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Role of Neurexin-1β and Neuroligin-1 in Cognitive Dysfunction After Subarachnoid Hemorrhage in Rats

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Background and Purpose—Neurexin-1β and neuroligin-1 play an important role in the formation, maintenance, and regulation of synaptic structures. This study is to estimate the potential role of neurexin-1β and neuroligin-1 in subarachnoid hemorrhage (SAH)-induced cognitive dysfunction.

Methods—In vivo, 228 Sprague–Dawley rats were used. An experimental SAH model was induced by single blood injection to prechiasmatic cistern. Primary cultured hippocampal neurons were exposed to oxyhemoglobin to mimic SAH in vitro. Specific small interfering RNAs and expression plasmids for neurexin-1β and neuroligin-1 were exploited both in vivo and in vitro. Western blot, immunofluorescence, immunoprecipitation, neurological scoring, and Morris water maze were performed to evaluate the mechanism of neurexin-1β and neuroligin-1, as well as neurological outcome.

Results—Both in vivo and in vitro experiments showed SAH-induced decrease in the expressions of neurexin-1β and neuroligin-1 and the interaction between neurexin-1β and neuroligin-1 in neurons. In addition, the interaction between neurexin-1β and neuroligin-1 was reduced by their knockdown and increased by their overexpression. The formation of excitatory synapses was inhibited by oxyhemoglobin treatment, which was significantly ameliorated by overexpression of neurexin-1β and neuroligin-1 and aggravated by the knockdown of neurexin-1β and neuroligin-1. More importantly, neurexin-1β and neuroligin-1 overexpression ameliorated SAH-induced cognitive dysfunction, whereas neurexin-1β and neuroligin-1 knockdown induced an opposite effect.

Conclusions—Enhancing the expressions of neurexin-1β and neuroligin-1 could promote the interaction between them and the formation of excitatory synapses, which is helpful to improve cognitive dysfunction after SAH. Neurexin-1β and neuroligin-1 might be good targets for improving cognitive function after SAH. **(*Stroke*. 2015;46:2607-2615. DOI: 10.1161/STROKEAHA.115.009729.)**

Key Words: neurexin-Ibeta ■ neuroligin 1 ■ stroke ■ subarachnoid hemorrhage ■ synapses

As an emergency scenario, aneurysmal subarachnoid hemorrhage (SAH) causes severe cases of rupture of cerebral blood vessels in the clinic and produces a high mortality and disability rate.[1–3] Despite the recent progress in microsurgical and endovascular surgical techniques, the outcome of patients who suffer a SAH remains unsatisfactory.[3,4] Cognitive impairment is the main obstacle for SAH patients to return to normal life.[5–8] It is well known that synapses are the basic structural and functional units of neurotransmission, which is the mechanism by which cognitive functions are formed.

As a transmembrane protein, neurexin-1β shows widespread expression in brain and is present in the presynaptic membrane of neurons.[9] Previous studies demonstrated that the combination of neurexin-1β and postsynaptic membrane protein neuroligin-1 plays a central role in the formation of synapses in the central nervous system.[10] Neurexin-1β and neuroligin-1 induce synaptic differentiation and regulate the transfer of neurotransmitters between neurons.[10] In addition, it has been reported that neuroligin-1 mutation lacking bind site for neurexin-1β failed to induce synapse formation.[11] In conclusion, the expression of neurexin-1β and neuroligin-1 and the interaction between them are closely related to cognitive function. However, until now, no study has investigated the contribution of neurexin-1β and neuroligin-1, especially

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the interaction between them, to SAH-induced cognitive dysfunction.

The aim of this study was to investigate the role of neurexin-1β and neuroligin-1 and the effect of interaction between neurexin-1β and neuroligin-1 on cognitive function after SAH and to explore the underlying mechanisms of SAH-induced cognitive impairment.

Materials and Methods

Animals

Two hundred twenty-eight adult male Sprague–Dawley rats weighing between 350 and 400 g were purchased from the Animal Center of Chinese Academy of Sciences, Shanghai, China. The animal experimental protocols, including all use, care, and operative procedures, were approved by the Animal Care and Use Committee of Soochow University and complied with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. All animal experiments were performed in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Every effort was made to minimize the number of animals used and their suffering.

Rat SAH Model

Experimental SAH model was induced by single blood injection to prechiasmatic cistern as reported previously.[12]

In this model, the inferior basal temporal lobe of SAH group was stained with blood (shown as shadow areas in Figure IA in the online-only Data Supplement).

Experimental Design

The in vivo experiments were divided into 2 parts. In experiment 1, 54 rats (68 rats were used, but only 54 rats survived after the surgery) were randomly assigned to 9 groups of 6 rats each, a sham group, and 8 experimental groups arranged by time: 3, 6, 12, 24, 48 and 72 hours, 1, and 2 weeks after SAH. At the indicated time point after SAH, rats were euthanized, and the cortex and hippocampus tissues were separated and taken for analysis (Figure IB in the online-only Data Supplement).

In experiment 2, 144 rats (160 rats were used, but only 144 rats were survived) were randomly divided into 9 groups: SAH group, SAH+scramble small interfering RNA (siRNA) group, SAH+neurexin-1β siRNA group, SAH+neuroligin-1 siRNA group, SAH+neurexin-1β siRNA+neuroligin-1 siRNA group, SAH+empty vector group, SAH+neurexin-1β plasmid group, SAH+neuroligin-1 plasmid group, and SAH+neurexin-1β plasmid+neuroligin-1 plasmid group (n=16 for each group). After the indicated treatments, rats were euthanized, the cortex and hippocampus tissues were separated and taken for analysis, and Morris water maze task was performed to evaluate the cognitive changes of the experimental rats (Figure IC in the online-only Data Supplement).

In vitro, primary hippocampal neurons were exposed to 20 μmol/L oxyhemoglobin to mimic the effect of SAH shown in Figure ID in the online-only Data Supplement.

Transfection of siRNA in Rat Brain

Specific siRNAs against neurexin-1β and neuroligin-1 were obtained from GenScript. To improve the knockdown efficiency, siRNA used in this study is a pool of 3 different siRNA duplexes.
Neurexin-1β siRNA sequences:

1. Sense: 5′ GCUGGAGUUUCAUAACAUA dTdT 3′
   Antisense: 3′ dTdT CGACCUCAAAGUAUUGUAU 5′
2. Sense: 5′ CCAGAAACUUAGACCUCAA dTdT 3′
   Antisense: 3′ dTdT GGUCUUUGAAUCUGGAGUU 5′
3. Sense: 5′ GGACUCAUGUUCAAAUCAA dTdT 3′
   Antisense: 3′ dTdT CCUGAGUACAAGUUUAGUU 5′

Neuroligin-1 siRNA sequences:

1. Sense: 5′ CCGGAAUGCCACUCAGUUU dTdT 3′
   Antisense: 3′ dTdT GGCCUUACGGUGAGUCAAA 5′
2. Sense: 5′ GCAGAAGAAGCCUUACAAA dTdT 3′
   Antisense: 3′ dTdT CGUCUUCUUCGGAAUGUUU 5′
3. Sense: 5′ CCGGAUGAUGUUCCUUUAA dTdT 3′
   Antisense: 3′ dTdT GGCCUACUACAAGGAAAUU 5′

For details of siRNA transfection in rat brain, please see the online-only Data Supplement.

Plasmid Transfection in Rat Brain

Specific expression plasmids for neurexin-1β and neuroligin-1 were obtained from GenScript. For details, please see the online-only Data Supplement.

Intracerebroventricular Injection

In in vivo transfection, both siRNAs and plasmids were given via intracerebroventricular injection. After anesthetization, rats were placed in a stereotactic frame, and intracerebroventricular injection was performed as described previously.[13] Briefly, a burr hole was drilled into the skull 1.0 mm lateral to and 1.5 mm posterior to the bregma over the left hemisphere. The needle of 100-μL Hamilton syringe was slowly inserted through the burr hole into the left lateral ventricle 4.0 mm below the dural surface. A reagent was infused into the left lateral ventricle at a rate of 0.5 μL/min.

Cell Cultures

A culture of primary hippocampal neurons was prepared as described previously.[14] For details, please see the online-only Data Supplement.

Transfection of siRNA and Plasmid in Cultured Neurons

siRNAs and plasmids were obtained as described above. Both siRNA transfection and plasmid transfection in cultured neurons were performed using Lipofectamine 3000 Transfection Reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. For details, please see the online-only Data Supplement.

Western Blot

Western blot assay was performed as described previously.[15] For details, please see the online-only Data Supplement.

Immunoprecipitation Analysis

Immunoprecipitation analysis was performed as described previously.[16] For details, please see the online-only Data Supplement.

Immunofluorescence Analysis

Immunofluorescence analysis was performed as described previously.[15] For details, please see the online-only Data Supplement.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Staining

To detect cell apoptosis in brain, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed according to the manufacturer's protocol (DeadEnd Fluorometric kit,

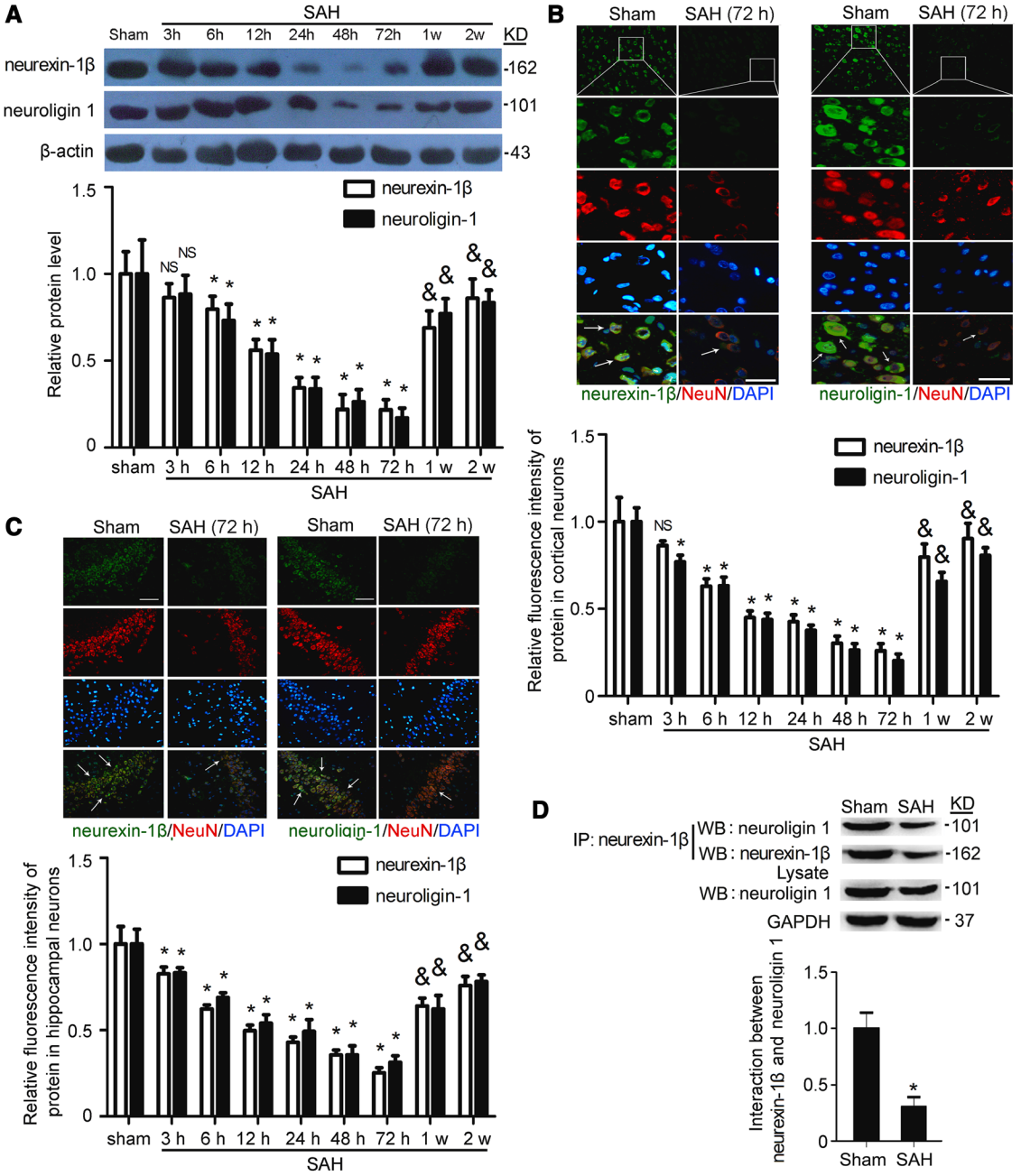

Figure 1. Subarachnoid hemorrhage (SAH) downregulated neurexin-1β and neuroligin-1 expression and decreased the interaction between them. **A**, Time course of neurexin-1β and neuroligin-1 expression in the rat brain tissues after SAH. **Upper**, Representative Western blot (WB) bands of neurexin-1β and neuroligin-1 are shown. **Bottom**, Quantitative analysis of the relative protein level is shown, and the mean value of sham group was normalized to 1.0. Double-immunofluorescence analysis was performed with antibodies for neurexin-1β or neuroligin-1 (green) and neuron marker (NeuN, red), and nuclei were fluorescently labeled with DAPI (blue). Arrows point to neurexin-1β–positive or neuroligin-1–positive neurons. Representative images of the expressions of neurexin-1β and neuroligin-1 in rat cortical neurons (**B**) and hippocampal neurons (**C**) at 72 hours after SAH. Scan bar was 64 μm in **B** and was 100 μm in **C**. **Bottom**, Quantification of the relative fluorescence intensity is shown. The mean fluorescence intensity of the sham group was normalized to 1.0. **D**, Immunoprecipitation (IP) analysis of the interaction between neurexin-1β and neuroligin-1 at 72 hours after SAH. WB analysis of cell lysates incubated with antibody of neurexin-1β, which then underwent IP. All values are means±SEM, **P*<0.05 compared with sham group and &*P*<0.05 compared with SAH group evaluated at 72 hours. NS indicates no significant difference, n=6.

Promega, WI). Three sections per rat were examined and were photographed in parallel for TUNEL-positive cell counting.

Neurological Scoring

Neurological scoring was performed using a scoring system and monitored for appetite, activity, and neurological defects (Table I in the online-only Data Supplement), as described previously.[17]

Morris Water Maze

Morris water maze were performed as described previously.[18,19] For details, please see the online-only Data Supplement.

Statistical Analysis

All data were presented as mean±SEM. Graph pad prism 5 was used for all statistical analysis. All data were analyzed using 1-way

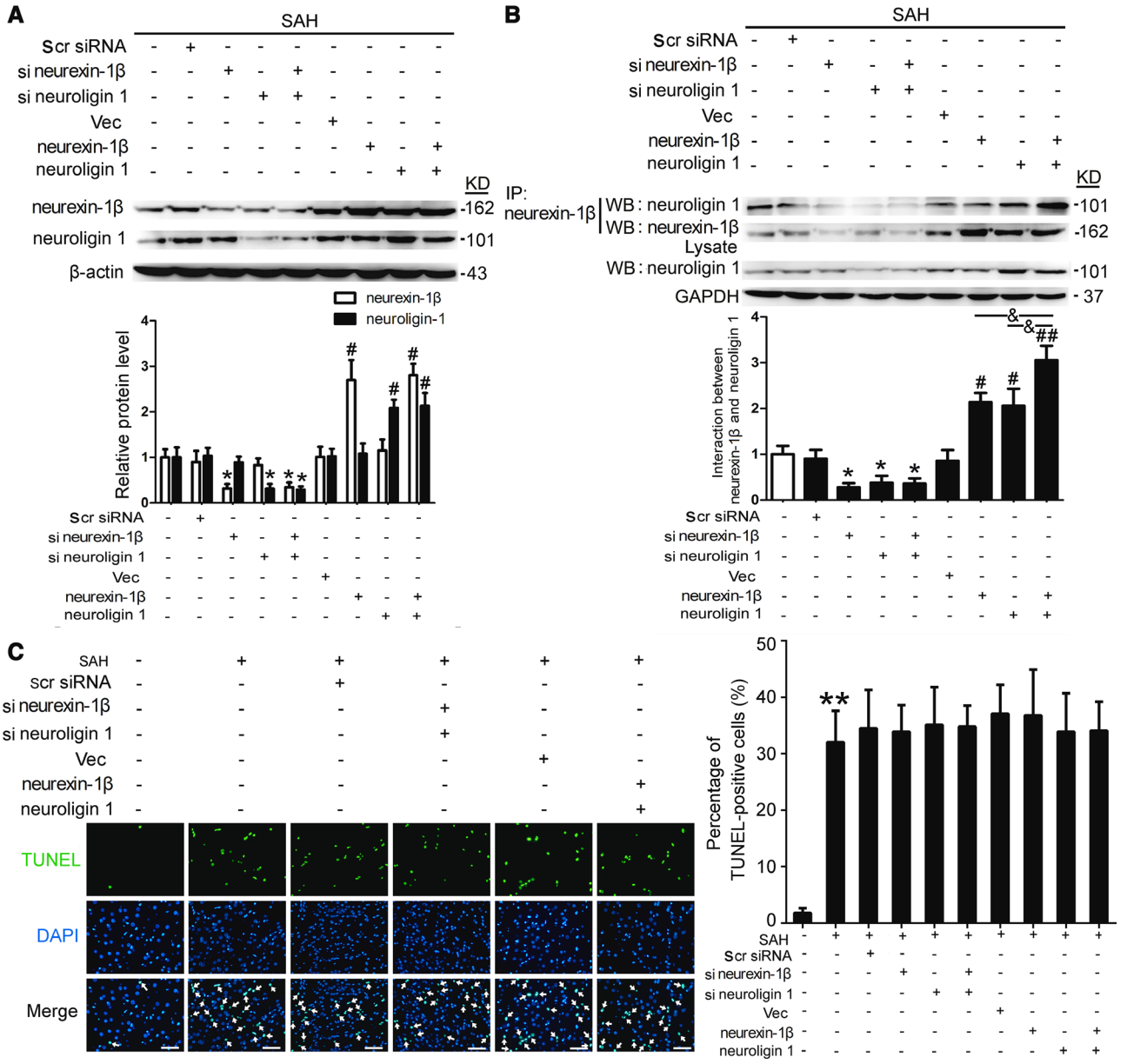

Figure 2. Effects of neurexin-1β and neuroligin-1 knockdown and overexpression on the interaction between them and cell death in the brain of SAH rats. Subarachnoid hemorrhage (SAH) rats accepted intracerebroventricular injection of small interfering RNAs (siRNAs) or plasmids as indicated. **A**, Western blot (WB) analysis of neurexin-1β and neuroligin-1 knockdown and overexpression efficiency in the brain of SAH rats. Quantification of relative protein levels of neurexin-1β and neuroligin-1 is shown below. Data are presented as mean±SEM. **P*<0.05 compared with SAH+scramble siRNA group and #*P*<0.05 compared with SAH+empty vector group, &*P*<0.05, n=3. **B**, Immunoprecipitation analysis of the effect of neurexin-1β and neuroligin-1 knockdown and overexpression on the interaction between them in the brain of SAH rats. Quantification of relative protein levels of neurexin-1β and neuroligin-1 is shown below. Data are presented as mean±SEM. **P*<0.05 compared with SAH+scramble siRNA group and #*P*<0.05, ##*P*<0.01 compared with SAH+empty vector group, &*P*<0.05, n=3. **C**, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Arrows point to TUNEL-positive cells. Scale bar=64 μm. **Right**, Percentage of TUNEL-positive cells was shown. Data are mean±SEM. ***P*<0.01, n=6.

ANOVA. The significance of differences among experimental groups was determined by Fisher's least significant difference (LSD) post-test, and *P*<0.05 was considered to be a significant difference.

Results

SAH-Induced Decrease in the Expressions of Neurexin-1β and Neuroligin-1 and the Interaction Between Them in Rat Brain

The results of Western blot demonstrated that when compared with the sham group, the expressions of neurexin-1β and neuroligin-1 in the brain were reduced significantly from 3 hours after SAH, reached the lowest point at 72 hours, and then rebounded gradually, and the expressions were similar to that in the sham group at 1 and 2 weeks (Figure 1A). Immunofluorescence assay further verified the SAH-induced decrease in the expressions of neurexin-1β and neuroligin-1 in cortical and hippocampal neurons (Figure 1B and 1C), which is described in detail in Figures II and III in the online-only Data Supplement. In addition, SAH also decreased the interaction between neurexin-1β and neuroligin-1 at 72 hours after SAH (Figure 1D).

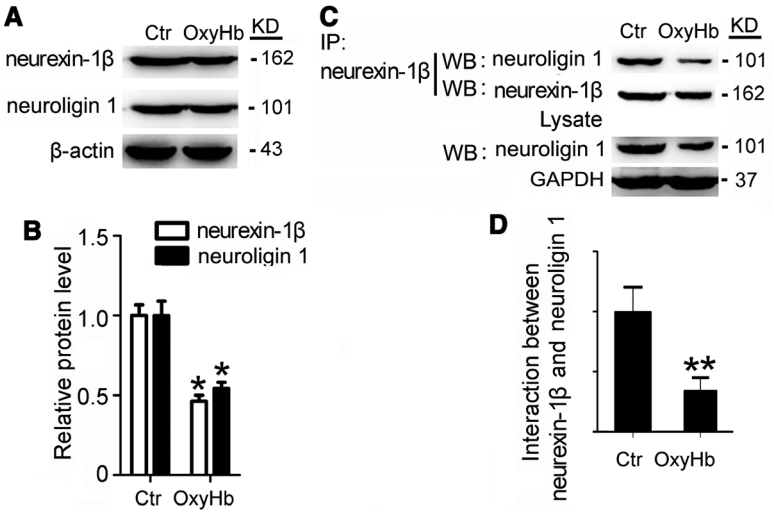

Figure 3. Effects of oxyhemoglobin (OxyHb) on the expressions of neurexin-1β and neuroligin-1, and the interaction between them in cultured hippocampal neurons under OxyHb treatment. **A**, Western blot (WB) analysis of the protein levels of neurexin-1β and neuroligin-1 in cultured primary hippocampal neurons exposed to OxyHb. **B**, Quantification of relative protein levels of neurexin-1β and neuroligin-1. Data are presented as mean±SEM. **P*<0.05 compared with control (Ctr) group, n=3. **C**, Immunoprecipitation analysis of the effect of OxyHb on the interaction between neurexin-1β and neuroligin-1 in cultured primary hippocampal neurons exposed to OxyHb. **D**, Quantification of relative protein levels of neurexin-1β and neuroligin-1. Values are mean±SEM. ***P*<0.01 compared with control (Ctr) group, n=6.

Effects of Knockdown and Overexpression of Neurexin-1β and Neuroligin-1 on the Interaction Between Them and on Brain Cell Death in SAH Rats

Western blot assay showed that the protein levels of neurexin-1β and neuroligin-1 were significantly decreased by siRNA transfection and increased by plasmid transfection (Figure 2A). The interaction between neurexin-1β and neuroligin-1 was also significantly decreased by the knockdown of neurexin-1β and neuroligin-1 and increased by the overexpression of neurexin-1β and neuroligin-1 (Figure 2B). In addition, TUNEL staining showed that neither the knockdown nor the overexpression of neurexin-1β and neuroligin-1 could affect cell death induced by SAH (Figure 2C; Figure IV in the online-only Data Supplement).

Oxyhemoglobin-Induced Decrease in the Expression of Neurexin-1β and Neuroligin-1 and the Interaction Between Them

Consistent with the in vivo data, Western blot and immunoprecipitation assay showed that both the protein levels of neurexin-1β and neuroligin-1 and the interaction between them were significantly decreased by oxyhemoglobin treatment (Figure 3).

Effects of Knockdown and Overexpression of Neurexin-1β and Neuroligin-1 on the Interaction Between Them and on Cell Death in Cultured Neurons

The efficiency of siRNA-mediated knockdown, as well as expression plasmid-mediated overexpression of neurexin-1β and neuroligin-1, in cultured hippocampal neurons was also verified by Western blot (Figure 4A). Consistently, the interaction between neurexin-1β and neuroligin-1 was also significantly decreased by the knockdown of neurexin-1β and neuroligin-1 and increased by the overexpression of neurexin-1β and neuroligin-1 (Figure 4B). In addition, TUNEL staining showed that neither the knockdown nor the overexpression of neurexin-1β and neuroligin-1 could affect oxyhemoglobin-induced neuron apoptosis. (Figure 4C; Figure V in the online-only Data Supplement).

Critical Role of Neurexin-1β and Neuroligin-1 in the Formation of Excitatory Synapses in Cultured Hippocampal Neurons Under Oxyhemoglobin Treatment

We next observed the effects of neurexin-1β and neuroligin-1 knockdown or overexpression on the number of excitatory synapses in cultured hippocampal neurons under oxyhemoglobin treatment (Figure 5). The results showed oxyhemoglobin-induced decrease in the number of excitatory synapses, as defined by synapsin (a presynaptic marker)-positive postsynaptic density protein 95 clusters, which was aggravated by the knockdown of neurexin-1β and neuroligin-1 and ameliorated by the overexpression of neurexin-1β and neuroligin-1. The results also showed that there were no significant differences between neurexin-1β siRNA+neuroligin-1 siRNA group and neurexin-1β siRNA group or neuroligin-1 siRNA group, whereas there were significant differences between neurexin-1β plasmid+neuroligin-1 plasmid group and neurexin-1β plasmid group or neuroligin-1 plasmid group.

Effects of Neurexin-1β and Neuroligin-1 on SAH-Induced Behavioral and Cognitive Dysfunction in Rats

SAH-induced neurological behavior impairment and whether knockdown or overexpression of neurexin-1β and neuroligin-1 affects impairment were tested (Figure 6A). Compared with the sham group, neurological score in the SAH group was significantly higher, suggesting a remarkable neurological defect induced by SAH. Scramble siRNA and empty vector treatment did not affect neurological outcome. Neurexin-1β and neuroligin-1 when silenced either individually or simultaneously could significantly aggravate SAH-induced neurological defect. Given the overexpression of neurexin-1β or neuroligin-1, the neurological behavior impairment was significantly reversed, whereas the overexpression of both neurexin-1β and neuroligin-1 simultaneously had a greater effect than the overexpression of neurexin-1β or neuroligin-1 individually.

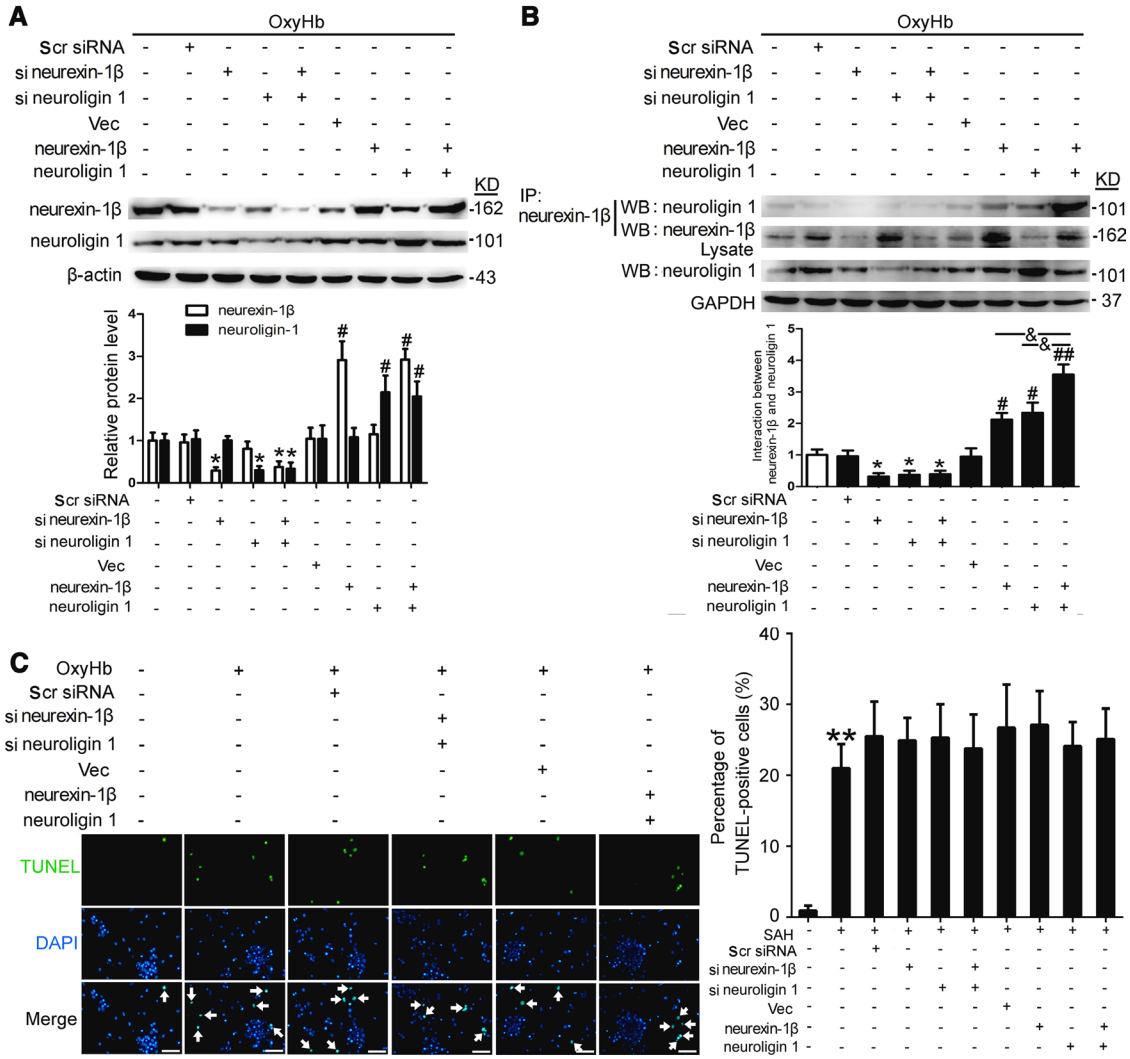

Figure 4. Effects of neurexin-1β and neuroligin-1 knockdown and overexpression on the interaction between them and cell death in cultured hippocampal neurons under oxyhemoglobin (OxyHb) treatment. Cultured primary hippocampal neurons were transfected with small interfering RNAs (siRNAs) or plasmids as indicated. **A**, Western blot (WB) analysis of neurexin-1β and neuroligin-1 knockdown and overexpression efficiency in cultured primary hippocampal neurons exposed to OxyHb. Quantification of relative protein levels of neurexin-1β and neuroligin-1 is shown below. Data are presented as mean±SEM. **P*<0.05 compared with OxyHb+scramble siRNA group and #*P*<0.05, ##*P*<0.01 compared with OxyHb+empty vector group, &*P*<0.05, n=3. **B**, Immunoprecipitation analysis of the effect of neurexin-1β and neuroligin-1 knockdown and overexpression on the interaction between them in cultured primary hippocampal neurons exposed to OxyHb. Quantification of relative protein levels of neurexin-1β and neuroligin-1 is shown below. Data are presented as mean±SEM. **P*<0.05 compared with OxyHb+scramble siRNA group and #*P*<0.05, ##*P*<0.01 compared with OxyHb+empty vector group, &*P*<0.05, n=3. **C**, TUNEL staining. Arrows point to TUNEL-positive cells. Scale bar=64 μm. **Right**, Percentage of TUNEL-positive cells are shown. Data are mean±SEM. ***P*<0.01, n=3.

The water maze performance demonstrated no difference in swimming speed between the experimental groups, which suggested that motor abilities did not grossly differ between the groups (data not shown). Rats from the SAH group showed longer latency and swim path length when compared with sham group, whereas no significant differences were observed between the SAH group and SAH+scramble siRNA group or SAH+empty vector group. In addition, SAH-induced increase in latency and swim path length was significantly ameliorated by the overexpression of neurexin-1β and neuroligin-1 and aggravated by the silencing of neurexin-1β and neuroligin-1. And, the overexpression of both genes simultaneously had more significant effect than the overexpression of the 2 genes individually (Figure 6B–6D; Figures VI–VIII in the online-only Data Supplement).

Discussion

In this study, we found that there was no rescue of cells by the overexpression of neurexin-1β and neuroligin-1 in SAH-induced brain cell death. However, the overexpression of neurexin-1β and neuroligin-1 could increase the interaction between them and the formation of excitatory synapses and improve SAH-induced cognitive dysfunction, whereas knockdown of neurexin-1β and neuroligin-1 led to the opposite effect. In addition, on the formation of excitatory synapses of neurons and the cognitive changes of the experimental rats, silencing neurexin-1β and neuroligin-1 individually or

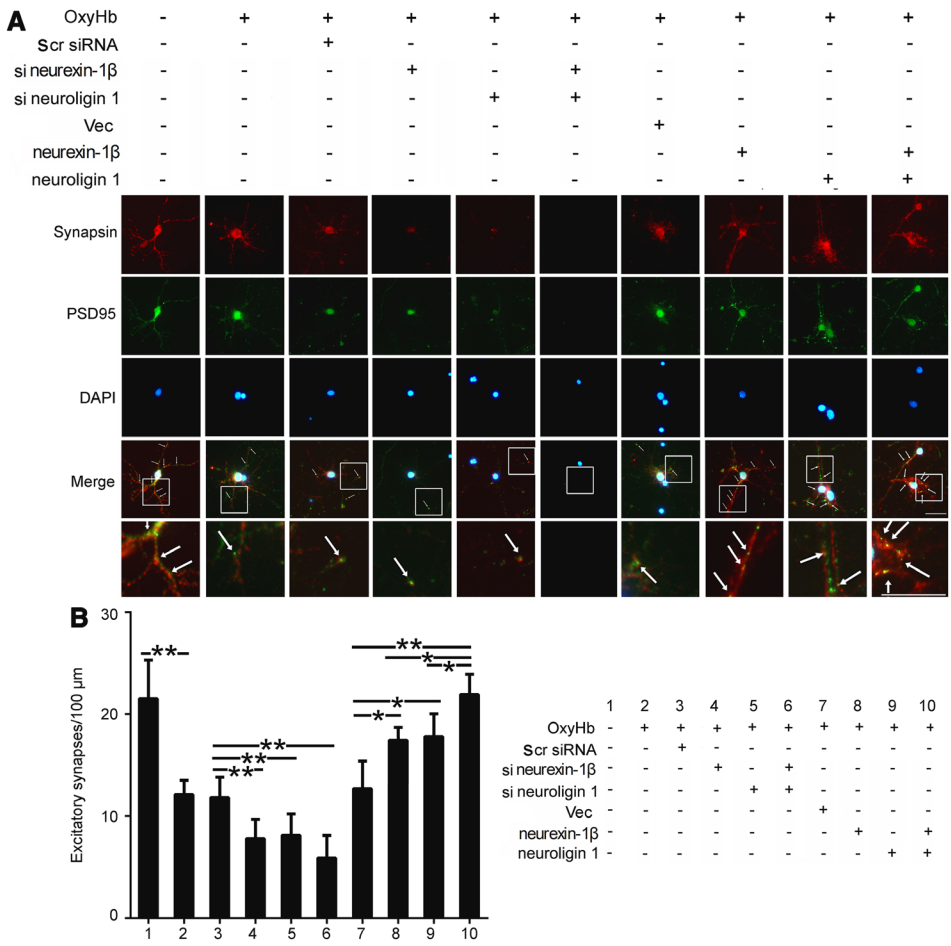

Figure 5. Effects of neurexin-1β and neuroligin-1 knockdown and overexpression on the formation of excitatory synapses in cultured hippocampal neurons under oxyhemoglobin (OxyHb) treatment. **A**, Cultured primary hippocampal neurons were transfected with small interfering RNAs (siRNAs) or plasmids as indicated and stained for postsynaptic density protein 95 (PSD-95;an excitatory postsynaptic marker) and synapsin (a presynaptic marker). Scale bar=32 μm. **B**, Quantification of the number of excitatory synapses per 100 μm of neurite, as defined by synapsin-positive PSD-95 clusters and indicated by arrows. Data are presented as mean±SEM. **P*<0.05, ***P*<0.01; n=3.

simultaneously exerts similar effects, whereas the overexpression of neurexin-1β and neuroligin-1 simultaneously exerts a more powerful effect than the overexpression of neurexin-1β and neuroligin-1 individually. These results suggested that neurexin-1β and neuroligin-1 worked cooperatively and played a critical role in the formation of excitatory synapses under SAH condition. Neurexin-1β and neuroligin-1 might be potential targets for improving SAH-induced cognitive dysfunction.

To our surprise, crystal structures of neuroligin-1 in isolation and in complex with neurexin-1β have been identified, which will help us to relate neurexin-1β/neuroligin-1 complex structure to function at the synapse and will provide molecular insights for understanding how to regulate the interaction between them.[20,21] In addition, there are commercial full-length protein and protein fragment of neurexin-1β and neuroligin-1 (such as Abcam products: ab160463, ab132182, and ab161450). Based on these researches, new medicines targeting neurexin-1β/neuroligin-1 complex or protein therapy will become available to patients.

Neuronal damage plays a major role in the pathogenesis of early brain injury after SAH. We hypothesized that, synapses, which are the key structures for transmitting information in neurons, were also damaged after SAH. Therefore, we focused our attention on changes in neurexin-1β and neuroligin-1 in the brain tissues of rats in a model of experimental SAH. The expression of both proteins in the brain cortex and hippocampus was reduced obviously as expected, as determined by Western blot. This suggests that the synaptic structure in hippocampal neurons might also be affected after SAH. The hippocampus plays a crucial role in cognitive function; thus, we speculate that the downregulation of neurexin-1β and neuroligin-1 affects cognitive function. The results of Morris water maze experiments are consistent with this hypothesis. The current in vitro experiments also suggested that excitatory synapses were reduced significantly in oxyhemoglobin-treated hippocampal neurons. However, the overexpression of neurexin-1β and neuroligin-1 increased the number of excitatory synapses compared with the oxyhemoglobin-treated group. This suggested that the overexpression of neurexin-1β and neuroligin-1 might reverse cognitive dysfunction after SAH, although this should be clarified in future studies.

The current study has some limitations. In vitro experiments showed that oxyhemoglobin maybe an incentive for the decrease in the expressions of neurexin-1β and neuroligin-1. However,

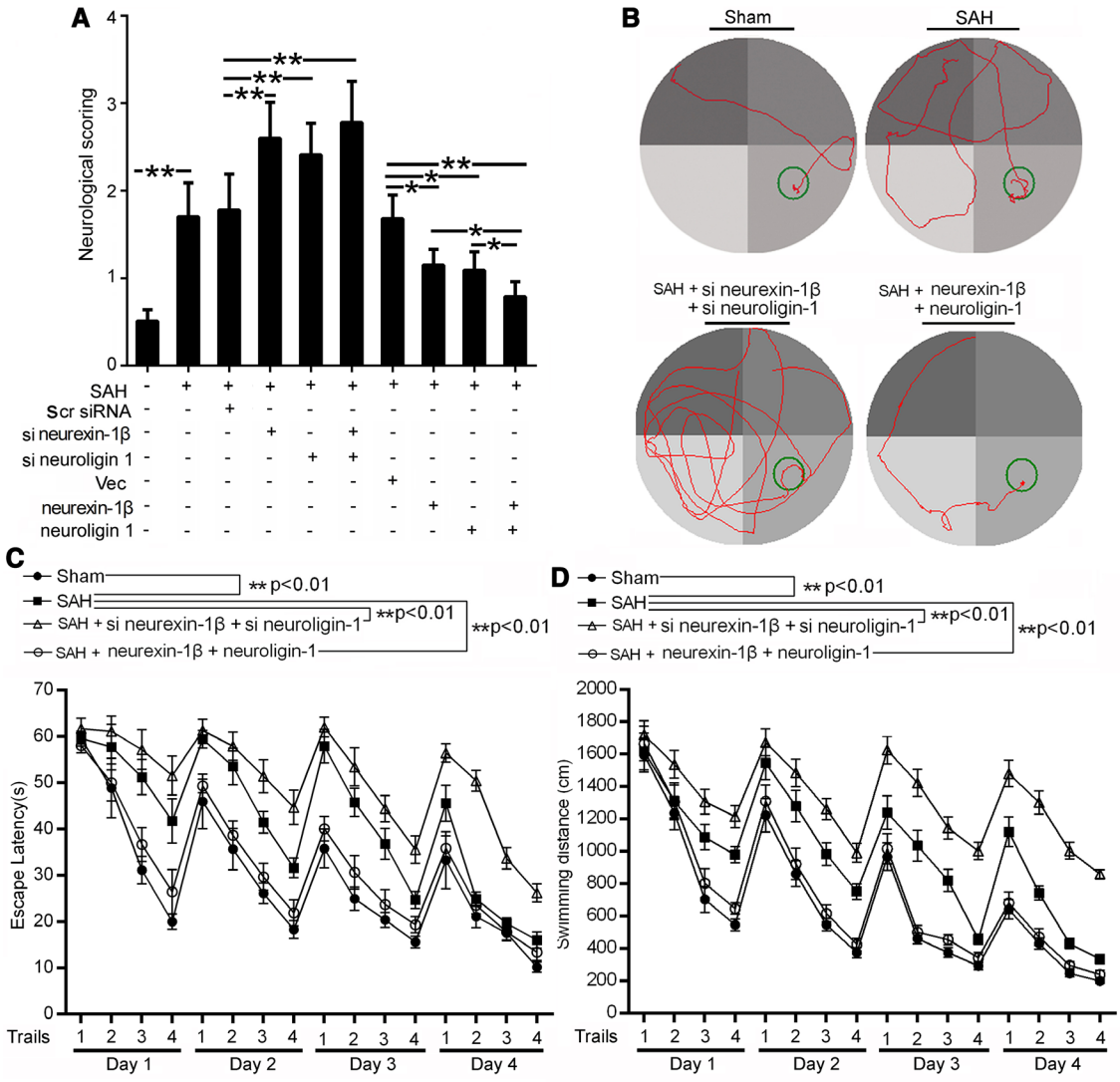

Figure 6. Effects of neurexin-1β and neuroligin-1 knockdown and overexpression on behavioral and cognitive function in subarachnoid hemorrhage (SAH) rats. **A**, Neurological behavior scores in each group. Data are presented as mean±SEM. **P*<0.05, ***P*<0.01; n=16. **B**, Representative tracing images from the Morris water maze test. **C**, Escape latency and (**D**) swim path length of 4 trials per day for 4 days, n=10.

because of the lack of studies of the other contents of hematoma, this study cannot infer the mechanism underlying SAH-induced decrease in the expressions of neurexin-1β and neuroligin-1. In addition, whether SAH-induced decrease in the expressions leads to SAH-induced decrease in the interaction between neurexin-1β and neuroligin-1 is also not answered in this study.

Summary

This study demonstrated for the first time that decrease in the expressions of neurexin-1β and neuroligin-1 and in the interaction between them occurred after SAH in brain tissues, and extraneous overexpression of neurexin-1β and neuroligin-1 improved cognitive function. Neurexin-1β/neuroligin-1 might be a promising treatment target for cognitive dysfunction after SAH.

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Disclosures

None.

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