematoxylin. At the end, images were observed under the ECLIPSE Ti2 microscope (Ti2-U, Nikon, Japan).

2.12. Statistical analysis

Data were analyzed using SPSS 19.0 (SPSS, Chicago, IL, USA), and expressed as mean ± standard deviation (SD). Differences between two groups were compared through the Student’s t-test. Differences among multiple groups were compared through one-way analysis of variance (ANOVA), and post hoc test was used for post-tests. Multiple comparisons between two groups at the same time point were analyzed by Tukey post-test. P < 0.05 was considered to be statistically significant.

3. Results

3.1. The SETD3 expression was up-regulated in PSD rat model

Firstly, we constructed PSD rat model by ET-1 injection combined with chronic unpredictable mild stress. Depression-like behaviors were evaluated through the sucrose consumption test, forced swim test and open field test. Compared with normal rats, the sucrose preference of PSD rats was severely decreased (Fig. 1A). Furthermore, the immobility time of the PSD rats was increased compared with normal rats (Fig. 1B). In addition, the locomotor and rearing activities of PSD rats were decreased compared with normal rats in the Con group (Fig. 1C, D). Moreover, we found that the expression of SETD3 mRNA and protein in the hippocampus were higher in the PSD group than that in the Con group (Fig. 1E, F). From these data, we concluded that SETD3 expression was up-regulated in PSD rat model.

3.2. SETD3 knockdown relieved the symptom of PSD

Then, we silenced SETD3 in PSD rats, and investigated the function of SETD3 knockdown in PSD. The knockdown efficiency of SETD3 was confirmed in Fig. 2A. In PSD rats, the sucrose preference was enhanced after silencing SETD3 (Fig. 2B). The immobility time was significantly reduced in PSD rats by silencingSETD3 (Fig. 2C). Furthermore, the higher locomotor and rearing activities were found in PSD rats after SETD3 knockdown (Fig. 2D, E). Repression of SETD3 significantly weakened SETD3 protein expression in the hippocampus of PSD rats (Fig. 2F). Taken together, SETD3 knockdown relieved the depressive symptom of PSD rats.

3.3. SETD3 knockdown promoted NSCs proliferation and differentiation

Next, the effects of SETD3 on neural stem cell proliferation and differentiation were explored. BrdU assay was carried out to observe the proliferation of NSCs. The results indicated that PSD rats exhibited a significant decrease in BrdU-positive cells compared with normal rats. SETD3 knockdown enhanced the BrdU-positive cells in PSD rats (Fig. 3A, B). Additionally, the NeuN, TuJ1 and Nestin-positive cells were also markedly decreased in the PSD rats, which was effectively enhanced by SETD3 suppression (Fig. 3B–H). These findings illustrated that SETD3 knockdown promoted NSCs proliferation and differentiation.
3.4. SETD3 knockdown up-regulated VEGF expression

Since many studies have also revealed the important role of VEGF in antidepressant, and SETD3 could negatively regulate VEGF, we hypothesized that SETD3 may affect PSD progression through VEGF. The mRNA and protein expression of VEGF was lower in PSD rats than that in normal rats. The up-regulated VEGF mRNA and protein expression was discovered in PSD rats after SETD3 knockdown (Fig. 4 A, B). Moreover, IHC assay also demonstrated that the VEGF levels in the hippocampus of PSD rats were increased by silencing of SETD3 (Fig. 4 C). These results suggested that SETD3 knockdown up-regulated VEGF expression.

3.5. SETD3 modulated NSCs proliferation and differentiation through VEGF

More experiments were conducted to verify whether SETD3 modulated the proliferation and differentiation of NSCs through regulating VEGF expression. The VEGF protein expression was up-regulated after SETD3 knockdown, while VEGF protein expression was down-regulated after overexpressing SETD3 (Fig. 5 A). In addition, SETD3 expression in the hippocampus of PSD rats was markedly reduced by SETD3 inhibition, whereas knockdown of VEGF had no effect on SETD3 expression. SETD3 silenced enhanced VEGF expression, while VEGF silencing repressed VEGF expression in the hippocampus of PSD rats. In particular, based on VEGF knockdown, SETD3 suppression had no significant effect on VEGF expression in PSD rats (Fig. 5B). The BrdU-positive cells in PSD rats was strengthened by SETD3 knockdown and weakened by VEGF knockdown. SETD3 knockdown-mediated inhibition of BrdU-positive cells was reversed by VEGF deficiency (Fig. 5C, D). These data indicated that SETD3 may also affect NSCs proliferation through other ways, not just through negative regulating VEGF. Furthermore, the NeuN, Tuj1 and Nestin-positive cells in PSD rats were enhanced by SETD3 knockdown. VEGF knockdown reduced NeuN, Tuj1 and Nestin-positive cells in PSD rats. Based on VEGF knockdown, co-suppression of SETD3 had no significant effect on the change of NeuN, Tuj1 and Nestin-positive cells (Fig. 5D, F). In summary, SETD3 modulated NSCs proliferation and differentiation through VEGF.

4. Discussion

PSD is one of the most frequent complications of stroke, and is relevant with the increased stroke morbidity and mortality as well as poor outcomes [21,22]. Abundant advances have been made in understanding PSD, but its pathophysiological mechanisms remain unclear. In animal model, the middle cerebral artery is the most commonly affected artery in stroke, and its occlusion can lead to depressive phenotype [23–25]. In this work, injection of ET-1 was utilized to establish the rat model of stroke, and then CUMS was applied to construct the rat model of PSD. Compared with the normal rats, PSD rats showed significantly decreased sucrose consumption, increased immobility time as well as the reduced locomotor and rearing activities. Therefore, these data showed that the PSD rat model used in our study was successfully established.

Previous studies have demonstrated that SETD3 is involved in the regulation of many diseases [12,14–19], but it has not been reported to be related to PSD progression. In present work, we discovered that SETD3 expression was up-regulated in PSD rat model. Additionally, SETD3 knockdown relieved the depressive symptom of PSD through enhancing the sucrose preference, higher locomotor and rearing activities as well as reducing the immobility time of PSD rats.

Depression is a common disorder worldwide, and has been linked to the increased risk of suicide, social withdrawal, and the impaired social
More and more studies have supported that there is a strong relationship between the neurogenesis of adult hippocampal and the formation of new neurons in the dentate gyrus and depression [27,28]. Depression patients usually display a decrease in hippocampal neurogenesis, which leads to hippocampal atrophy [29,30]. Neurogenesis contains the NSCs proliferation, differentiation (mainly to astrocytes and neurons), and the functional integration of newly formed cells [31,32]. Previous study proved that the proliferation of NSCs and the differentiation of the newly formed cells into neurons are critical factors in reversing depressive behavior [33]. Depression leads to the decreased neurogenesis, and effective antidepressant targets have been revealed to improve depression-like behavior and strengthen neurogenesis [34]. However, the molecular regulatory mechanisms and useful targets of neurogenesis still need more exploration. In our work, SETD3 knockdown promoted proliferation and differentiation of neural stem cells.

Many studies have illustrated that VEGF is an important mediator of the neurogenesis and behavioral effects of various antidepressants [35,36]. Recent literatures have shown that the neurogenic and neuroprotective effects of VEGF can affect hippocampal-dependent processes, such as learning and memory [37–39]. VEGF is signaled through two high-affinity receptor tyrosine kinases (Flk-1 and Flt-1) [39,40]. The Flk-1 receptor is expressed in endothelial cells and neural progenitor cells and has been shown to mediate the mitogenic effects of VEGF in vitro [41]. Increasing studies have also revealed the important role of VEGF in antidepressants [42,43]. Interestingly, the researchers have discovered that SETD3 interacts with FoxM1, and the interaction between SETD3 and FoxM1 occurs at the VEGF promoter and SETD3 can transcriptionally regulate VEGF expression [19,44]. SETD3 inhibition blocks the viability and invasive phenotype of breast cancer cells, and SETD3 inhibition regulates the expression of different potential effector genes, such as FoxM1, β-actin, FOXM1, FBXW7, Fascin, eNOS, and MMP-2 [45]. SETD3 promotes DNA methylation levels DCLK1 promoter, and inhibit the transcription of DCLK1. SETD3 participates in the HCC metastasis via DCLK1/Pi3K/MMP-2 pathway [46].
Fig. 3. SETD3 knockdown promoted NSCs proliferation and differentiation. PSD rat model was constructed by Endothelin-1 injection combined with chronic unpredictable mild stress, or combined with injection of LV-shSETD3 or LV-shNC. The groups were divided into the Con, PSD, PSD + LV-shNC and PSD + LV-shSETD3 group. (A, B) The cell proliferation of NSCs was detected through BrdU IF assay. The NeuN (C, D), Tuj1 (E, F) and Nestin (G, H) positive cells were evaluated through IF assay. NeuN, a marker for neuron; Tuj1, a marker for immature neurons; Nestin, a marker for neural stem cells.

Fig. 4. SETD3 knockdown up-regulated VEGF expression. PSD rat model was constructed by Endothelin-1 injection combined with chronic unpredictable mild stress, or combined with injection of LV-shSETD3 or LV-shNC. The groups were divided into the Con, PSD, PSD + LV-shNC and PSD + LV-shSETD3 group. (A) The VEGF mRNA expression was assessed through RT-qPCR. (B) The VEGF protein expression was detected through western blot. (C) The VEGF levels were measured through IHC assay. $P < 0.01, \quad \dddot{P} < 0.001.$
relationship between SETD3 and VEGF in PSD and whether SETD3 can regulate VEGF methylation still needs further research. Thus, we speculated that SETD3 regulated PSD progression through regulating VEGF expression. Similar to these previous studies, results from our study demonstrated that SETD3 knockdown up-regulated VEGF expression. Moreover, further experiments verified that SETD3 modulated NSCs proliferation and differentiation through regulating VEGF expression.

5. Conclusion

In summary, our study first depicted that up-regulation of SETD3 contributed to PSD progression in rats through negatively regulating VEGF expression. However, data regarding the regulatory effects of SETD3 on PSD progression remains limited. In the future, more experiments will be done to further explore the role of SETD3 in PSD development.

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Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of Affiliated Hospital of Youjiang Medical University for Nationalities.

CRediT authorship contribution statement

Yun Feng and Xuebin Li designed the study, supervised the data collection, Jie Wang analyzed the data, interpreted the data, Lanqing Meng, Xionglin Tang, Xiaohua Wang, Jianmin Huang, Chongdong Jian prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Competing interests

The authors state that there are no conflicts of interest to disclose.
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Available data and materials

All data generated or analyzed in this study are included in this published article.

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Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2021.113564.

Appendix B. Supplementary material

Not applicable.

Appendix C. Supplementary material

Not applicable.