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microRNA-15b contributes to depression-like behavior in mice by affecting synaptic protein levels and function in the nucleus accumbens

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Running title: microRNA-15b-5p induces depression-like behavior

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Abstract

Major depression is a prevalent affective disorder characterized by recurrent low mood. It presumably results from stress-induced deteriorations of molecular networks and synaptic functions in brain reward circuits of genetically susceptible individuals through epigenetic processes. Epigenetic regulator microRNA-15b inhibits neuronal progenitor proliferation and is upregulated in the medial prefrontal cortex of mice that demonstrate depression-like behavior, indicating the contribution of microRNA-15 to major depression. Using a mouse model of major depression induced by chronic unpredictable mild stress (CUMS), here we examined the effects of microRNA-15b on synapses and synaptic proteins in the nucleus accumbens of these mice. The application of a microRNA-15b antagonir into the nucleus accumbens significantly reduced the incidence of CUMS-induced depression and reversed the attenuations of excitatory synapse and syntaxin-binding protein 3 (STXBP3A /vesicle-associated protein 1 (VAMP1) expression. In contrast, the injection of a microRNA-15b analog into the nucleus accumbens induced depression-like behavior as well as attenuated excitatory synapses and STXBP3A/VAMP1 expression similarly to the downregulation of these processes induced by the CUMS. We conclude that microRNA-15b-5p may play a critical role in chronic stress-induced depression by decreasing synaptic proteins, innervations and activities in the nucleus accumbens. We propose that the treatment of anti-microRNA-15b-5p may convert stress-induced depression into resilience.

Introduction

Major depression is a low mood disorder, in which patients show recurrent anhedonia, interest loss and low self-esteem. The interaction between chronic stress and genetic susceptibility is presumably its etiology (1-5). Chronic stress that leads to negative emotional outcomes in genetic susceptible individuals may dysfunction their neurons in the reward neural circuit, such as the ventral tegmental area, nucleus accumbens and prefrontal cortex (6-13). However, most people do not suffer from depression in response to chronic stress, i.e., resilience (14). The elucidation of
endogenous mechanisms underlying major depression versus resilience should shed light on developing therapeutic strategies to convert depression into resilience. Recently, epigenetic process is thought to be a key switch from chronic stress to cellular and molecular pathology of major depression and resilience, especially microRNA (15-28). As microRNA interacts with mRNA, by which the binding of microRNAs with their dicers degrade mRNAs and weaken their translation (29-32), the role of microRNAs in major depression and resilience is based presumably on their influence on the expression levels of cell proteins. These molecular and cellular targets acted by microRNAs for major depression versus resilience remain to be addressed.

The nucleus accumbens has been placed in the category of reward neural circuits (33-38), which regulates emotional reactions and cognitive processes (36,39-41). For instance, structural elements and functional activities in neural circuits that include the nucleus accumbens are involved in the regulation of reward and fear memory (37,42-47). Pathologically, some cell functions and signaling molecules in the nucleus accumbens are impaired in affective disorders (25,38,40,48-52). The dysfunction of GABAergic neurons in the nucleus accumbens is relevant to major depression (51,53-55). GABAergic synaptic transmission in the nucleus accumbens is deteriorated in depression-like mice, compared to resilience mice (55). These results indicate that GABAergic synapses and neurons in the nucleus accumbens may be cellular targets in major depression. In terms of molecular triggers, amygdala microRNA-15a is involved in the chronic stress (56).

microRNA-15b has been found to inhibit neuronal progenitor proliferation (57) and to be upregulated in the medial prefrontal cortex of depression-like mice by acting to synapse-relevant proteins (22). These results indicate that microRNA-15 may be an initiator of molecular and cellular changes in major depression. In present study, we intend to examine how microRNA-15b in the nucleus accumbens works to be one of essential epigenetic molecules that impair GABAergic neurons and lead to depressive disorder.

Strategies to reveal the essential role of microRNA-15b in major depression to chronic unpredicted mild stress (CUMS) as well as its molecular and cellular targets in the nucleus accumbens are presented below. The tests of sucrose preference, Y-maze and forced swimming were used to identify CUMS-induced depression-like behavior. Anti-microRNA-15b was done by microinjecting microRNA-15b antagonim into the nucleus accumbens. The effect of microRNA-15b was mimicked by microinjecting microRNA-15b agonim into this area. Synapse activities in the nucleus accumbens were monitored by electrophysiological whole-cell recording in brain slices. Axon projections and synapse innervations were analyzed by neural tracing with AAV-conjugated fluorescent proteins. The molecular targets of microRNA-15b were identified by quantitative RT-PCR, western-blot and dual luciferase reporter assay.

Results

**Chronic unpredictable mild stress leads to depression-like behavior and GABAergic downregulation in mice**

After an accommodation a week, two groups of mice were placed into the control house and the house with chronic unpredicted mild stress (CUMS) for three weeks, respectively. Their mood state was assessed by the sucrose preference test (SPT), Y-maze test (YMT) and forced swimming test (FST). Compared to control mice, the mice in the CUMS house appear an increased immobile time in the FST, a decreased sucrose preference and a decreased M-arm stay time in Y-arm maze. Values for immobile time are 142.8±9.14 seconds in CUMS-treated mice (red symbols in Figure 1B, n=21) and 88.05±7.7 second in control mice (hollow symbols; n=15, p<0.001, one-way ANOVA). SPT values in CUMS-treated mice are 68.31±2.1% after the CUMS (red symbols in Figure 1C) and 88.42±1.06% before the CUMS (red symbols; n=21, p<0.001, paired t-test). The ratios of the stay time in the M-arm to the stay time in total arms in CUMS-treated mice are 35.33±2.29% after the CUMS (red symbols in Figure 1D) and 46.72±2.02% before the CUMS (red symbols; n=21, p<0.01, paired t-test). Therefore, the CUMS induces the decreases in sucrose preference and social interaction as well as an increase of immobility in the forced swim, i.e., depression-like behavior in mice. We then
examined cellular and molecular changes in the nucleus accumbens.

Excitatory synaptic transmission was studied at GABAergic neurons in the nucleus accumbens included in the coronal section of brain slices. Spontaneous excitatory postsynaptic currents (sEPSC) were recorded under the whole-cell voltage-clamp. sEPSC amplitudes and frequencies appear to be decreased on GABAergic neurons from CUMS-induced depression mice, in comparison with control mice (Figure 2A). Figure 2B illustrates the cumulative probabilities of sEPSC amplitudes in CUMS-induced depression group (red symbols, n=14 cells from 5 mice) and in control group (hollows, n=15 cells from 5 mice). Inset shows that sEPSC amplitudes at 67% cumulative probability are 8.43±0.34 pA in control mice and 6.17±0.44 pA in CUMS-induced depression mice (p<0.001). Figure 2C shows the cumulative probabilities of inter-sEPSC intervals in CUMS-induced depression group (red symbols, n=14 from 5 mice) and in control group (hollow symbols, n=15 cells from 5 mice). Inset shows that inter-sEPSC intervals at 67% cumulative probability are 526±51.20 ms in control mice and 1144±104.7 ms in CUMS-induced depression mice (p<0.001). The decreased sEPSC amplitude and frequency indicates that excitatory synapses on GABAergic neurons in the nucleus accumbens are deteriorated in CUMS-induced depression mice.

Excitatory synapse innervation from the medial prefrontal cortex to neurons in the nucleus accumbens was analyzed in CUMS-induced depression mice and control mice. GABAergic neurons in mouse brain were genetically labeled by green fluorescent protein (GFP). mCherry carried by adeno-associated viruses (AAV) was injected into the medial prefrontal cortex (please see Methods in details). Subsequently, these mice were placed in either a CUMS house or a control house for three weeks. The contacts (yellow) between presynaptic axonal boutons (red) from the medial prefrontal cortex and postsynaptic GABAergic neurons (green) in the nucleus accumbens were counted to be synapse innervations. As showed in Figure 3A-B, excitatory synapse innervations on GABAergic neurons are lower in CUMS-induced depression mice than controls. The numbers of boutons on GABAergic neurons are 12.67±0.64 per cell in control group (hollow symbols in Figure 3C; n=27 cells from 5 mice) and 9.57±0.4 per cell in CUMS-induced depression group (red symbols; n=35 cells from 5 mice; p<0.001, one-way ANOVA). This result indicates that excitatory synapse innervation to GABAergic neurons in the nucleus accumbens from the medial prefrontal cortex is lowered in CUMS-induced depression mice.

To the functional and morphological downregulations of excitatory synapses onto GABAergic neurons, we assumed that the expression of genes and proteins relevant to synapse structure and function was attenuated. Genes VAMP1 and STXBP3A that encode vesicle-associated membrane protein and syntaxin binding protein (58-61) were selected to examine the molecular basis of synapse downregulation. The reason for examining the changes of presynaptic targets is based on the results above that presynaptic transmitter release and synapse innervations onto nucleus accumbens neurons are lowered in CUMS-induced depression. Their mRNAs were analyzed by quantitative RT-PCR, which were harvested from tissues of the nucleus accumbens in CUMS-induced depression mice and controls. The expression level of VAMP1 decreases from 100.16±2.01% in control mice (hollow symbols in Figure 4A, n=6) to 41.60±6.42% in CUMS-induced depression mice (red symbols; n=6, p<0.001, one-way ANOVA). The expression level of STXBP3A attenuates from 100.1±1.62% in control mice (hollow symbols in Figure 4B, n=6) to 55.98±5.24% in CUMS-induced depression mice (red symbols; n=6, p<0.001, one-way ANOVA). In addition, proteins VAMP1 and STXBP3A were investigated by western-blot, which were harvested from tissues of the nucleus accumbens from CUMS-induced depression mice and controls. The level of VAMP1 decreases from 100.0±8.22% in control mice (hollow symbols in Figure 4D, n=6) to 28.65±5.73% in CUMS-induced depression mice (red symbols; n=6, p=0.0021, one-way ANOVA). The level of STXBP3A attenuates from 100.0±4.81% in control mice (hollow symbols in Figure 4E, n=6) to 52.72±8.04% in CUMS depression-like mice (red symbols; n=6, p=0.0072, one-way ANOVA). Based on the quantitative
analyses of mRNA and proteins, genes and proteins in relevance to the fusion of synaptic vesicles with presynaptic membrane for transmitter release are downregulated in the nucleus accumbens from CUMS-induced depression mice.

The results above indicate that the CUMS downregulates synapse-relevant proteins (VAMP1 and STXBP3A), excitatory synapse innervations and synaptic transmission in the nucleus accumbens, which in turn induces major depression. This indication needs to be validated. Initiative factor from the CUMS to molecular and cellular changes remains to be figured out. Previous studies indicate the involvement of microRNAs in major depression (15–19,21,23). mRNA and microRNA interact each other, by which the binding of microRNA to dicers degrades mRNA to weaken mRNA translation (30,31). microRNA-15b-5p is elevated in CUMS-induced depression (22). microRNA-15b-5p directly acts to mRNAs VAMP1 and STXBP3A (Figure 5). We hypothesize that microRNA-15b-5p may be one of CUMS-acted initiative factors that induce pathological alternations in synapse molecules, morphology and functions. If it is a case, anti-microRNA-15b-5p is expected to rescue the downregulations of synapse-related proteins and synapses in the nucleus accumbens of CUMS-induced depression mice, and microRNA-15b-5p derivatives should induce depression-like behavior and synapse-relevant changes.

**Anti-microRNA-15b-5p significantly rescues CUMS-induced depression and synapse downregulation**

In addition to control and CUMS-treated mice, another two groups of mice were taken into our study that received the microinjections of microRNA-15b-5p antagonir and microRNA-15b-5p antagonir-control into the nucleus accumbens, respectively. The reason for examining the role of microRNA15b is based on our previous studies that microRNA-15b is upregulated in the medial prefrontal cortex from CUMS-induced depression mice (22,62) as well as the change of presynaptic targets has been seen where presynaptic transmitter release and synapse innervations onto nucleus accumbens neurons from the medial prefrontal cortex are lowered in CUMS-induced depression. These two groups of mice with microinjections were placed in the CUMS house for three weeks, similarly to CUMS-treatment alone. Their mood state was assessed by SPT, YMT and FST. Compared with control, CUMS and microRNA-15b-5p antagonir-control plus CUMS groups, mice that have received microRNA-15b-5p antagonir plus the CUMS demonstrate a significant improvement of CUMS-induced depression-like behavior. The values of immobile time in the FST are 88.05±7.71 seconds in control mice (hollow symbols in Figure 6A; n=15), 142.8±9.14 seconds in CUMS-treated mice (red symbols; n=21), 150.5±7.1 seconds in microRNA-15 antagonir-control plus CUMS mice (circled dot symbols; n=15) and 111.8±10.1 seconds in microRNA-15b-5p antagonir plus CUMS mice (blue symbols; n=18, p<0.01, one-way ANOVA). SPT values are 89.03±1.00% in control mice (hollow symbols in Figure 6B; n=15), 68.31±2.08% in CUMS-treated mice (red symbols; n=21), 71.89±2.50% in microRNA-15 antagonir-control plus CUMS mice (circled dot symbols; n=15) and 82.26±2.03% in microRNA-15b-5p antagonir plus CUMS mice (blue symbols; n=18, p values are less than 0.001 to 0.05, one-way ANOVA). The ratios of stay time in the M-arm to stay time in total arms are 47.92±1.59% in control mice (hollow symbols in Figure 6C; n=15), 35.33±2.29% in CUMS-treated mice (red symbols; n=21), 34.13±1.43% in microRNA-15b-5p antagonir-control plus CUMS mice (circled dot symbols; n=15) and 44.37±3.21% in microRNA-15b-5p antagonir plus CUMS mice (blue symbols; n=18, p values are less than 0.001 to 0.05, one-way ANOVA). This result indicates that anti-microRNA-15b-5p in the nucleus accumbens significantly rescues CUMS-induced depression-like behavior. We subsequently examined cellular and molecular mechanisms underlying this rescue.

The influence of microRNA-15b-5p downregulation on cell functions was examined by recording sEPSCs on GABAergic neurons in the nucleus accumbens from control mice, CUMS-induced depression mice and microRNA-15b-5p antagonir-injection plus CUMS-treatment mice. microRNA-15b-5p antagonir in the nucleus accumbens appears to rescue the decrease of excitatory synaptic transmission induced by the CUMS (Figure 7A). Figure 7B illustrates the
microRNA-15b-5p induces depression-like behavior

Cumulative probability of sEPSC amplitudes in control group (hollow symbols; n=15 cells from 5 mice), CUMS-induced depression group (red symbols, n=14 cells from 5 mice) and microRNA-15b-5p antagonist-injection plus CUMS group (blue symbols, n=14 cells from 5 mice). Inset illustrates that sEPSC amplitudes at 67% cumulative probability are 8.43±0.34 pA in control mice (hollow symbols), 6.17±0.44 pA in CUMS-induced depression mice (red symbols) and 7.32±0.34 pA in microRNA-15b-5p antagonist-injection plus CUMS-treated mice (blue symbols; p<0.001 and 0.05, respectively). Figure 7C shows the cumulative probability of inter-EPSC intervals in control group (hollow symbols; n=15 cells from 5 mice), CUMS-induced depression group (red symbols, n=14 cells from 5 mice) and microRNA-15b-5p antagonist-injection plus CUMS group (blue symbol, n=14 cells from 5 mice). Inset shows that inter-sEPSC intervals at 67% cumulative probability are 526±51.2 ms in control mice (hollow symbols), 1144±104.7 ms in CUMS-induced depression mice (red symbols) and 729.8±70.4 ms in microRNA-15b-5p antagonist-injection plus CUMS-treated mice (blue symbols, p values are less than 0.001 to 0.05). The CUMS-induced downregulation of excitatory synaptic transmission on GABAergic neurons in the nucleus accumbens is reversed by anti-microRNA-15b-5p. That is, microRNA-15b-5p is required for the downregulation of synaptic transmission in CUMS-induced depression.

The influence of microRNA-15b-5p downregulation on synapse innervations was studied by neural tracing in the nucleus accumbens from control mice, CUMS-induced depression mice and microRNA-15b-5p antagonist-injection plus CUMS-treated mice. AAV-carried mCherry was injected and expressed in the medial prefrontal cortex. microRNA-15b-5p antagonist was injected into the nucleus accumbens. After various treatments, their mood state was assessed by SPT, YMT and FST. mCherry-labeled axon boutons onto GABAergic neurons were analyzed in the nucleus accumbens by scanning slice sections under a confocal microscope. microRNA-15b-5p antagonist appears to reverse the decrease of synapse innervation from the medial prefrontal cortex to GABAergic neurons in the nucleus accumbens induced by the CUMS (Figure 8A-C).

Figure 8D illustrates that boutons per GABAergic neuron are 12.67±0.64 in control group (hollow symbols; n=27 cells from 5 mice), 9.57±0.40 in CUMS-induced depression group (red symbols, n=35 cells from 5 mice) and 11.70±0.47 in microRNA-15b-5p antagonist-injection plus CUMS group (blue symbols, n=30 cells from 5 mice, p<0.01, one-way ANOVA). The CUMS-induced decrease of excitatory synapse innervations onto GABAergic neurons in the nucleus accumbens is reversed by anti-microRNA-15b-5p. In other words, microRNA-15b-5p is required for the downregulation of synapse innervations in CUMS-induced depression.

The effect of microRNA-15b-5p downregulation on synapse-relevant genes VAMP1 and STXBP3A was tested by quantitative RT-PCR in the nucleus accumbens from control mice, CUMS-induced depression mice and microRNA-15b-5p antagonist-injection plus CUMS-treated mice. microRNA-15b-5p antagonist was injected into the nucleus accumbens. After various treatments, their mood was assessed by SPT, YMT and FST. The tissues of the nucleus accumbens were harvested from these mice for qRT-PCR. The expression of VAMP1 decreases from 100.16±2.01% in control mice (hollow symbols in Figure 9A; n=6) to 41.60±6.42% in CUMS-induced depression mice (red symbols; n=6), which is reversed to 67.25±5.19% in microRNA-15b-5p antagonist-injection plus CUMS-treated mice (blue symbols; n=6, p<0.01, one-way ANOVA). The expression of STXBP3A attenuates from 100.1±1.62% in control mice (hollow symbols in Figure 9B; n=6) to 55.98±5.24% in CUMS-induced depression mice (red symbols, n=6), which is reversed to 73.93±4.83% in microRNA-15b-5p antagonist-injection plus CUMS-treated mice (blue symbols; n=6, p<0.05, one-way ANOVA). The CUMS-induced decrease of synapse-relevant genes VAMP1 and STXBP3A in the nucleus accumbens is significantly but partially reversed by anti-microRNA-15b-5p.

The effect of microRNA-15b-5p downregulation on synapse-relevant proteins VAMP1 and STXBP3A was tested by western-blot in the nucleus accumbens from control mice, CUMS-induced depression mice and microRNA-15b-5p antagonist-injection plus CUMS-treated mice. microRNA-15b-5p antagonist was injected
into the nucleus accumbens. The tissues of the nucleus accumbens were harvested from these mice for western-blot. As illustrated in Figure 9C, microRNA-15b-5p antagonist appears to reverse the decrease of VAMP1 and STXBP3A in the nucleus accumbens induced by the CUMS. The expression of VAMP1 decreases from 100±8.22% in control mice (hollow symbols in Figure 9D; n=6) to 28.65±5.73% in CUMS-induced depression mice (red symbols, n=6), which is reversed to 63.43±8.48% in microRNA-15b-5p antagonist plus CUMS-treated mice (blue symbols; n=6, p<0.05, one-way ANOVA). The expression of STXBP3A changes from 100±4.81% in control mice (hollow symbols in Figure 9E; n=6) to 52.7±8% in CUMS-induced depression mice (red symbols, n=6), which is reversed to 88.12±6.3% in microRNA-15b-5p antagonist plus CUMS-treated mice (blue symbols; n=6, p<0.05, one-way ANOVA). The CUMS-induced decrease of synapse-relevant proteins VAMP1 and STXBP3A in the nucleus accumbens is significantly but partially reversed by anti-microRNA-15b-5p. In summary, microRNA-15b-5p is required for the downregulation of genes and proteins (VAMP1 and STXBP3A) in CUMS-induced depression.

**microRNA-15b-5p agomir mimics CUMS-induced depression and synapse downregulation**

In addition to control and CUMS-treatment groups, other two groups of mice were taken into our study that received microinjections of microRNA-15b-5p agomir and microRNA-15b-5p agomir-control into the nucleus accumbens, respectively. The mice with microinjections were placed in control house for three weeks. Their mood was assessed by SPT, YMT and FST. The values of immobile time in the FST are 88.05±7.71 seconds in control mice (hollow symbols in Figure 10A; n=15), 142.8±9.14 seconds in CUMS-treated mice (red symbols; n=21), 92.36±9.10 seconds in microRNA-15 agomir-control mice (circled saltire symbols; n=15) and 130.2±8.81 seconds in microRNA-15b-5p agomir-injection mice (yellow symbols; n=20, p<0.01, one-way ANOVA). SPT values are 89.03±1.00% in control mice (hollow symbols in Figure 10B; n=15), 68.31±2.08% in CUMS-treated mice (red symbols; n=21), 90.76±0.89% in microRNA-15b-5p agomir-control mice (circled saltire symbols; n=15) and 72.66±2.37% in microRNA-15b-5p agomir-injection mice (yellow symbols; n=20, p values are less than 0.001 to 0.05, one-way ANOVA). The ratios of stay time in the M-arm to stay time in total arms are 47.92±1.59% in control mice (hollow symbols in Figure 10C; n=15), 35.33±2.3% in CUMS-treated mice (red symbols; n=21), 45.32±2.28% in microRNA-15b-5p agomir-control mice (circled saltire symbols; n=15) and 32.53±3.09% in microRNA-15b-5p agomir-injection mice (yellow symbols; n=20, p values are less than 0.001 to 0.05, one-way ANOVA). This result indicates that microRNA-15b-5p in the nucleus accumbens leads to depression-like behavior similarly to those induced by the CUMS. We also studied cellular and molecular mechanisms underlying this mimic.

The influence of microRNA-15b-5p upregulation on cellular function was examined by recording sEPSCs on GABAergic neurons in the nucleus accumbens from control mice, CUMS-induced depression mice and microRNA-15b-5p agomir-injection mice. microRNA-15b-5p agomir in the nucleus accumbens appears to lower excitatory synaptic transmission similarly to those by the CUMS (Figure 11A). Figure 11B shows the cumulative probability of sEPSC amplitudes in control group (hollow symbols; n=15 cells from 5 mice), CUMS-induced depression group (red symbols, n=14 cells from 5 mice) and microRNA-15b-5p agomir-injection group (yellow symbols, n=12 cells from 5 mice). Inset shows that sEPSC amplitudes at 67% cumulative probability are 8.43±0.34 pA in control mice (hollow symbols), 6.17±0.44 pA in CUMS-induced depression mice (red symbols) and 6.69±0.41 pA in microRNA-15b-5p agomir-injection mice (yellow symbols; p<0.01). Figure 11C illustrates the cumulative probability of inter-sEPSC intervals in control group (hollow symbols; n=15 cells from 5 mice), CUMS-induced depression group (red, n=14 cells from 5 mice) and microRNA-15b-5p agomir-injection group (yellow, n=12 cells from 5 mice). Inset shows that inter-sEPSC intervals at 67% cumulative probability are 526±51.2 ms in control mice (hollow symbols), 1144±104.7 ms in CUMS-induced depression mice (red symbols) and 1029±87.63 ms in microRNA-15b-5p agomir-injection mice (yellow symbols, p<0.001). The
microRNA-15b-5p induces depression-like behavior

CUMS-induced decrease of excitatory synaptic transmission on GABAergic neurons in the nucleus accumbens is mimicked by microRNA-15b-5p derivatives. In other words, microRNA-15b-5p sufficiently induces the downregulation of synaptic transmission similarly to that in CUMS-induced depression.

The effect of microRNA-15b-5p upregulation on synapse innervations was examined by neural tracing in the nucleus accumbens from control mice, CUMS-induced depression mice and microRNA-15b-5p agomir-injection mice. AAV-carried mCherry was injected and expressed in the medial prefrontal cortex, and microRNA-15b-5p agomir was injected in the nucleus accumbens. After various treatments, their mood state was assessed by SPT, YMT and FST. The expression of microRNA-15b-5p appears to attenuate synapse innervations from the medial prefrontal cortex to GABAergic neurons in the nucleus accumbens similarly to that by the CUMS (Figure 12A–C). Figure 12D shows that boutons per GABAergic neuron are 12.67±0.64 in control group (hollow symbols; n=27 cells from 5 mice), 9.57±0.40 in CUMS-induced depression group (red symbols, n=35 cells from 5 mice) and 9.03±0.5 in microRNA-15b-5p agomir-injection group (yellow symbols; n=31 cells from 5 mice, p<0.001, one-way ANOVA). The CUMS-induced decrease of excitatory synapse innervations on GABAergic neurons in the nucleus accumbens is mimicked by microRNA-15b-5p derivatives. In other words, microRNA-15b-5p sufficiently induces for the impairment of synapse innervations similarly to that in CUMS-induced depression.

The influence of microRNA-15b-5p upregulation on synapse-relevant genes VAMP1 and STXBP3A was studied by qRT-PCR in the nucleus accumbens from control mice, CUMS-induced depression mice and microRNA-15b-5p agomir-injection mice. microRNA-15b-5p agomir was injected into the nucleus accumbens. After various treatments, their mood was assessed by SPT, YMT and FST. The tissues of the nucleus accumbens were harvested from these mice. The expression of VAMP1 changes from 100.0±8.22% in control mice (hollow symbols in Figure 13B; n=6) to 28.65±5.73% in CUMS-induced depression mouse (red symbols, n=6) and to 20.59±5.2% in microRNA-15b-5p agomir-injection mouse (yellow symbols; n=6, p<0.01, one-way ANOVA). The expression of STXBP3A changes from 100.0±4.81% in control mice (hollow symbols in Figure 13E; n=6) to 52.72±8.04% in CUMS-induced depression mouse (red symbols, n=6) and to 37.19±5.43% in microRNA-15b-5p agomir-injection mouse (yellow symbols; n=6, p<0.01, one-way ANOVA). The CUMS-induced downregulation of synapse proteins VAMP1 and STXBP3A in the nucleus accumbens is mimicked by microRNA-15b-5p derivatives. That is, microRNA-15b-5p sufficiently suppresses the expression of genes and proteins (VAMP1 and STXBP3A) similar to that in CUMS-induced depression.

Discussion

Chronic unpredictable mild stress (CUMS) induces depression-like behavior, e.g., an increase of immobile time in the forced swim test as well as
the decreases in sucrose preference and social interaction. This CUMS also weakens excitatory synaptic transmission and synapse innervation on GABAergic neurons in the nucleus accumbens from the medial prefrontal cortex as well as decreases the expression of genes and proteins relevant to the synapses, such as VAMP1 and STXBP3A. In other words, the CUMS induces major depression by downregulating the excitatory synapses, e.g., their presynaptic targets, on GABAergic neurons in the nucleus accumbens. In terms of the initiative factor from the CUMS to these molecular and cellular changes in the nucleus accumbens, our study shows that the upregulation of microRNA-15b-5p induces depression-like behavior and the downregulation of microRNA-15b-5p significantly rescues CUMS-induced depression by influencing these cellular and molecular changes in relevance to GABAergic neurons in the nucleus accumbens. Therefore, the requirement and sufficiency of microRNA-15b for CUMS-induced depression and its relevant cellular and molecular changes indicate microRNA-15b-5p is essential for initiating CUMS-induced depression as well as anti-microRNA-15b-5p may be used to rescue major depression. The chain reaction that leads to CUMS-induced depression includes the CUMS, the elevated microRNA-15b-5p expression, the downregulated VAMP1 and STXBP3A as well as the deteriorated presynaptic components of excitatory synapses on GABAergic neurons in the nucleus accumbens.

The deterioration of GABAergic neurons in the nucleus accumbens is the basis of depression-like behavior since the depression seems to be rescued by upregulating their synapse innervations/functions as well as induced by their downregulation. By manipulating microRNA-15b-5p-regulated genes and proteins in relevance to presynaptic targets, our data strengthen implications from those studies that GABAergic neurons in the nucleus accumbens are associated with stress-induced depression (51,53,54), and that mice in the resilience to chronic mild stress demonstrate the relatively normal function of GABAergic neurons in the nucleus accumbens (55). As the nucleus accumbens is presumably the important region of reward circuits (33-38), its dysfunction shifts the emotion balance toward the negative end, leading to anhedonia, interest loss and low self-esteem.

In terms of the molecular mechanism underlying the CUMS-induced dysfunction of GABAergic neurons in the nucleus accumbens, our data indicate that the attenuated expression of VAMP1 and STXBP3A genes and proteins is associated with CUMS-induced depression (Figure 4) and that this effect is based on the direct inhibitory interaction of microRNA-15b-5p to mRNAs in relevance to encoding these two proteins (Figure 5). Furthermore, microRNA-15b-5p antagonir significant rescues CUMS-induced depression-like behavior as well as preserves synapse function and innervations by upregulating VAMP1 and STXBP3A (Figures 6–9). microRNA-15b-5p derivative mimics CUMS-induced depression-like behavior and synapse deterioration by downregulating VAMP1 and Stxbp3 (Figures 10–13). Our studies suggest that microRNA-15b-5p by inhibiting vesicle-release proteins VAMP1 and STXBP3A may impair excitatory synapses on GABAergic neurons in the nucleus accumbens and induces depression-like behavior. Other molecular and cellular processes regulated by microRNA-15b in the nucleus accumbens may be involved in major depression, which remains to be tested.

It is noteworthy that other molecules in the nucleus accumbens have been found to be related to depression-like behavior. For instance, Tet1 in the nucleus accumbens opposes depression-like behavior (49). In the nucleus accumbens, the downregulations of serotonergic and dopaminergic synapses as well as of the MAPK and calcium signaling pathways are associated with CUMS-induced depression. The upregulations of PI3K-Akt and cAMP signaling pathways and amino acid metabolism are associated with CUMS-induced depression (25). As all these molecules in the nucleus accumbens may be associated with major depression, microRNA-15b-5p partially preserves CUMS-induced depression by upregulating the expression of synaptic proteins and the excitatory driving force on GABAergic neurons in the nucleus accumbens (Figures 10-13). Moreover, our study by upregulating and downregulating microRNA-15b-5p to conclude its essential role in major depression is compelling, in comparison with those previous studies that show the
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associated changes between molecules and behavior only.

In addition, previous study indicates the involvement of amygdala microRNA-15a in chronic stress (56). microRNA-19b associates with Ago2 in the amygdala after chronic stress and regulates adrenergic β1-receptor (63). microRNA-15b-5p is upregulated in the cerebral cortices of depression-like mice by acting to synapse-relevant proteins (22). How microRNA-15a, microRNA-19b and microRNA-15b-5p in the amygdala and nucleus accumbens coordinately lead to major depression remains to be investigated. Moreover, numerous microRNAs and mRNAs in the nucleus accumbens, amygdala and ventral tegmental area are associated with depression-like behavior (24,25,27). How these microRNAs and mRNAs relevant to synapses and neural signaling pathways among these brain areas coordinately induce major depression remains to be investigated. As microRNA-15b-5p significantly leads to depression-like behavior, it should be one of critical molecules for the incidence of major depression.

The nucleus accumbens is considered as an important region of brain reward circuits (33-38), especially as important structure to regulate emotion reaction and cognitive events (36,39-41). The nucleus accumbens possesses the mutual synapse innervations with many brain regions, such as the ventral tegmental area, the prefrontal cortex and the amygdala. Its physiological roles presumably involve various psychological processes, such as emotion, motivation, cognition and action, as well as encode the reward feeling from the food taking and drug addiction (13,36,43,64-66). As the nucleus accumbens receives synapse innervations from the ventral tegmental area and the prefrontal cortex for the reward-driven events, the downregulation of presynaptic proteins VAMP1 and Stxbp3 by anti-microRNA-15b may affect all of these presynaptic terminals onto nucleus accumbens neurons. Their dysfunction by activity silence may lead to anhedonia, interest loss and low motivation in major depression (53). Taken these data together, we suggest that the induction of major depression may be due to lack of reward that leads to the poor activation of brain reward circuits (55), which is being tested.

Although the chronic stress is a main etiology of major depressive disorder (1-5,67-69), most of the individuals after experiencing the chronic stress do not suffer from major depression, i.e., a resilience to the chronic stress (14). It has been found that the functions of GABAergic neurons in the nucleus accumbens are close to normal in CUMS-resilience mice (55), indicating that there may be endogenous anti-depression mechanisms in the nucleus accumbens for the animals to be resilient to chronic stress. In the nucleus accumbens, the downregulations of chemokine signaling pathway, synaptic vesicle cycle and nicotine addiction and the upregulations of calcium signaling pathway and tyrosine metabolism are associated with CUMS-resilience (25,70). An induction of DeltaFosB in the nucleus accumbens in response to chronic social defeat stress was both necessary and sufficient for resilience (71). Taken these previous studies together with our results that microRNA-15b-5p downregulation of and its preservation to the low expression of VAMP1 and STXBP3A appear to significantly reduce the incidence of CUMS-induced depression, we assume that an endogenous mechanism for animals to be resistance to chronic stress is based on a molecular network. The elucidation of the endogenous mechanism for the resilience to the chronic stress should shed light on developing therapeutic strategies for major depression. The ventral tegmental area, nucleus accumbens, amygdala and medial prefrontal cortex are accounted into the brain reward circuit (72-74). These structures contain GABAergic neurons, especially the core area of the amygdala and the nucleus accumbens mainly include the cluster of GABAergic neurons. The impairment of GABAergic neurons in the limbic system is associated with major depression (55,62,75-89). Whether the intact function of the GABAergic neurons in these regions beyond the nucleus accumbens is also associated with resilience to chronic stress remains to be examined in order to get a general view that the function state of GABAergic neurons in the brain is correlated with the resilience versus susceptibility to chronic stress for major depression.

Experimental procedures
Subjects were male C57 GAD67-GFP mice (3 weeks of age) housed in cages (32×16×16 cm, L×W×H) with free access to food and water and maintained in a well conditioned room with controlled temperature (22±2°C), humidity (55±5%) and a light/dark cycle of 12 hours (illumination at 7:00-19:00). Experimental procedures were accredited by Institutional Animal Care and Use Committee (B10831). Experiments were conducted in compliance with the rules and guidelines of the Administration Office of Laboratory Animals at Beijing China.

The mouse model of major depression induced by chronic unpredictable mild stress (CUMS)

Strain C57 GAD67-GFP mice (90) were used for the study of cell-specific mechanism of NAc-related depression, for the reason that the GABAergic neurons of this strain were genetically labeled by green fluorescent protein (GFP). Postnatal 21 days C57 male mice were used for our experiments that was based on the studies that young individuals were more susceptible to chronic stress (27,55,88). After one week accommodation, their performance on locomotion, sucrose preference and Y-maze tests were recorded as the self-control data. Mice at postnatal day 28 that had consistent behavioral values (within mean ±2SD of three tests) were randomly divided into the control, CUMS, antagonir injection plus CUMS and agonir injection groups. The CUMS treatment were absent for the control and agonir injection mice. C57 male mice were used on the basis of previous research (91-93).

The CUMS was used to induce major depression in mice (22,62,88). Mice lived in inescapable stressful environment constantly experienced defeated outcomes. Some mice showed depression-like symptoms like anhedonia and low self-esteem after exposure to CUMS, which induce repetitive negative memory that driven the mice to feel cognitive disability and emotional frustration (13,24,25,27,66,94,95). The paradigms of 3 week CUMS consisted of social isolation, empty cage, tilted cage, wet sawdust cage, white noise, restraint, circadian disturbance and strobe light (22,55,62,88). Except for the social isolation, other stressors were randomly given to mice individually or in combination everyday (please refer Table 1 in (88) for detail).

The CUMS-induced depression-like symptoms such as anhedonia and low self-esteem were examined in days 36~39. Anhedonia was measured by sucrose preference test (SPT). Loss of interest for social interaction was evaluated by Y-maze test (YMT). The state of self-esteem was assessed by forced swimming test (FST) (7,88,96,97). During the four hours SPT, the ingestion of 1% sucrose water and normal water were measured. Sucrose preference values were calculated as the percentage of the ingested sucrose water to total ingested water. The YMT was performed by recording the mouse stay time in a special arm and other two arms. At the terminal of special arm (defined as M-arm), a female mouse caged in the glass box (punched with many round holes) was placed to attract subject mouse for social interaction. After three minutes recording, M-arm stay time was presented by the percentage of stay time in M-arm to three arms. The FST was conducted by recording the length of immobility when mice floated in a water cylinder (diameter=10 cm, depth=19 cm, 25°C). The SPT, YMT were conducted before and after the CUMS, while the FST were done once. Before the SPT, mice were deprived of water and food for three hours to invoke their eagerness to drink water. After each YMT, three arms were cleaned by 70% ethanol and then water to erase the residual odor. Before the behavior tests, mice were absent from stressors and were allowed to accommodate themself in the testing environment. All behavior test were conducted in a quiet room with controlled temperature (25°C) and humanity (55±5%) (22,62,88).

After comparison with the behavioral performance of themself (day 0) and the control mice, depression-like behavior of mice were verified by the reduction in sucrose preference, M-arm stay time and the increase length of immobility. The behavior performance of each mice would be regarded as significant change if the sucrose preference and M-arm stay time reduced above 20% when compared with its self-control as well as the immobile time increased above 15% when compared with control group. These standards were set up on the basis of the averaged behavioral performance in mice during our previous tests (24,25,27,55,62,88).
The microinjection of microRNA-15b-5p antagonomir or agomir into the nucleus accumbens

miRNA antagonomir was the chemically modified cholesterol-conjugated single-stranded RNA analogues complementary to specific miRNAs. In previous study, the antagonomir designed for inhibiting miRNA was based on the rule of their base complementary, and their binding has been found to specifically inhibit miRNA expression (98). This approach has been proved to be well matured. In our study, microRNA-15b-5p antagonomir was a RNA analogue complementary to microRNA-15b-5p. microRNA-15b-5p agomir was a derivative of chemical synthesized microRNA-15b-5p. Antagomir or agomir control is miRNA that its sequence is based on the C. elegans. As it has no identifiable effects on mammalians, it has been served as the negative control of miRNA antagonomir or agomir. microRNA-15b-5p antagonomir, agomir and their negative control were purchased from Ruibo Biological Technology (Guangzhou, China). Mice were anesthetized by the intraperitoneal injection of 4% chloral hydrate (0.1 ml/10 g) and their heads were fixed at the stereotaxic device. 1 nM antagonomir, agomir or their negative control dissolved in 1 µl ACSF were slowly injected (30-40 minutes) into NAc (1.2 mm prior to the bregma, 1.0 mm lateral to the midline and 3.5 mm in the depth) for three times every three days. The glass pipettes were use for the microinjections by adding air pressure from the microsyringe (RWD Life science, Shenzhen, China).

Brain slices and neurons

To obtain more health neurons for whole-cell recordings, we prepared the brain slices including NAc by using the following protocols. Mice were anesthetized by inhaling isoflurane and their left ventricles were infused by the artificial cerebrospinal fluid (ACSF, 4°C, oxygenated by 95% O2 and 5% CO2) until their limbs turn pale. The concentrations (mM) of ACSF were 124 NaCl, 3 KCl, 26 NaHCO3, 1.2 NaH2PO4, 4 MgSO4, 0.5 CaCl2, 10 dextrose and 220 sucrose at pH 7.35. Mouse were quickly decapitated by guillotine and the head were submerged into oxygenated ACSF (4°C) to isolated the brain tissue. Brain tissue were sliced (300 m) in the oxygenated ACSF (4°C) in coronal direction by the Vibratome. The brain slices were transferred to another oxygenated ACSF (124 mM NaCl, 3 mM KCl, 1.2 mM NaH2PO4, 26 mM NaHCO3, 2 mM CaCl2, 2 mM MgSO4, 10 mM dextrose, and 5 mM HEPES, at pH 7.35) at 25°C to incubate for 2 hours. During the whole-cell recordings, brain slice was submerged in a chamber (Warner RC-26G) infused with oxygenated ACSF at 31°C (99-101). The chemical reagents were from Sigma.

GFP-labeled GABAergic neurons in the NAc for whole-cell recording were confirmed under DIC-fluorescent microscope (Nikon FN-E600, Tokyo Japan). The excitation wavelength of GFP is 488 nm. GABAergic neurons were featured by expressing fast spikes with less adaptive frequency and amplitude, which is typical for the interneurons (102-106).

Whole-cell recording for synaptic activities

The synaptic activity was recorded by MultiClamp-700B amplifier under the voltage-clamp and intrinsic property was analyzed by current-clamp. Electrical signals were collected and analyzed by pClamp-10 (Axon Instrument Inc.). The output frequency of amplifier bandwidth was maintained at 3 KHz. The micropipette solution used for recording synaptic transmission included (mM) 150 K-gluconate, 5 NaCl, 5 HEPES, 0.4 EGTA, 4 Mg-ATP, 0.5 Tris-GTP, and 5 phosphocreatine (pH 7.35; (107-109). The micropipettes were newly made and internal solution was freshly prepared and filtered (0.1 μm). The osmolarity was 295–305 mOsmol and the resistance of micropipette tip was 5–6 MΩ.

The electrophysiological function of GABAergic neurons was evaluated via their excitatory synapses activity (110,111). To isolate sEPSCs from synaptic currents, slices was infused by 10 μM bicuculline to eliminate GABA_AR-mediated inhibitory synaptic currents. To confirm that sEPSCs are mediated by ionotropic glutamate receptors (112,113), 40 μM D-amino-5-phosphonovanolenic acid (DAP5) and 10 μM 6-Cyano-7-nitroquinoxaline-2,3-(1H,4H)-dione (CNQX) that blocked sEPSCs were blended in the ACSF before the end of recording.

The whole-cell recording data were anlayzed only if the recorded neurons had the resting membrane potentials below -60 mV and action
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Potential amplitudes above 90 mV, and also included less than 5% fluctuation of spike amplitudes, resting membrane potential and input resistances during each recording. To monitor the series and input resistances of recorded neurons, hyperpolarization pulses (5 mV/50 ms) were injected. The resistances were calculated as pulses voltages divided by instantaneous and steady-state currents.

**Morphological imaging of structural connections**

Structural connections from mPFC to NAc were traced by injecting anterograde tracer pAAV-SynaptoTag-mCherry-GFP (by courtesy of Dr. Tom Sudhof) into the mPFC and detecting its projection to the NAc (90). In terms of the working principle of this AAV, Synapsin-I promoter initiated the expression of EGFP-synaptobrevin-2 in presynaptic boutons and the expression of mCherry in entire neurons, especially axons (114). According to the map of mouse brain (115), glass micropipettes were positioned in mPFC (1.8 mm prior to the bregma, 0.4 mm lateral to midline and 1.5 mm in depth) during the pAAV injections. The excitation and emission wavelength of mCherry was 565 nm and 610 nm, that was used to identify axon projection from mPFC to the NAc. Four weeks after the injection, the axon projection from mPFC to the NAc neurons were visualized by the cofocal imaging.

After one week recovery from stereotaxic surgery, mice were exposed to the CUMS or control condition. Once all behavioral test were done, the mice were anesthetized by the intraperitoneal injection of urethane (1.5 g/kg), and their left ventricles then were slowly perfused with 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS) until their limbs turn stiff and pale. Brains were extracted and soaked in 4% paraformaldehyde PBS for 24 hours fixation. Brain tissues including NAc were sliced (80 μm) in the coronal direction by the Vibratome. Slices were gently washed in PBS for 10 minutes, air-dried and cover-slipped.

Morphological structure of mCherry-labeled boutons and GFP-labeled GABAergic neuron in the NAc were captured under the confocal microscopy (Nikon A1R plus, Tokyo Japan). Excitation wavelengths of mCherry and GFP were 565 nm and 488 nm. The optical grating for GFP and RFP were 500~550 nm and 570~620 nm. The merged images illustrated the synapse contacts. The resolution of confocal scanning was maintained at 0.05 μm/pixel (116-118).

To improve the clarity of boutons and neurons, brain slices were soaked in ScaleA2 solution for 24 hours (119,120). The morphology and density of synapse contacts were visualized and counted in the shell and core of the NAc by using softwares ImageJ (version 1.8.0; National Institute of Health, USA) and Imaris (version 9.3; Bitplane, England). Structural contacts between mCherry-labeled boutons and GFP-labeled GABAergic neurons were identified in the merged images under confocal microscopy were presumed to be synapses. We counted the number of synaptic contacts per neurons in the NAc.

**Quantitative RT-PCR to validate the regulation of microRNA-15b-5p to mRNAs**

SYBR Green-based quantitative PCR were used for mRNA expression assay of genes VAMP1 and STXBP3A. Total RNAs were extracted from NAc tissues by using the Trizol Kit. cDNAs for mRNA expression analysis was reverse trancribed by using the PrimeScript RT Reagent Kit (TaKaRa, RR037A, Kusatsu, Japan). Reverse transcription of mRNA was performed in a 20μl reactions comprised of 1μl template cDNA, 0.5μl of forward and 0.5 μl reverse primers (10 nmol/l), 10μl 2×qPCR Mastermix (SYBR Green), and 8μl ddH2O. Real-time qPCR was performed on the Bio-Rad CFX96 Touch. The reaction started with initial denaturation for 2 minutes at 95°C, followed by 40 cycles of denaturation for 15 seconds at 95°C, annealing and extension for 30 seconds at 60°C, and ended with obtaining the melt curve from 65°C to 95°C (increment 0.5°C per 5 seconds ). The expression of miR-15b-5p targeted mRNAs was normalized to the internal control β-actin. The results were calculated with the 2^ΔΔCt method (121). All real-time qPCR of each treatment was ran in triplicates.

**Dual luciferase reporter assay**

The sequence carrying the targeted sites of miR-15b-5p targeting gene VAMP1 and STXBP3A were amplified, cut by NotI and XhoI and restructured into the luciferase reporter vector.
psiCHECK2 (22,62). The miR-15b-5p targeting sites were site-directed mutated by QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the instructions of manufacturer. Luciferase reporter detection assays were conducted following the previously description (62). HEK293T cells were planted at 24-well plates (5×10^4 cells per well) and cultured in DMEM added with 10% FBS. After 24 hours, HEK293T cells were transfected by 50 ng wild-type psiCHECK2 or mutant luciferase reporter plasmids, 50 nM miRNAs mimic or negative control by using Lipofectamine® 2000 transfection reagent (Invitrogen, Waltham, Massachusetts, USA). After 48 hours, the activities of Renilla and firefly luciferase were measured by Dual-Glo® Luciferase Assay System (Promega, Madison, Wisconsin, USA) under the instructions of manufacturer. Each treatment was ran in triplicates in three independent experiments.

**Western-blot to quantify protein**

The NAc tissues extracted from mice brain were gently washed 20 seconds in PBS (4°C) and quickly transferred to 1 ml RIPA Lysate buffer (4°C) added with 1 mM PMSF (Beyotime Biotechnology, China) for complete homogenization. Homogenized tissues were placed into a fresh tube, held on ice for 30 minutes, and centrifuged at 12000 g/min for 15 minutes at 4°C. Collected the supernatant into a new tube. Total protein concentration of supernatant was estimated by using BCA protein assay under the instruction of manufacturer (Beyotime Biotechnology, Shanghai, China). Prepared the 5% and 12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE gel) for electrophoresis to separate VAMP1 and STXBP3A from each sample. Twenty micrograms of total proteins per sample and molecular weight marker were loaded into the wells of the SDS-PAGE gel, run the electrophoresis for 1 hour. Electrically transferred the protein from the gel to the nitrocellulose membranes (0.2 μm). The membranes were blocked by 5% non-fat milk solution (dissolved in 1×TBS, added with 0.1% Tween 20) at 25°C for 60 minutes. Incubated the membrane with primary antibodies (1:1000 in dilution) of VAMP1 (13151S, Cell Signaling Technology, USA), STXBP3A (13764-1-AP, Proteintech, Wuhan, China) and β-actin (AC026, ABclonal Technology, Wuhan, China) overnight. After incubation with primary antibody, the membranes were washed by PBS for 10 minutes (3 times). Incubated the membrane with secondary antibodies DyLight 680 (5470S, Thermo Fisher Scientific, Massachusetts, USA) for one hour. Bands of VAMP1, STXBP3A and β-actin were visualized by using infrared imaging system Odyssey® CLx (LI-COR, Lincoln, Nebraska, USA). The fluorescence of each band was determined after the adjustment of background for relative quantitative analysis. The fluorescence of each band was normalized to the internal control β-actin and were analyzed by using software ImageJ (version. 1.47; National Institute of Health, USA).

**Statistical analyses**

The data of behavioral tests, electrophysiological recordings and protein chemistry are presented as mean±SEM. Comparisons of behavioral data in each of the mice before and after CUMS was made by Paired t-test. Comparisons of neuronal function, structural connection and molecular expression among control, CUMS-induced depression mice, microRNA-15b-5p antagonir plus CUMS and microRNA-15b-5p agomir was made by One-way ANOVA. Animal experiments and data analysis were assigned to different investigators in a blinded way to ensure the unawareness of information about the group and manipulation. Statistical data were analyzed by using Origin (version 8; Northampton, MA, USA) and GraphPad Prism (version 7.00 ; La Jolla, California, USA) softwares.

**Data availability**

All data are contained within the manuscript.
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Abbreviations and nomenclature

The abbreviations used are:

AAV    Adeno-Associated Virus
CUMS   Chronic Unpredictable Mild Stress
FST    Forced Swimming Test
GABA   \gamma\text{-Aminobutyric Acid}
GAD    Glutamic Acid Decarboxylase
GFP    Green Fluorescent Protein
NAc    Nucleus Accumbens
qPCR   Quantitative Polymerase Chain Reaction
sEPSC  Spontaneous Excitatory Postsynaptic Current
STXB3A Syntaxin-Binding Protein 3
VAMP1  Vesicle-Associated Membrane Protein 1
YMT    Y-Maze Test
Figure Legends

**Figure 1** Chronic unpredictable mild stress (CUMS) leads the mice to express depression-like behavior. **A)** shows the procedures produced depression-like mice including the adaptation for 1 week, the CUMS for 3 weeks, and the behavioral tests in 6 days. **B)** illustrates immobile time of staying in the water cylinder in the forced swim test (FST) from control mice (hollow symbols, n=15) and CUMS-treated mice (red symbols, n=21). **C)** shows the sucrose preference test (SPT) values (%) from control mice (hollow symbols, n=15) and CUMS-treated mice (red symbols, n=21). **D)** illustrates the ratios of stay time in M-arm to stay time in 3 arms by the Y-maze test (YMT) from control mice (hollow symbols, n=15) and CUMS-treated mice (red symbols, n=21). Two asterisks show $P<0.01$ and three asterisks show $P<0.001$. One-way ANOVA was used for statistical comparisons between control mice and CUMS-treated mice. Paired- $t$ test was used for statistical comparisons before and after the CUMS.
Figure 2  Excitatory synaptic transmission is downregulated in GABAergic neurons of the nucleus accumbens from CUMS-induced depression-like mice. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded under voltage-clamp in the brain slices in the presence of 10 μM bicuculline. **A**) shows sEPSCs from a control mouse (left traces) and a CUMS-induced depression mouse (right traces), Calibration symbols: vertical, 20 pA; horizontal, 1 second (top) and 80 milliseconds (bottom) in horizontal. **B**) shows cumulative probability versus sEPSCs amplitudes from control group (hollow symbols, n=15 cells from 5 mice) and CUMS-induced depression group (red symbols, n=14 cells from 5 mice). Dash-lines indicate sEPSCs amplitudes at cumulative probability to 67% (CP_{67}). The inset shows a comparison of sEPSCs amplitudes at CP_{67} from control mice (hollow symbols) and CUMS-induced depression mice (red symbols). **C**) shows cumulative probability versus inter-sEPSCs intervals from control group (hollow symbols, n=15 cells from 5 mice) and CUMS-induced depression group (red symbols, n=14 cells from 5 mice). Dash-lines indicate sEPSCs intervals cumulative probability to 67% (CP_{67}). The inset shows a comparison of sEPSCs intervals at CP_{67} from control mice (hollow symbols) and CUMS-induced depression mice (red symbols). Three asterisks show P<0.001. One-way ANOVA was used for statistical comparisons between control mice and CUMS-induced depression-like mice.
**Figure 3** Excitatory synapse innervations from the mPFC to GABAergic neurons in the NAc are lowered in CUMS-induced depression-like mice. **A~B** illustrate innervations (yellow) from mPFC to GABAergic neurons (green) of NAc in control mice (3A) and CUMS-induced depression mice (3B). **C** shows the innervations from mPFC to GABAergic neurons of NAc in control group (hollow symbols, n=27 cells from 5 mice) and CUMS-induced depression group (red symbols, n=35 cells from 5 mice). Three asterisks show $P<0.001$. One-way ANOVA was used for statistical comparisons between control mice and CUMS-induced depression-like mice. Arrows indicate synaptic contacts between presynaptic boutons and GABAergic cell bodies.
Figure 4 Genes and proteins in relevance to the fusion of synaptic vesicles with presynaptic membrane for releasing transmitters are downregulated in the nucleus accumbens from CUMS-induced depression mice. The expression of mRNA VAMP1 and STXB3P3A were analyzed by quantitative RT-PCR. The expression of proteins VAMP1 and STXB3P3A were measured by western-blot. A–B) show the relative value of mRNAs VAMP1 (4A) and STXB3P3A (4B) in control mice (hollow symbols, n=6) and CUMS-induced depression mice (red symbols, n=6), in which internal control is β-actin. C) shows the expressions of VAMP1 and STXB3P3A from control mice and CUMS-induced depression mice, in which internal control is β-actin. D–E) illustrate the normalized ratios of VAMP1 (4D) and STXB3P3A (4E) to β-actin from control mice (hollow symbols, n=6) and CUMS-induced depression mice (red symbols, n=6). The relative ratios for control mice are normalized to be one. Two asterisks show $P<0.01$ and three
asterisks show $P<0.001$. One-way ANOVA was used for statistical comparisons between control mice and CUMS-induced depression-like mice.

**Figure 5** The miR-15b-5p targeted messenger RNAs (mRNAs) for synaptic vesicles fusion related proteins are validated by Luciferase reporter assay. (A-B) Luciferase reporter assay is performed by the co-transfection of luciferase reporter containing wild or mutant 3'-untranslated repeat (UTR) of vesicle-associated membrane protein 1 (VAMP1) and syntaxin binding protein 3a (STXBP3A) mRNA with miR-15b-5p mimic and negative control into HEK293T cells. Luciferase activity was determined 48h after co-transfection. $P<0.01$, $P<0.001$. 
Figure 6 Anti-microRNA-15b-5p rescues CUMS-induced depression-like behavior. A) illustrate immobile time in the forced swim test from control mice (hollow symbols, n=15), CUMS-induced depression mice (red symbols, n=21), microRNA-15b-5p antagonir-control plus CUMS mice (circled dot symbols, n=15) and microRNA-15b-5p antagonir-injection plus CUMS mice (blue symbols, n=18). B) shows values of the sucrose preference test (%) in control mice (hollow symbols, n=15), CUMS-induced depression mice (red symbols, n=21), microRNA-15b-5p antagonir-control plus CUMS mice (circled dot symbols, n=15) and microRNA-15b-5p antagonir-injection plus CUMS mice (blue symbols, n=18). C) illustrates the ratios of stay time in M-arm to stay time in three arms by the Y-maze test from control mice (hollow symbols, n=15), CUMS-induced depression mice (red symbols, n=21), microRNA-15b-5p antagonir-control plus CUMS mice (circled dot symbols, n=15) and microRNA-15b-5p antagonir-injection plus CUMS mice (blue symbols, n=18). An asterisk shows $P<0.05$, two asterisks show $P<0.01$ and three asterisks show $P<0.001$. One-way ANOVA was used for statistical comparisons among control mice, CUMS-induced depression mice, microRNA-15b-5p antagonir-control plus CUMS mice and microRNA-15b-5p antagonir-injection plus CUMS mice.
Figure 7 Anti-microRNA-15b-5p rescues excitatory synaptic transmission in GABAergic neurons of the nucleus accumbens impaired by CUMS. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded under voltage-clamp in the brain slices in the presence of 10 μM bicuculline. A) shows sEPSCs from a control mouse (top traces), a CUMS-induced depression mouse (middle traces) and a microRNA-15b-5p antagonir-injection plus CUMS mouse (bottom traces). Calibration symbols: vertical, 20 pA; horizontal, second (top) and 80 milliseconds (bottom) in horizontal. B) shows cumulative probability versus sEPSCs amplitudes from control group (hollow symbols, n=15 cells from 5 mice), CUMS-induced depression group (red symbols, n=14 cells from 5 mice) and microRNA-15b-5p antagonir-injection plus CUMS group (blue symbols, n=14 cells from 5 mice). Dash-lines indicate sEPSCs amplitudes at cumulative probability to 67% (CP67). The inset shows a comparison of sEPSCs amplitudes at CP67 from control mice (hollow symbols), CUMS-induced depression mice (red symbols) and microRNA-15b-5p antagonir-injection plus CUMS mice (blue symbols). C) shows cumulative probability versus intersEPSCs intervals from control group (hollow symbols, n=15 cells from 5 mice), CUMS-induced depression group (red symbols, n=14 cells from 5 mice) and microRNA-15b-5p antagonir-injection plus CUMS group (blue symbols, n=14 cells from 5 mice). Dash-lines indicate sEPSCs intervals cumulative probability to 67% (CP67). The inset shows a comparison of sEPSCs intervals at CP67 from control mice (hollow symbols), CUMS-induced depression-like mice (red symbols) and microRNA-15b-5p antagonir-injection plus CUMS mice (blue symbols). One asterisk shows $P<0.05$, two asterisks show $P<0.01$ and three asterisks show $P<0.001$. One-way ANOVA was used for statistical comparisons among control mice, CUMS-induced depression mice and microRNA-15b-5p antagonir-injection plus CUMS mice.
microRNA-15b-5p induces depression-like behavior

**Figure 8** The decrease of synapse innervations from the mPFC to GABAergic neurons in the NAc is rescued by anti-microRNA-15b-5p. **A–C** illustrate the innervations (yellow) from mPFC to GABAergic neurons (green) of the NAc in control mice (7A), CUMS-induced depression-like mice (7B) and microRNA-15b-5p antagonir-injection plus CUMS mice (7C). **D** shows the innervations from mPFC to GABAergic neurons of NAc from control group (hollow symbols, n=27 cells from 5 mice), CUMS-induced depression group mice (red symbols, n=35 cells from 5 mice) and microRNA-15b-5p antagonir-injection plus CUMS group (blue symbols, n=30 cells from 5 mice). Two asterisks show $P<0.01$ and three asterisks show $P<0.001$. One-way ANOVA was used for statistical comparisons among control mice, CUMS-induced depression mice and microRNA-15b-5p antagonir-injection plus CUMS mice. Arrows indicate synaptic contacts between presynaptic boutons and GABAergic cell bodies.
microRNA-15b-5p induces depression-like behavior

**Figure 9** Anti-microRNA-15b-5p rescues the decrease of VAMP1 and STXBP3A in the NAc induced by the CUMS. The expression of mRNAs *VAMP1* and *STXBP3A* were analyzed by quantitative RT-PCR. The expression of proteins VAMP1 and STXBP3A were measured by western-blot. A~B) show the relative value of mRNA for *VAMP1* (8A) and *STXBP3A* (8B) in control mice (hollow symbols, n=6), CUMS-induced depression mice (red symbols, n=6) and microRNA-15b-5p antagonir-injection plus
CUMS mice (blue symbols, n=6), in which internal control is β-actin. C) shows the expressions of VAMP1 and STXBP3A from control mice, CUMS-induced depression mice and microRNA-15b-5p agomir-injection plus CUMS mice, in which internal control is β-actin. D-E) show the normalized ratios of VAMP1 (8D) and STXBP3A (8E) expression to β-actin from control mice (hollow symbols, n=6), CUMS-induced depression mice (red symbols, n=6) and microRNA-15b-5p agomir-injection plus CUMS mice (blue symbols, n=6). The relative ratios for control mice are normalized to be one. An asterisk shows \( P < 0.05 \), two asterisks show \( P < 0.01 \) and three asterisks show \( P < 0.001 \). One-way ANOVA was used for statistical comparisons among control mice, CUMS-induced depression-like mice and microRNA-15b-5p agomir-injection plus CUMS mice.

**Figure 10** microRNA-15b-5p in the nucleus accumbens evokes depression-like behavior in mice similarly to those induced by the CUMS. A) illustrate the immobile time in the FST from control mice (hollow symbols, n=15), CUMS-induced depression mice (red symbols, n=21), microRNA-15b-5p agomir-control mice (circled saltire symbols, n=15) and microRNA-15b-5p agomir-injection mice (yellow symbols, n=20). B) shows the SPT values (%) from control mice (hollow symbols, n=15), CUMS-induced depression mice (red symbols, n=21), microRNA-15b-5p agomir-control mice (circled saltire symbols, n=15) and microRNA-15b-5p agomir-injection mice (yellow symbols, n=20). C) illustrates the ratios of stay time in M-arm to stay time in three arms by the YMT from control mice (hollow symbols, n=15), CUMS-induced depression mice (red symbols, n=21), microRNA-15b-5p agomir-control mice (circled saltire symbols, n=15) and microRNA-15b-5p agomir-injection mice (yellow symbols, n=20). Two asterisks show \( P < 0.01 \) and three asterisks show \( P < 0.001 \). One-way ANOVA was used for statistical comparisons among control mice, CUMS-induced depression mice, microRNA-15b-5p agomir-control mice and microRNA-15b-5p agomir-injection mice.
**Figure 11** microRNA-15b-5p impairs synaptic transmission in GABAergic neurons of the NAc similarly to that induced by the CUMS. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded under voltage-clamp in the brain slices in the presence of 10 μM bicuculline. **A** shows sEPSCs from a control mouse (top traces), a CUMS-induced depression mouse (middle traces) and a microRNA-15b-5p agomir-injection mouse (bottom traces). Calibration symbols: vertical symbols, 20 pA; horizontal, 1 second (top) and 80 milliseconds (bottom). **B** shows cumulative probability versus sEPSCs amplitudes from control group (hollow symbols, n=15 cells from 5 mice), CUMS-induced depression group (red symbols, n=14 cells from 5 mice) and microRNA-15b-5p agomir-injection group (yellow symbols, n=12 cells from 5 mice). Dash-lines indicate sEPSCs amplitudes at cumulative probability to 67% (CP67). The inset shows a comparison of sEPSCs amplitudes at CP67 from control mice (hollow symbols), CUMS-induced depression-like mice (red symbols) and microRNA-15b-5p agomir-injection mice (yellow symbols). **C** shows cumulative probability versus inter-sEPSCs intervals from control group (hollow symbols, n=15 cells from 5 mice), CUMS-induced depression group (red symbols, n=14 cells from 5 mice) and microRNA-15b-5p agomir-injection group (yellow symbols, n=12 cells from 5 mice). Dash-lines indicate sEPSCs intervals cumulative probability to 67% (CP67). The inset shows a comparison of sEPSCs intervals at CP67 from control mice (hollow symbols), CUMS-induced depression mice (red symbols) and microRNA-15b-5p agomir-injection mice (yellow symbols). Two asterisks show $P<0.01$ and three asterisks show $P<0.001$. One-way ANOVA was used for statistical comparisons among control mice, CUMS-induced depression mice and microRNA-15b-5p agomir-injection mice.
**Figure 12** microRNA-15b-5p attenuates synapse innervations similarly to that induced by the CUMS. A~C) show innervations (yellow) from mPFC to GABAergic neurons (green) of NAc in control mice (11A), CUMS-induced depression mice (11B) and microRNA-15b-5p agomir-injection mice (11C). D) shows the innervations from mPFC to GABAergic neurons of NAc in control group (hollow symbols, \( n=27 \) cells from 5 mice), CUMS-induced depression group (red symbols, \( n=35 \) cells from 5 mice) and microRNA-15b-5p agomir-injection group (yellow symbols, \( n=31 \) cells from 5 mice). Three asterisks show \( P<0.001 \). One-way ANOVA was used for statistical comparisons among control mice, CUMS-induced depression mice and microRNA-15b-5p agomir-injection mice.
**Figure 13** microRNA-15b-5p attenuates the expression of VAMP1 and STXBP3A similarly to that induced by the CUMS. The expression level of mRNA *VAMP1* and *STXBP3A* were analyzed by quantitative RT-PCR. The expression of proteins VAMP1 and STXBP3A were measured by western-blot. 

**A~B** show the relative value of mRNA for *VAMP1* (12A) and *STXBP3A* (12B) in control mice (hollow symbols, n=6), CUMS-induced depression mice (red symbols, n=6) and microRNA-15b-5p agomir-injection mice (yellow symbols, n=6), in which internal control is β-actin. 

**C** shows the expressions of VAMP1 and STXBP3A from control mice, CUMS-induced depression mice and microRNA-15b-5p agomir-injection mice, in which internal control is β-actin. 

**D~E** illustrate the normalized ratios of VAMP1 (12D) and STXBP3A (12E) expression to β-actin from control mice (hollow symbols, n=6),
CUMS-induced depression mice (red symbols, n=6) and microRNA-15b-5p agomir-injection mice (yellow symbols, n=6). The relative ratios for control mice are normalized to be one. An asterisk shows $P<0.05$, two asterisks show $P<0.01$ and three asterisks show $P<0.001$. One-way ANOVA was used for statistical comparisons among control mice, CUMS-induced depression mice and microRNA-15b-5p agomir-injection mice. Arrows indicate synaptic contacts between presynaptic boutons and GABAergic cell bodies.
microRNA-15b contributes to depression-like behavior in mice by affecting synaptic protein levels and function in the nucleus accumbens

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