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

Haitao Shen, MM*; Zhouqing Chen, MM*; Yang Wang, MM*; Anju Gao, MM; Haiying Li, PhD; Yonghua Cui, MD; Li Zhang, MD; Xiang Xu, MM; Zhong Wang, MD; Gang Chen, MD, PhD

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-lbeta  ■  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.4,5 Cognitive impairment is the main obstacle for SAH patients to return to normal life.4,5 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...
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. 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 IA.

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 and neuroligin-1 were obtained from GenScript. Three different siRNA duplexes from GenScript were purchased for transfection with the plasmid group. To improve the knockdown efficiency, siRNA used and neuroligin-1 were obtained from GenScript. Three different siRNA duplexes from GenScript were purchased for transfection with the plasmid group.

Neuroligin-1 siRNA sequences:

1. Sense: 5’ CCAGGAGUUCUAACUCAA dTdT 3’
   Antisense: 3’ dTdT GCCGAGCUUCAAGUGUAU 5’
2. Sense: 5’ CCAGGAGUUCUAACUCAA dTdT 3’
   Antisense: 3’ dTdT GGCCGAGCUUCAAGUGUAU 5’
3. Sense: 5’ CCAGGAGUUCUAACUCAA dTdT 3’
   Antisense: 3’ dTdT GGCCGAGCUUCAAGUGUAU 5’

Neuroligin-1 siRNA sequences: 1. Sense: 5’ CCAGGAGUUCUAACUCAA dTdT 3’
   Antisense: 3’ dTdT GCCGAGCUUCAAGUGUAU 5’
2. Sense: 5’ CCAGGAGUUCUAACUCAA dTdT 3’
   Antisense: 3’ dTdT GGCCGAGCUUCAAGUGUAU 5’
3. Sense: 5’ CCAGGAGUUCUAACUCAA dTdT 3’
   Antisense: 3’ dTdT GGCCGAGCUUCAAGUGUAU 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. 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. 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. For details, please see the online-only Data Supplement.

Immunoprecipitation Analysis

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

Immunofluorescence Analysis

Immunofluorescence analysis was performed as described previously. 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,
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

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.

Morris Water Maze
Morris water maze were performed as described previously.14,19 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
ANOV A. 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 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.
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.
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
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,
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.

**Sources of Funding**

This work was supported by grants from the National Natural Science Foundation of China (No. 81371279, 81422013, 81471196, and 81400949), Jiangsu Province’s Outstanding Medical Academic Leader program (No. LJ201139), Scientific Department of Jiangsu Province (No. LCZK201301, SZZS201413 and SYS201332), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

**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.

**Disclosures**

None.

**References**

1. Athar MK, Levine JM. Treatment options for cerebral vasospasm in aneurysmal subarachnoid hemorrhage. *Neurotherapeutics*. 2012;9:37–43. doi: 10.1007/s13311-011-0098-1.
2. Emiman N, Buchholz BA, Dreier R, Bruckner P, Torner JC, Steiger HJ, et al. Cerebral aneurysms: formation, progression, and developmental chronology. *Transl Stroke Res*. 2014;5:167–173. doi: 10.1007/s12975-013-0294-x.
3. Tso MK, Macdonald RL. Subarachnoid hemorrhage: a review of experimental studies on the microcirculation and the neurovascular unit. *Transl Stroke Res*. 2014;5:174–189. doi: 10.1007/s12975-014-0323-4.
4. Fuji M, Yan J, Rolland WB, Soejima Y, Caner B, Zhang JH. Early brain injury, an evolving frontier in subarachnoid hemorrhage research. *Transl Stroke Res*. 2013;4:432–446. doi: 10.1007/s12975-013-0257-2.

5. Al-Khindi T, Macdonald RL, Schweitzer TA. Cognitive and functional outcome after aneurysmal subarachnoid hemorrhage. *Stroke*. 2010;41:e519–e536. doi: 10.1161/STROKEAHA.110.581975.

6. Kreiter KT, Copeland D, Bernardini GL, Bates JE, Peery S, Claassen J, et al. Predictors of cognitive dysfunction after subarachnoid hemorrhage. *Stroke*. 2002;33:200–208.

7. Haug T, Sorteberg A, Sorteberg W, Lindegaard KF, Lundar T, Finset A. Cognitive outcome after aneurysmal subarachnoid hemorrhage: time course of recovery and relationship to clinical, radiological, and management parameters. *Neurosurgery*. 2007;60:649–56; discussion 656. doi: 10.1227/01.NEU.0000255414.70807.A0.

8. Mavaddat N, Sahakian BJ, Hutchinson PJ, Kirkpatrick PJ. Cognition following subarachnoid hemorrhage from anterior communicating artery aneurysm: relation to timing of surgery. *Neuron*. 1999;22:489–496.

9. Sugita S, Khvostochev M, Südhof TC. Neurexins are functional α-latrotoxin receptors. *Neuron*. 1999;9:1–12.

10. Ma Vaddat N, Sahakian BJ, Hutchinson PJ, Kirkpatrick PJ. Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. *Proc Natl Acad Sci U S A*. 2005;102:6137–6142. doi: 10.1073/pnas.0502038102.

11. Chubykin AA, Liu X, Comoletti D, Tsigelny I, Taylor P, Südhof TC. Dissection of synapse induction by neuroligins: effect of a neuroligin mutation associated with autism. *J Biol Chem*. 2005;280:22365–22374. doi: 10.1074/jbc.M410723200.

12. Wang Z, Ma C, Meng CJ, Zhu GQ, Sun XB, Huo L, et al. Melatonin activates the Nr2f2-ARE pathway when it protects against early brain injury in a subarachnoid hemorrhage model. *J Pineal Res*. 2012;53:129–137. doi: 10.1111/j.1600-079X.2012.00978.x.

13. Chen Y, Zhang Y, Tang J, Liu F, Hu Q, Luo C, et al. Norrin protected blood-brain barrier via frizzled-4/β-catenin pathway after subarachnoid hemorrhage in rats. *Stroke*. 2015;46:529–536. doi: 10.1161/STROKEAHA.114.007265.

14. Kato A, Fukazawa Y, Ozawa F, Inokuchi K, Sugiyama H. Activation of ERK cascade promotes accumulation of Vesl-1S/Homer-1a immunoreactivity at synapses. *Brain Res Mol Brain Res*. 2003;118:33–44.

15. Li H, Gao A, Feng D, Wang Y, Zhang L, Cui Y, et al. Evaluation of the protective potential of brain microvascular endothelial cell autophagy on blood-brain barrier integrity during experimental cerebral ischemia-reperfusion injury. *Transl Stroke Res*. 2014;5:618–626. doi: 10.1007/s12975-014-0354-x.

16. Wang L, Li H, Zhang J, Lu W, Zhao J, Su L, et al. Phosphatidylethanolamine binding protein 1 in vacular endothelial cell autophagy and atherosclerosis. *J Physiol*. 2015;591(Pt 20):5005–5015. doi: 10.1113/jphysiol.2015.262667.

17. Yamaguchi M, Zhou C, Nanda A, Zhang JH. Ras protein contributes to cerebral vasospasm in a canine double-hemorrhage model. *Stroke*. 2004;35:1750–1755. doi: 10.1161/01.STR.0000129898.68350.9F.

18. Wurm F, Keiner S, Kunze A, Witte OW, Redecker C. Effects of skilled forelimb training on hippocampal neurogenesis and spatial learning after focal cortical infarcts in the adult rat brain. *Stroke*. 2007;38:2833–2840. doi: 10.1161/STROKEAHA.107.485524.

19. Jeon H, Ai J, Sabri M, Tariq A, Macdonald RL. Learning deficits after experimental subarachnoid hemorrhage in rats. *Neuroscience*. 2010;169:1805–1814. doi: 10.1016/j.neuroscience.2010.06.039.

20. Araç D, Boucard AA, Ozkan E, Strop P, Newell E, Südhof TC, et al. Structures of neuroligin-1 and the neuroligin-1/neurexin-1 beta complex reveal specific protein-protein and protein-Ca2+ interactions. *Neuron*. 2007;56:992–1003. doi: 10.1016/j.neuron.2007.12.002.

21. Levinson JN, El-Husseini A. A crystal-clear interaction: relating neuroligin/neurexin complex structure to function at the synapse. *Neuron*. 2007;56:937–939. doi: 10.1016/j.neuron.2007.12.003.
Role of Neurexin-1β and Neuroligin-1 in Cognitive Dysfunction After Subarachnoid Hemorrhage in Rats
Haitao Shen, Zhouqing Chen, Yang Wang, Anju Gao, Haiying Li, Yonghua Cui, Li Zhang, Xiang Xu, Zhong Wang and Gang Chen

Stroke. 2015;46:2607-2615; originally published online July 28, 2015;
doi: 10.1161/STROKEAHA.115.009729

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/46/9/2607
Free via Open Access

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2015/07/28/STROKEAHA.115.009729.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL.
Role of neurexin-1β and neuroligin-1 in cognitive dysfunction after subarachnoid hemorrhage in rats

Supplemental Methods
Rat SAH model
After intraperitoneal anesthesia using chloral hydrate (36mg/100g body weight), the heads of rats were fastened in a stereotactic frame with the mouthpiece at 0°. The body temperature of the animals was maintained at 37.5±0.5°C using an automatic heating pad. The tail artery was cannulated to gauge mean arterial blood pressure and to gain blood samples. The animals were placed in a stereotaxic frame with the mouthpiece at 0°. A laser Doppler flowmeter was used to monitor the cerebral blood flow supplied by the middle cerebral artery in the area of the cerebral cortex constantly. To place the LDF probes, a bur hole was bored 5-mm left lateral and 1-mm posterior to the bregma without injuring to the dura mater.

Then, experimental SAH model was induced by single blood injection to prechiasmatic cistern as reported previously. Briefly, a total of 0.3 ml of non-heparinized fresh autologous arterial blood from femoral artery was injected evenly into the prechiasmatic cistern over 20 s using a syringe pump under aseptic conditions. Sham animals were injected in the same manner with 0.3 ml saline. The animals were allowed to recover for 45 min after the operation. After the operation, 5 ml of 0.9% NaCl was injected subcutaneously to prevent dehydration. It was here observed that the inferior basal temporal lobe of SAH group was stained with blood (shown as shadow areas in Figure IA).

Transfection of si RNA in Rat Brain
According to the manufacturer’s instructions for Entranster-in vivo RNA transfection reagent (Engreen, 18668-11-1), the transfection complex of siRNA was prepared as follows. Briefly, 500 pmol neurexin-1β siRNA, 500 pmol neuroligin-1 siRNA, 500 pmol neurexin-1β siRNA and 500 pmol scramble siRNA were respectively dissolved in 5 μL RNase-free water. Then, 10 μL Entranster-in vivo RNA transfection reagent was added to 5 μL siRNA or 5 μL scramble siRNA immediately. The solution was mixed for 15 minutes at room temperature. Finally, 15 μL Entranster-in vivo–siRNA mixture was injected intracerebroventricularly 48 h before SAH.

Plasmid transfection in Rat Brain
In vivo plasmid transfection in rat brain was performed according to the manufacturer’s instructions for Entranster-in vivo DNA transfection reagent (Engreen, 18668-11-2). Briefly, 5 μg neurexin-1β plasmid, 5 μg neuroligin-1 plasmid, 5 μg neurexin-1β plasmid and 5 μg neuroligin-1 plasmid, and 5 μg empty vector were respectively dissolved in 5 μL endotoxin-free water. Then, 10 μL Entranster-in vivo DNA transfection reagent was added to 5 μL plasmid or 5 μL empty vector immediately. The solution was mixed for 15 minutes at room temperature. Finally, 15 μL Entranster-in vivo–plasmid mixture was injected intracerebroventricularly 48 h before SAH.

Cell cultures
Briefly, hippocampus pairs were isolated from SD rats at 18 days of gestation, and treated with papain (100 mg/ml, Worthington, USA) for 10 min at 37°C. Dissociated neurons were plated at a density of 20,000 cells/cm² onto plates (Corning, USA) precoated with 0.1 mg/ml
poly-D-lysine (Sigma, USA), cultured in Neurobasal-A medium supplemented with 2% B-27 and 0.5 mM GlutaMAX™-I (all from Invitrogen, Grand Island, NY), and maintained at 37°C under humidified conditions and 5% CO₂ for 14–19 days. Half of the media were exchanged for fresh media every three days.

**Transfection of si RNA and plasmid in cultured neurons**

Both si RNA transfection and plasmid transfection in cultured neurons were performed using Lipofectamine® 3000 Transfection Reagent (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. Cells were transfected with scramble siRNA, neurexin-1β siRNA, neuroligin-1 siRNA, neurexin-1β siRNA + neuroligin-1 siRNA, empty vector, neurexin-1β plasmid, neuroligin-1 plasmid, neurexin-1β plasmid + neuroligin-1 plasmid, respectively. Twenty-four hours after tranfection, the cells were stimulated with 20 μmol/L oxyhemoglobin (OxyHb) for an additional 24 h. After the indicated treatments, cells were harvested for further analysis.

**Western blot**

The frozen samples or cells were lysed mechanically in lysis buffer containing 20 mmol/L Tris (pH 7.6), 0.2% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 0.11 IU/ml aprotinin (all from Sigma–Aldrich, USA). The lysates were centrifuged at 12,000g for 20 min at 4°C, and the concentration was measured by the bicinchoninic acid (BCA) method using enhanced BCA protein assay kit (Beyotime, China). The samples were separated using 10% SDS–PAGE and electrotransferred onto nitrocellulose membrane (Bio-Rad, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature, and were then incubated with primary antibodies against neurexin-1β and neuroligin-1 (Santa cruz, USA) in 5% BSA (in TBS+0.1%Tween 20) overnight at 4°C. The GAPDH or β-actin (Santa cruz, USA) was used as a loading control. The membranes were washed three times for 5 min each in TBS+0.1%Tween 20, and were then incubated in the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa cruz, USA) for 2 h at room temperature. Finally, the protein bands were visualized using enhanced chemiluminescence (ECL). The relative quantity of proteins was analyzed using Image J and normalized to that of loading controls.

**Immunoprecipitation analysis**

Firstly, the brain samples were lysed in ice-cold RIPA lysis buffer (Beyotime, China). For immunoprecipitation, the lysate was incubated with specific antibodies or rabbit IgG (negative control) overnight at 4°C with agitation. Protein A+G Sepharose beads were then added to each immune complex and the lysate-bead mixture was incubated for 4 h at 4°C with rotary agitation. SDS-PAGE and immunoblotting were then performed for further protein separation and detection.

**Immunofluorescence analysis**

The brain samples were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 4 μm sections, and examined by immunofluorescence staining. Briefly, sections were stained with primary antibodies and appropriate secondary antibodies as described. Normal rabbit IgG was used as a negative control for immunofluorescence assay (data not shown). Finally, sections were observed by a fluorescence microscope (OLYMPUS BX50/BX-FLA/DP70; Olympus Co., Japan). At least three random sections from each rat were examined. The relative fluorescence intensity was analyzed by use of Image J program.
Neurological scoring
At 72 h after SAH, all the rats involved in part 2 of in vivo experiment were examined for behavioral impairment using a previously published scoring system and monitored for appetite, activity, and neurological defects (Table I).

Morris water maze
Briefly, rats were trained in the Morris water maze on days 3 to 6 postsurgery (4 trials per day). The tank had an altitude of 50 cm, a diameter of 180 cm, and was filled with 20°C to 22°C water to a height of 30 cm. The platform was placed 2 cm beneath the surface of the water. Starting points were changed everyday. Each trial lasted either until the rat found the platform or for 90 seconds. After each trial, rats were allowed to rest for 20 seconds on the platform. Swimming speed, latency and swim path length were recorded.

Supplemental Tables
Table I Behavior and activity scores

| Category   | Behavior                               | Score |
|------------|----------------------------------------|-------|
| Appetite   | Finished meal                          | 0     |
|            | Left meal unfinished                   | 1     |
|            | Scarcely ate                           | 2     |
| Activity   | Walk and reach at least three corners of the cage | 0     |
|            | Walk with some stimulations            | 1     |
|            | Almost always lying down               | 2     |
| Deficits   | No deficits                            | 0     |
|            | Unstable walk                          | 1     |
|            | Impossible to walk                     | 2     |
Supplemental Figures and Figure Legends

**Figure Ⅰ** Experimental design. (A) Representative images of the brains of sham group and SAH group. The inferior basal temporal lobe of SAH group stained with blood was shown as shadow areas. (B) and (C) Experimental design for *in vivo* experiment; (D) Experimental design for *in vitro* experiment.
Figure II: Representative images of time course of neurexin-1β and neuroligin-1 expression in the rat cortex after SAH. Immunofluorescence was performed for neurexin-1β (A) and neuroligin-1 (B); cells were counter-stained with NeuN (red; neuronal marker) and DAPI (blue; nuclei). Scale bar = 64 μm.
Figure III Representative images of time course of neurexin-1β and neuroligin-1 expression in the rat hippocampus after SAH. Immunofluorescence was performed for neurexin-1β (A) and neuroligin-1 (B); cells were counter-stained with NeuN (red; neuronal marker) and DAPI (blue; nuclei). Scale bar = 100 μm.

Figure IV TUNEL staining tested the effects of neurexin-1β and neuroligin-1 knockdown and overexpression on cell death in the brain of SAH rats. SAH rats accepted intracerebroventricular injection of siRNAs or plasmids as indicated. Arrows point to TUNEL-positive cells. Scale Bar = 64 μm.
Figure V TUNEL staining tested the effects of neurexin-1β and neuroligin-1 knockdown and overexpression on cell death in cultured hippocampal neurons under OxyHb treatment. Cultured primary hippocampal neurons were transfected with siRNAs or plasmids as indicated. Arrows point to TUNEL-positive cells. Scale Bar = 64 μm.

Figure VI Representative tracing images from the Morris water maze test.
Figure VII Escape latency of each group in Morris water maze test, n=10.
Figure VIII Swim path length of each group in Morris water maze test, n=10.

Supplemental References
1. Wang Z, Ma C, Meng CJ, Zhu GQ, Sun XB, Huo L, et al. Melatonin activates the nrf2-are pathway when it protects against early brain injury in a subarachnoid hemorrhage model. Journal of pineal research. 2012;53:129-137
2. Li H, Gao A, Feng D, Wang Y, Zhang L, Cui Y, et al. Evaluation of the protective potential of brain microvascular endothelial cell autophagy on blood-brain barrier integrity during experimental cerebral ischemia-reperfusion injury. Translational stroke research. 2014;5:618-626