miR-135a-5p mediates memory and synaptic impairments via the Rock2/Adducin1 signaling pathway in a mouse model of Alzheimer’s disease

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Aberrant regulation of microRNAs (miRNAs) has been implicated in the pathogenesis of Alzheimer’s disease (AD), but most abnormally expressed miRNAs found in AD are not regulated by synaptic activity. Here we report that dysfunction of miR-135a-5p/Rock2/Add1 results in memory/synaptic disorder in a mouse model of AD. miR-135a-5p levels are significantly reduced in excitatory hippocampal neurons of AD model mice. This decrease is tau dependent and mediated by Foxd3. Inhibition of miR-135a-5p leads to synaptic disorder and memory impairments. Furthermore, excess Rock2 levels caused by loss of miR-135a-5p plays an important role in the synaptic disorder of AD via phosphorylation of Ser726 on adducin 1 (Add1). Blocking the phosphorylation of Ser726 on Add1 with a membrane-permeable peptide effectively rescues the memory impairments in AD mice. Taken together, these findings demonstrate that synaptic-related miR-135a-5p mediates synaptic/memory deficits in AD via the Rock2/Add1 signaling pathway, illuminating a potential therapeutic strategy for AD.
Alzheimer’s disease (AD) is the most common neurodegenerative disorder in aged people with an increasing incidence with age in the population worldwide. The best characterized clinical manifestation of AD is progressive cognitive decline, which appears at the very early stages of AD, in the absence of the two pathological hallmarks: senile plaques and neurofibrillary tangles. Although the underlying mechanisms involved in the two pathological changes have been well studied, therapeutic strategies targeting these pathologies have failed. To date, there have been no approved drugs to block the decline in cognitive function in AD.

Synapses are considered the fundamental units in the brain, and synaptic activity can stimulate the maturation of mushroom-shaped spines and form new synapses such that synaptic strength can be adapted to environmental changes and in turn play an important role in learning and memory. In the brain of an individual with AD, both the disruption of synaptic activity and loss of synapses were found, especially at the early disease stage. Compared with senile plaques and neurofibrillary tangles, synaptic disorders, characterized by loss of synapses and decreased synaptic activity, have higher correlations with cognitive impairments in AD. Thus, understanding the underlying mechanisms of synaptic disorders will be beneficial to the development of therapeutic strategies for AD at an early stage.

MicroRNAs (miRNAs) are a group of small noncoding RNAs ~22 bp in length that silence targeted genes at the post-transcriptional level. miRNAs participate in many physiological processes and pathological pathways, including embryonic development, tumorigenesis, and cardiac diseases. The fine-tuning ability of miRNAs permits them to regulate the local translation of synaptic-associated proteins at synapses. In brain tissue or sera from individuals with AD, numerous miRNAs have been identified to be deregulated and proposed to be involved in the formation of senile plaques and neurofibrillary tangles. Recently, the abnormal regulation of miRNAs in the synaptic disorder of AD was also investigated. Some brain-enriched miRNAs, such as miR-124, miR-125b, and miR-132, were aberrantly expressed in AD brains and mediated impairments to synaptic plasticity. However, most of these miRNAs expression levels are not themselves regulated by synaptic activity. Thus, how these miRNAs contribute to synaptic disorders in AD remains elusive.

Here, we first screened for alterations in the levels of miRNAs associated with synaptic plasticity in the hippocampus of AD model mice and found that miR-135a-5p is abnormally down-regulated in excitatory pyramidal neurons. The loss of miR-135a-5p is tau dependent and mediated by the reduction of the transcriptional factor Forkhead box D3 (Foxd3). Artificial inhibition of miR-135a-5p results in synaptic disorder and memory impairments via activation of the Rho-associated coiled-coil containing protein kinase 2 (Rock2)/Adducin 1 (Add1) signaling pathway. Overexpressing miR-135a-5p or silencing Rock2 not only rescues dendritic spine maturation but also attenuates synaptic disorders and memory deficits in AD model mice. Finally, a peptide that can block the direct phosphorylation of Add1 exerts similar neuroprotective effects, which provides the basis for developing a novel therapeutic approach for AD.

Results
Alterations in synaptic activity-dependent miRNAs in the hippocampus of AD model mice. To understand the potential role of synaptic activity-associated miRNAs in the pathogenesis of AD, we first performed a systematic analysis of the expression profile of synaptic activity-related miRNAs in the hippocampus of AD model mice at different stages. We focused on 12 miRNAs related to synaptic plasticity as reported in previous literature and compared the expression of those miRNAs in the hippocampus of APP/PS1 AD model mice to that of wild-type mice at 9 months and 12 months. We found that at 12 months of age, the AD model mice showed apparent downregulation of miR-135a-5p and upregulation of miR-136-5p, miR-19a-3p, miR-125b-5p, and miR-26a-5p (Fig. 1a). However, at 9 months of age, only a decrease in miR-135a-5p and an increase in miR-125b-5p were detected (Fig. 1b). These data suggested that miR-135a-5p downregulation or miR-125b-5p upregulation may play an important role in the relatively early stages of AD. As the dysfunction of miR-125b-5p in AD has been well documented, we focused on the possible role of miR-135a-5p in the synaptic disorder of AD in this work.

The miR-135a-5p expression is abnormally reduced in the hippocampus of AD model mice and the human frontal cortex of patients with AD. We further examined changes in miR-135a-5p levels in the hippocampus of APP/PS1 mice of different ages. We found that the level of miR-135a-5p was not changed at 3 months in APP/PS1 mice but showed a decrease at 6 months (Fig. 1c). We also performed fluorescence in situ hybridization and detected that the decrease in miR-135a-5p expression is prominent in CaMKII-positive neurons (Fig. 1d, e) but not in GAD67-positive neurons (Supplementary Fig. 1a), suggesting that the loss of miR-135a-5p in AD mainly occurs in excitatory neurons. In the cultured primary neurons, the miR-135a-5p level was reduced upon overexpression of human Tau, APP, or PS1 (Fig. 1f, Supplementary Fig. 1b, c). However, in primary neurons from Tau knockout mice, only Tau overexpression could induce the reduction in miR-135a-5p (Supplementary Fig. 1d, e). In line with this, downregulation of miR-135a-5p was also detected in the hippocampus of P301S tau mice at 6 months (Supplementary Fig. 1f). Moreover, the extent of Tau expression was negatively correlated with miR-135a-5p levels in the hippocampus of AD model mice (Fig. 1g, h) but not the wild-type mice (Supplementary Fig. 1g, h). These data suggested that the loss of miR-135a-5p in AD is tau dependent, which leads to suppressed synaptic strength (Supplementary Fig. 1i–k). Finally, in the frontal cortex of patients with AD and AD mice, the level of miR-135a-5p is decreased when compared to age-matched control subjects (Fig. 1i, Supplementary Fig. 1l).

Loss of miR-135a-5p expression is mediated by the reduction of Foxd3. We then investigated why miR-135a-5p is downregulated in AD. Previous studies have shown that RNA degradation is increased in the brains of individuals with AD, and we first predicted that the loss of miR-135a-5p is caused by RNA degradation. To test this hypothesis, we examined the stability of miR-135a-5p by blocking new RNA synthesis with actinomycin D and measuring the loss of miR-135a-5p and U6 levels over a 12-h period in primary hippocampal neurons infected with adeno-associated virus (AAV) containing human Tau or empty vector (Fig. 2a, b). We found that the half-life of miR-135a-5p in Tau-overexpressing neurons was identical to that in control virus-infected neurons (Fig. 2b). Our data are reliable because the half-life of miR-132-3p in our system was approximately 4 h (Fig. 2a), which is consistent with a previous study. Thus, the suppression of miR-135a-5p by Tau overexpression was not caused by dysfunction in RNA degradation. Next, we considered whether the transcription of miR-135a-5p is suppressed in AD. To address this, we measured the levels of primary miR-135a-5p transcript (pri-miR-135a-1/2) in the nuclear fractions of primary hippocampal cultures with Tau overexpression. We found that pri-miR-135a-1 but not pri-miR-135a-2 was reduced in...
Fig. 1 Alteration of synaptic activity-associated miRNAs in the hippocampus of AD mice. a, b Fold changes of synaptic activity associated with microRNAs (miRNAs) in the hippocampus of 12-month-old (a) or 9-month-old (b) APP/PS1 and control mice (WT) (n = 4 for each group). c Relative expression of miR-135a-5p in the hippocampus of APP/PS1 and control mice (WT) at different months as indicated (n = 4 for each group, two-way ANOVA, p < 0.0001). d The distribution of miR-135a-5p in glutamatergic excitatory neurons in hippocampal slices of 9 months APP/PS1 and control mice (WT) by co-immunofluorescence experiments with the antibody of CamKII (Green) and FISH of miR-135a-5p (Red). The amplification images were shown at the bottom of each group. Scale bar = 20 μm (upper), 5 μm (lower) (n = 3 for each group). e Quantification of fluorescence intensity for miR-135a-5p in CamKII positive neurons from (d) (n = 11, 17 for WT, APP/PS1, p < 0.0001). f qRT-PCR was used to evaluate the expression of miR-135a-5p in the primary culture neurons at DIV 10 after overexpression of AAV-hTau (Tau) or control virus (Con) for 72 h (n = 3 for each group). g A representative immunofluorescence staining of FISH for miR-135a-5p (Red) and immunofluorescence experiments with the antibody of Tau-5 (Tau, Green) in hippocampus of 9 months APP/PS1 mice. Scale bar = 20 μm (left), 5 μm (right). h The fluorescence correlation of intensities of miR-135a-5p and Tau-5 in APP/PS1 mice as shown in panel (g) (n = 68 neurons from 5 mice). i The expression of miR-135a-5p in the frontal cortex from patients with AD and age-matched controls was measured by qRT-PCR (n = 4 for each group). (Data are presented as mean ± S.E.M. and two-tailed t tests were used unless otherwise specified. Source data are provided as a Source Data file. *p < 0.05, **p < 0.01, ***p < 0.001 vs. WT/Con).
Tau-overexpressing neurons (Fig. 2c). Consistent with that result, pri-miR-135a-1 was reduced in the hippocampus of APP/PS1 AD model mice from 6 months (Fig. 2d, Supplementary Fig. 2a, b). These data suggested that the transcription of pri-miR-135a-1 is inhibited, which possibly leads to the loss of miR-135a-5p in AD. To determine how this transcription was inhibited, we cloned different fragments (1 kb, 2 kb, 3 kb) of the promoter region of pri-miR-135a-1 and inserted them into the pGL3-reporter luciferase construct (Fig. 2e). We found that the construct containing the 1 kb promoter region exhibited the highest luciferase intensity (Fig. 2f). We further analyzed the potential transcriptional factor binding sites within the promoter region of pri-miR-135a-1.
Inhibition of miR-135a-5p induces synaptic disorder in vivo. Because hippocampal synaptic plasticity is believed to be the basis of learning memory 67, we then examined alterations in synaptic plasticity in S-135a-treated mice. We first evaluated hippocampal LTP in the CA3–CA1 circuit and found that the I–O curve in S-135a-infected mice was lower than that in control virus-infected mice (Fig. 4a), indicating that infection of S-135a altered basal synaptic transmission. In addition, S-135a-infected mice displayed impairment in LTP because the normalized fEPSP slope made during the search stages for the target hole than control mice (Fig. 3j–n). Finally, in the fear conditioning test, S-135a-infected mice showed a significant reduction in freezing time in the contextual conditioning paradigms, while only a slight difference was detected in tone conditioning (Fig. 3o, p), which relies on the ventral part of the hippocampus 55,56. Together, these results indicated that loss of miR-135a-5p expression induced Alzheimer-like learning and memory deficits in mice.

Loss of miR-135a-5p expression results in the activation of the Rock2 signaling pathway. It is known that miRNAs control gene expression by either inhibiting the translation or promoting the degradation of their target mRNAs 29. Based on this premise, we sought to identify the direct target of miR-135a-5p that mediates synaptic disorders in AD. We used TargetScan 40 and miRDB 31 databases to predict the potential targets of miR-135a-5p (Supplementary Fig. 4a, Supplementary Data 4 and 5) and chose candidates with a Pct over 0.6 or miRDB scores over 70. Then, we selected 194 common candidate genes and conducted a KEGG
analysis (Supplementary Fig. 4b). We found that 15 signal pathways were highly enriched, and the adjusted p value was less than 0.05 (Supplementary Table 1). Among the candidates in the 15 signal pathways, four of them (Rock1, Rock2, Cacna1d, and Pik3r2) were the most attractive because they exist in at least four pathways and are associated with actin cytoskeleton dynamics or calcium signaling. Given that Rock1 protein is mainly distributed in nonneuronal cells and showed no change in the hippocampus of 9-month-old APP/PS1 mice (Fig. 5a), we excluded the possible involvement of Rock1 in miR-135a-5p loss-induced memory/synaptic disorder though it plays an important role in actin remodeling and spine density. We also detected
alterations in ROCK2, voltage-dependent L-type calcium channel subunit alpha-1D isoform a (encoded by Cacna1d) and p85β (encoded by Pik3r2) in the hippocampus of APP/PS1 mice. We found that only ROCK2 expression was dramatically upregulated (Fig. 5a), which indicates that ROCK2 is the potential downstream target that mediates the synaptic disorder induced by the loss of miR-135a-5p expression.

To verify the direct regulation of ROCK2 by miR-135a-5p, we constructed the wild-type (WT) 3′UTR region of ROCK2 (NM_009072.2), which contains the miR-135a-5p binding site, and a 3′UTR variant with a mutation at the miR-135a-5p binding site; cloned them both in a luciferase reporter gene and cotransfected the reporter vectors into HEK293T cells with either miR-135a-5p mimic or scrambled control (Fig. 5b). We found that the miR-135a-5p mimic suppressed the luciferase intensity in cells transfected with the WT construct but not the mutated construct (Fig. 5c). Meanwhile, by transfecting the mimic and inhibitor of miR-135a-5p into Neuro2a cells, we found that the protein levels of ROCK2 were downregulated and upregulated, respectively, while mRNA levels were unaffected (Supplementary Fig. 4c, e). These data suggested the direct posttranscriptional regulation of ROCK2 by miR-135a-5p. More importantly, we detected a negative correlation of miR-135a-5p and ROCK2 in the hippocampus of APP/PS1 and C57 mice (Supplementary Fig. 4f, g).

Next, we explored the potential downstream effectors of ROCK2 activation in mediating synaptic disorders in AD. Considering the above data, ROCK2 activation may result in morphological changes in dendrites and dendritic spines, and LTD (Supplementary Fig. 8c-m). These data suggested that restoring the miR-135a-5p/ROCK2 signaling pathway rescues memory impairments and synaptic disorders in AD model mice. Finally, we tested whether blocking the phosphorylation of Add1 signaling pathway rescues memory impairments and synaptic disorders in AD model mice. According to our previous experience43, we selected Add1, Add2, and Crmp2 as candidates for further biological experiments. We found that in the hippocampus of APP/PS1 mice at 9 months, phosphorylation at Thr445 and Ser726 of Add1 and at Thr555 of Crmp2 were dramatically increased (Fig. 5d). However, in cultured hippocampal neurons, application of the miR-135a-5p inhibitor only markedly increased the phosphorylation of Add1 at Thr445 and Ser726, but not the Crmp2 at Thr555 (Fig. 5e), nor the phosphorylation of Limk1/2 (Supplementary Fig. 4h, i), two most important proteins downstream Rock regulating actin stabilization and remodeling37,38. Furthermore, in the frontal cortex of patients with AD and AD mice, the levels of phosphorylated Ser726-Add1 are increased, which are accompanied by an increase in ROCK2 expression (Fig. 5f, Supplementary Fig. 4j, k). Importantly, we detected a relatively strong negative correlation of miR-135a-5p and both ROCK2 and phospho-S726-Add1 but a positive correlation of ROCK2 and phospho-S726-Add1 (Fig. 5g-i). Thus, the loss of miR-135a-5p expression results in aberrant ROCK2 activation and the subsequent deregulation of the cellular cytoskeleton via phosphorylation of Add1 at Ser726.

Overexpression of miR-135a-5p or silencing of ROCK2 rescues memory impairments and synaptic disorders in AD model mice. We then asked whether upregulation of miR-135a-5p or silencing of ROCK2 could rescue memory impairments and synaptic disorders in AD model mice. We used 8-month-old APP/PS1 mice for the injection, because the memory impairment was apparent in 9 months but not 6 months mice39,40, and the agomir of miR-135a-5p only lasts up to 6 weeks41,42. Upon infusion of the agomir of miR-135a-5p or the lentivirus containing ROCK2-specific shRNA into the hippocampus of 8-month-old APP/PS1 mice (Supplementary Fig. 5a), we found that both agents were able to not only suppress ROCK2 expression and the phosphorylation of Add1 at Ser726 (Fig. 6a, Supplementary Fig. 5a) but also restore the density and maturation of dendritic spines (Fig. 6b–d, Supplementary Fig. 5b–d) and the complexity of neuronal dendrites (Fig. 6e–g, Supplementary Fig. 5e–g). Moreover, the LTP/LTD deficits (Fig. 6h, i, Supplementary Fig. 5h, i, Supplementary Fig. 6) and memory impairments in AD model mice could also be partially rescued (Fig. 6j–m, the original data was shown in Supplementary Fig. 7, Supplementary Fig. 5j–m). These data suggested that restoring the miR-135a-5p/ROCK2 signaling pathway rescues memory impairments and synaptic disorders in AD.

A peptide blocking the phosphorylation of Add1 rescues memory impairments and synaptic disorders in AD model mice. Finally, we tested whether blocking the phosphorylation of Add1 can rescue memory impairments and synaptic disorders in AD model mice. According to our previous experience43, we generated a series of peptides of different lengths that overlap the phosphorylation sites (Supplementary Fig. 8a). We found that only peptide 3 could attenuate the phosphorylation of Add1 in vitro (Fig. 7a, Supplementary Fig. 8b); thus, we named this peptide siP-Add1 and administered it to 9-month-old APP/PS1 or wild type mice at a dose of 15 mg/kg for 14 consecutive days via i.p. injection (Fig. 7b). We observed that siP-Add1 had no effect on WT mice, including the bodyweight, open field test, dendritic spines and trees, and LTD (Supplementary Fig. 8c–m). Our results showed reduced levels of phosphorylated S726-Add1 expression (Fig. 7f, Supplementary Fig. 4j, k). Importantly, we detected a relatively strong negative correlation of miR-135a-5p and both ROCK2 and phospho-S726-Add1 but a positive correlation of ROCK2 and phospho-S726-Add1 (Fig. 5g-i). Thus, the loss of miR-135a-5p expression results in aberrant ROCK2 activation and the subsequent deregulation of the cellular cytoskeleton via phosphorylation of Add1 at Ser726.

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in vivo (Fig. 7c). Moreover, siP-Add1 administration not only significantly restored the density of dendritic spines (Fig. 7d–f) and the complexity of neuronal dendrites (Fig. 7g–i) but also ameliorated LTP and LTD deficits (Fig. 7j, k, Supplementary Fig. 8k–m) and memory impairments (Fig. 7l–o) in AD model mice. Thus, blocking Rock2-mediated phosphorylation of Add1 effectively rescues AD-like synaptic and memory impairments in mice.
Discussion
In this study, we identified that miR-135a-5p, a synaptic-associated miRNA, is abnormally downregulated in AD. Loss of miR-135a-5p expression results in an increase in Rock2 activity and hyperphosphorylation of Add1 at Ser726, which in turn leads to dendritic abnormalities and memory impairments. Blocking the miR-135/Rock2/p-Add1 axis effectively rescued the AD-like synaptic and memory deficits in the APP/PS1 mouse model (Fig. 8).

Recently, we have demonstrated the critical roles of multiple miRNAs in mediating the synaptic disorders of AD, but most of them are not associated with synaptic activity. Here, we first screened the alterations in miRNAs associated with the synaptic disruptions of AD.
synaptic activity, and of those miRNAs, miR-135a-5p was of particular interest because it showed the most dramatic change in expression in the hippocampus of AD model mice at 9 months. Although the expression profile of miR-135a-5p in the hippocampus of patients with AD is not clear, some of the high throughput data suggested that miR-135a-5p was downregulated in the gray matter and serum of AD patients. miR-135a-5p is upregulated by long-term depression (LTD), a form of synaptic plasticity proposed as the primary cellular substrate of learning/memory. Our data demonstrated that the downregulation of miR-135a-5p in AD is tau dependent because the loss of miR-135a-5p expression in MAPT KO neurons can be induced by tau overexpression but not by either APP or PS1. In line with this, tau pathology (hyperphosphorylation of Tau) in the hippocampus and cortex of APP/PS1 mice occurs no earlier than 6 months, at which point miR-135a-5p levels began to decrease and the mice displayed significant synaptic disturbance. Soluble tau aggregates inhibit synaptic LTD in vivo, and Tau is required for the synaptic toxicity of Aβ. Therefore, tau pathology might play a critical role in mediating miR-135a-5p suppression in AD. Moreover, we also found no negative correlation of Tau with miR-135a-5p in normal mice, suggesting that the synergy of Aβ and Tau might be important to mediate the downregulation of miR-135a-5p in AD. In addition, we found that the decrease in miR-135a-5p expression is prominent in excitatory neurons and caused by a decrease in Fox3 levels in AD. Fox3 is a well-known transcriptional factor that is important for the development of the vertebrate nervous system, including the determination, migration, and differentiation of neural crest lineages. There are many synaptic-related (or brain-enriched) genes, including many miRNAs that are transcriptionally regulated by Fox3. Importantly, a study of genome-wide identification of the binding region of Tau in neurons found that an AG-rich motif was present within Tau-binding regions, and Tau acted as a repressor of genes related to Tau-binding. Meanwhile, we found that two of the variant AG-rich motif (GGAGGGAGAG, GGA-GAGAGAG) were present in the promoter region of Fox3 at −3807 to −3793 in the mouse genome. It is thus possible that Tau protein especially the pathological Tau could bind and repress the transcription of Fox3, resulting in a reduction of Fox3 protein. Consistently, the direct transcriptional regulation of miR-135a-5p by Fox3 was identified in our study based on a series of experiments and previous array data. Thus, the loss of miR-135a-5p expression in AD is Tau dependent and mediated by Fox3.

Numerous reports have revealed the crucial roles of miRNAs in controlling neuronal morphology and maintaining synaptic plasticity. An image-based miRNAome-wide functional miRNA screen in an induced neuronal differential cell model suggested that miR-135a-5p is the potent stimulator for developmental axon growth and branching, cortical neuronal migration, and axon regeneration. Intravitreal injection of miR-135a/b mimic promoted the axon regeneration of retinal ganglion cell (RGC) after optic nerve injury in adult mice. While miR-135a/b sponge further destroyed the axon regeneration of RGC. This was consistent with our result that miR-135a-5p promoted dendritic complexity of hippocampal pyramidal neurons. Administration of miR-135a inhibitors effectively blocked NMDA-induced spine retraction. These lines of evidence strongly suggest that miR-135a-5p deregulation is correlated with abnormalities in neuronal morphology. In this study, we found that the loss of miR-135a-5p expression results in a decrease in dendritic complexity and spine maturation, while overexpression of miR-135a-5p in vivo rescues those abnormalities. Moreover, loss of miR-135a-5p expression induces the reduction in LTP and basal transmission in the CA3–CA1 projection. This inhibition seems to be a postsynaptic process because we did not observe any differences in the PPF experiment. However, a recent study reported that overexpression of pre-miR-135a leads to a significant reduction in the number of spines in mouse primary hippocampal neurons. We propose that this discrepancy is due to the different circumstances between the in vitro and in vivo experiments, and the CMV or constitutively active mutant Me2-vp16 driven overexpression of pre-miR-135a would overwhelm the endogenous miRNA processing machinery and result in significant increases of both miR-135a-5p and miR-135a-1/2-3p, which is different from our findings here showing upregulation of miR-135a-5p alone. Similar to our research, Liu et al. reported decreased miR-135a-5p in the hippocampus of APP/PS1 mice and suggested that this is responsible for the overproduction of Aβ, the prime contributor to the synaptic disorder in AD. In another study, miR-135a-5p knockdown enhances the frequency of eEPSC events in BLA pyramidal neurons, suggesting a regulatory role of miR-135a-5p in presynaptic function in the amygdala. Here, we demonstrated the critical role of miR-135a-5p in regulating postsynaptic maturation in the hippocampus. Interestingly, targeted knockdown of miR-135a-5p in the amygdala results in anxiety-like behavior in mice. As anxiety is one of the most prevalent psychiatric manifestations in the early stage of AD, administration of miR-135a-5p might be a therapeutic approach for both emotional and cognitive disorders relating to AD. Besides, a series of important studies have demonstrated several other microRNAs, including miR-132-3p, were differentially expressed in response to LTP induction and regulated synaptic proteins relevant to persistent LTP.
the important roles of miRNA in the fine-tuning of gene regulation\(^6\), targeting these synaptic activity-regulated miRNAs might be promising therapeutic approaches for synaptic disorder-related neuropsychiatric diseases.

Our study further demonstrated the important role of Rock2-mediated hyperphosphorylation of Add1 in the synaptic/memory disorder caused by reduced miR-135a-5p expression in AD. It is well known that posttranscriptional regulation by miRNAs has pleiotropic effects, and it is reasonable to speculate that miR-135a-5p participates in synaptic/memory disorders by targeting different target mRNAs. A previous KEGG analysis in the rat hippocampus suggested that the predicted target genes of...
miR-135a-5p might be important for LTP and the synaptic vesicle cycle of glutamatergic synapses in AD68. Here, we pursued Rock2 as a direct target of miR-135a-5p based on a series of bioinformatics analyses and biochemical validation. The in vivo experiments further confirmed the remedial effects of silencing Rock2 on dendritic abnormalities and synaptic/memory deficits. Our study demonstrated that the miR-135a-5p/Rock2 signaling axis is important for actin cytoskeleton dynamics in dendrites and is consistent with a previous in vitro study69. Coincidentally, overexpression of miR-135a-5p results in the downregulation of Rock1 and Rock2 expression, which in turn suppresses the formation of dendritic spines in AD70. Therefore, miR-135a-5p/Rock2 signaling pathway in mediating synaptic disorders in AD. As decreased miR-135a-5p levels have been observed in the gray matter of patients with AD46 and Rock2 levels are increased in the earliest stages of AD and remain elevated throughout disease progression, our study proposed a therapeutic target for overcoming synaptic and memory deficits in AD.

Methods

Animals and cells. Male APP/PS1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, NO. 034832). Adult male C57BL/6 mice were purchased from the National Resource Center of Model Mice (Nanjing, China). The Tau-Knockout (Tau−/−) mice was generated by CRISPR–Cas9 system by specifically knocking out the sequence of exon 5 (Beijing Biocytogen Co., Ltd., Beijing, China). Genotyping for Tau−/− mice was performed by multiplex polymerase chain reaction using a pair of primers (Forward primer: 5′-GCTACAGTGTGAGTAGGAGTTCTAGC-3′; Reverse primer: 5′-GAACACAGCTGCTTAGGGGAAAAC-3′). The genetic background of Tau−/− mice is C57BL/6. All these mice and their nontransgenic littermates were bred in the Experimental Animal Central of Tongji Medical College, Huazhong University of Science and Technology. Mouse N2a cells and Human 293T cells were bought from the American Type Culture Collection (ATCC) bank (Manassas, VA, USA).

Plasmids and viruses. miR-135a mimic/inhibitor/agomir and the scrambled control were purchased from Ribobio (Guangzhou, China). Mimic and inhibitor was used for transfection on cells, and agomor was used for stereotaxic injection in mice. 3′UTR of Rock2 was amplified and cloned into psCHECK-2 (Promega, Madison, WI). The promoter sequence of miR-135a-5p was obtained from UCSC, and the 3′, 5′, and 3′ kb, 1 kb sequence immediately upstream to the transcription start site were cloned into pGL3-Basic vector. The coding sequences for the miRNAs of Fox3d, Gain-1, and Foxo1 were cloned into pdNA3.1(−) or pdNA3.1(−)3xFlag. Rock2 was cloned into a pcDNA4 vector. AAVs for miR-135a-5p, Adenovirus for Rock2 short hairpin RNA was purchased from OriBio Technology (Shanghai, China). The target sequence of Rock2 shRNA was 5′-AACAACTAGAGCTCAAGAAT-3′.

Morris water maze. The Morris water maze was performed as previously described45. Briefly, Marks of different shapes were pasted around the swimming
tank, the temperature of the water was kept between 20 and 22 °C and was made opaque with moderate titanium dioxide powder. The mice were trained for six consecutive days in the afternoon to find a platform hidden that submerged 1 cm below the water surface. Mice were trained from three different quadrants to find the platform for up to 60 s every day. Each mouse remained on the platform for 15 s if they found it or they were guided to the platform and stayed for 15 s. The movements of mice were recorded by a video camera connected to a computer. There was no task on the seventh day and mice got a rest. On the eighth day, the hidden platform was removed, mice were tested to find the platform from the opposite quadrant for 60 s (probe trial). The latency to reach the place of the platform, the percent time spent in the target quadrant, the crossing times to the platform regions, and the swimming velocity was recorded.
Fig. 7 A peptide blocking the phosphorylation of Add1 rescues the memory impairment and synaptic disorder in AD mice. a HEK293T cells were co-transfected with Add1 and Rock2 for 24 h. Then, the cells were treated with siP-Add1 at 0–20 μM as indicated for another 3 h. The phosphorylation level of Ser726-Add1 (S726-A1) was detected by western blotting. Upper panel, the representative blots; lower panel, the quantification analysis data (n = 4 for each group, Welch's one-way ANOVA, p = 0.0063, Dunnett's T3 post hoc p < 0.05, **p < 0.01 vs. 0 μM). b Schematic diagram for the experimental procedure. 9-month-old APP/PS1 mice were intraperitoneally injected with siP-Add1 or scrambled peptides (Scr) (15 mg/kg per day) for 2 weeks. Then mice were subjected to Morris water maze, electrophysiological recordings, western blotting, and Golgi staining. c Western blotting was used to analyze the expression of S726-Add1 in hippocampal homogenates from APP/PS1 injected with siP-Add1 or scrambled peptides (n = 4 for each group). d-f Golgi staining was used to examine the dendritic spines. The representative images for the dendritic spines in CA1 region (d) and quantitative analysis of the spine density (per 10 μm) (e) and percentage of the mushroom spine (f) (n = 30 neurons from 5 mice for each group, one-way ANOVA with Tukey's post hoc, p < 0.0001). g The representative images of dendritic trees in the hippocampal CA1 neurons. Bar = 20 μm. h-l The Sholl analysis (h) and Dendritic Complexity Index (DCI) analysis (l) were performed to evaluate the dendritic complexity (n = 30 neurons from 5 mice for each group, Welch's one-way ANOVA with Dunnett's T3 post hoc, p = 0.0001). j, k The electrophysiological recording was performed to examine the LTP in the CA3-CA1 projections. Representative traces and normalized IEPSP slopes were shown in (j). The quantitative analysis (k) was calculated from last 5 min recording (n = 9 slices for 4 mice for each group, one-way ANOVA, p < 0.0001, Tukey's post hoc, p < 0.0001). l-o The performances of mice in Morris water maze. Latencies in the learning stage from days 1–6 were recorded (l). The representative traces (m), first time to the platform region (n) and crossing times (o) at day 8 were analyzed and shown (n = 9 for each group, repeated measures two-way ANOVA with Tukey's post hoc, p < 0.0001 for (l), one-way ANOVA with Tukey's post hoc, p = 0.0082 for (n), p = 0.0106 for (o)). (Data are presented as mean ± S.E.M and two-tailed t tests were used unless otherwise specified. Source data are provided as a Source Data file. *p < 0.05, **p < 0.01, ***p < 0.001 A/P + Scr vs. WT, ##p < 0.01, ###p < 0.001 A/P + siP-Add1 vs. A/P + Scr).

Fig. 8 miR-135a-5p/Rock2/Add1 signal mediates the synaptic/memory impairments in AD. In normal conditions (left), miR-135a-5p was transcriptionally regulated by Fox3 and suppressed the Rock2 expression, which was essential for normal synaptic plasticity. In AD (right), loss of Fox3 results in the downregulation of miR-135a-5p and the overexpression of Rock2, which in turn caused the hyperphosphorylation of Add1 in the Ser726 site and the aberrant dendritic morphology, as well as the synaptic/memory impairments. Blocking the hyperphosphorylation of Add1 by siP-Add1 partially rescued those abnormalities.

Luciferase reporters assay. The pGL3 containing different promoter sequences of miR-135a was cotransfected into HEK293T cells with pRL-TK. And the vector containing 1 kb promoter of miR-135a was cotransfected with Fox3, Gata-1 or Fosos1 in addition to pRL-TK. The psiCHECK2 containing WT or mutated (Mut) 3'UTR of Rock2 was cotransfected into HEK293T with mimic or scramble of miR-135a. Cells were harvested after 48 h and cell lysates were collected with 1× PLB buffer for firefly and renilla luciferase activities using the dual-luciferase reporter assay system (Promega, USA) according to the manufacturer's protocol.

Primary hippocampal neuron culture. The isolated embryonic hippocampal neurons were cultured as previously described45. Briefly, pregnant mice between 15 and 17 days were anesthetized and hippocampi of fetal mice were isolated under a dissection microscope at 4 °C. The collected hippocampi were cut into small pieces with scissors, digested with 4 ml of 0.125% trypsin for 10–15 min, and stopped by adding 5 ml of the neuronal plating medium (10% fetal bovine serum in DMEM/F12). After centrifugation, resuspending, and trituration with the plating medium, the neurons were filtered with a 200-mesh sieve and plated onto a 60 mm plastic culture dish or 6-well cell culture plate coated with poly D-lysine. The neurons were incubated in the humidified incubator at 37 °C for 4 h with 5% CO2, and the medium was replaced with a Neurobasal medium supplemented with 2% B27 (maintenance medium). The medium was changed every 3 days for half volume.

Electrophysiological recording. The electrophysiological recording of hippocampal sections was performed as previously described45. Briefly, mice were decapitated and the brains were immediately immersed in ice-cold ACSF (125 mM NaCl, 2.0 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 26 mM NaHCO3, and 11 mM glucose), which was continuously bubbled with 95% O2 and 5% CO2. The mouse brain was cut into 300-μm-thick transverse slices in a horizontal plane with a vibrating microtome (Leica). Slices were preincubated in ACSF constantly with ACSF at 32 °C. I/O curves were obtained with an incremental standard high-frequency stimulation paradigm consisting of 2 trains of stimuli at 100 Hz (each for 1 s, pulse duration 200 μs) with 900 pulses for 15 min. For mEPSCs recordings, hippocampal neurons were held at −70 mV in voltage-clamp mode and were recorded in 10 s epochs for a total duration of at least 200 s per recording48. The data were collected and analyzed with pClamp 10 and Clampfit 10.2, respectively. Statistical analysis was performed by using the data from the last 5 min of recording.

Statistical analysis. All data are presented descriptively as the mean ± SEM and analyzed using SPSS 18.0 and Prism 6.0. The difference between the two groups was assessed using an unpaired Student’s t test. The variance among multiple groups was assessed by a one- or two-way analysis of variance with/without repeated measures followed by a post hoc test. All experiments were repeated three times except those specified, and p < 0.05 was considered statistically significant. All the statistical analysis data was supplied as Supplementary Table 4.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The source data for figures and supplementary figures of this paper are available in the source data file. Source data are provided with this paper.
Received: 25 June 2020; Accepted: 25 February 2021; Published online: 26 March 2021

References
1. Fratiglioni, L. & Qiu, C. Prevention of common neurodegenerative disorders in the elderly. Exp. Gerontol. 44, 46–50 (2009).
2. West, M. J., Kawas, C. H., Stewart, W. F., Budow, G. L. & Troncoso, J. C. Hippocampal neurons in pre-clinical Alzheimer’s disease. Neurobiol. Aging 25, 1205–1212 (2004).
3. Jessen, F. et al. A conceptual framework for research on subjective cognitive decline in preclinical Alzheimer’s disease. Alzheimers Dement. 10, 844–852 (2014).
4. Saneyoshi, T. et al. Activity-dependent synaptogenesis: regulation by a CaM-kinase kinase/CaM-kinase II/betaPIX signaling complex. Neuron 57, 94–107 (2008).
5. Ryan T. J., Roy D. S., Pignatelli M., Arons A., Tonegawa S. Memory. Engram 11, 228–234 (2009).
6. Banerjee, S., Neveu, P. & Kosik, K. S. A coordinated local translational control of neuroplasticity. Genes Brain Behav. 14, 100–112 (2015).
7. Winter, J., Jung, S., Keller, S., Gregory, R. I. & Diederichs, S. Many roads to synaptic plasticity: a review of functional microRNA targets. Nucleic Acids Res. 48, D127–D131 (2020).
8. Hashimoto, R. et al. Distribution of Rho-kinase in the bovine brain. Biochem. Biophys. Res. Commun. 263, 575–579 (1999).
9. Penzes, P., Buonanno, A., Passafaro, M., Sala, C. & Sweet, R. A. Developmental vulnerability of synapses and circuits associated with neuropsychiatric disorders. J. Neurochem. 126, 165–182 (2013).
10. Penzes, P. & Cahill, M. E. Deconstructing signal transduction pathways that regulate the actin cytoskeleton in dendritic spines. Cytoskeleton (Hoboken) 69, 426–442 (2011).
11. Amano, M. et al. Kinase-interacting substrate screening is a novel method to identify kinase substrates. J. Biol. Chem. 280, 895–912 (2015).
12. Xia, Q. et al. Phosphoproteomic analysis of human brain by calcium phosphate precipitation and mass spectrometry. J. Proteome Res. 7, 2845–2851 (2008).
13. Lin, T. et al. Rho-ROCK-LIMK-cofilin pathway regulates shear stress activation of steroid regulatory element binding proteins. Circ. Res. 92, 1296–1304 (2003).
14. Henderson, B. W. et al. Pharmacologic inhibition of LIMK1 provides dendritic spine resilience against beta-amyloid. Sci. Signal. 12, eaaw9318 (2019).
15. Chen, S. Q. et al. Age-related changes in brain metabolites and cognitive function in APP/PS1 transgenic mice. Behav. Brain Res. 235, 1–6 (2012).
16. Minkeviciene, R. et al. Age-related decrease in stimulated glutamate release and vesicular glutamate transporters in APP/PS1 transgenic and wild-type mice. J. Neurochem. 105, 584–594 (2008).
17. Landgraf, D. et al. Genetic disruption of circadian rhythms in the suprachiasmatic nucleus causes helplessness, behavioral despair, and anxiety-like behavior in mice. Biol. Psychiatry 80, 827–835 (2016).
18. Li, H. et al. A novel microRNA targeting HDAC3 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. J. Clin. Investig. 119, 3666–3677 (2009).
19. Su, Y. et al. MicroRNA-26a-death-associated protein kinase 1 signaling induces synucleinopathy and dopaminergic neuron degeneration in Parkinson’s disease. Biol. Psychiatry 85, 769–781 (2019).
20. Wang, X. et al. A novel MicroRNA-124-PTPN1 signal pathway mediates synaptic and memory deficits in Alzheimer’s disease. Biol. Psychiatry 83, 395–405 (2018).
21. Liu, D. et al. Targeting the HDAC2/HNF-4A/miR-101b/AMPK pathway rescues tauopathy and dendritic abnormalities in Alzheimer’s disease. Mol. Neurobiol. 55, 752–764 (2017).
22. Wang, X. W., Huang, Q., Hu, Y., Stromberg, A. J. & Nelson, P. T. Patterns of microRNA expression in normal and early Alzheimer’s disease temporal lobe neocortex. Brain, behavior and disease 22, 121 (2010).
23. Liu, G. et al. The FOXD3/miR-214/MED19 axis suppresses tumour growth and metastasis in human colorectal cancer. Br. J. Cancer 115, 1367–1378 (2016).
56. Xiang, J., Wen, F., Zhang, L. & Zhou, Y. FOXD3 inhibits SCN2A gene transcription in intractable epilepsy cell models. Exp. Neurol. 302, 14–21 (2018).
57. Benhelli-Mokrani, H. et al. Genome-wide identification of genic and intergenic neuronal DNA regions bound by Tau under physiological and stress conditions. Nucleic Acids Res. 46, 11405–11422 (2018).
58. Siegel, G., Saba, R. & Schratt, G. microRNAs in neurons: manifold regulatory roles at the synapse. Curr. Opin. Genet. Dev. 21, 491–497 (2011).
59. van Battum, E. Y. et al. An image-based miRNA screen identifies miRNA-135s as regulators of CNS axon growth and regeneration by targeting Kruppel-like factor 4. J. Neurosci. 38, 613–630 (2018).
60. Vangoor, V. R. et al. Antagonizing increased miR-135a levels at the chronic stage of experimental TLE reduces spontaneous recurrent seizures. J. Neurosci. 39, 50–59 (2019).
61. Chen, Y., Gao, D. Y. & Huang, L. In vivo delivery of miRNAs for cancer therapy: challenges and strategies. Adv. Drug Deliv. Rev. 81, 128–141 (2015).
62. Borel, F., Kay, M. A. & Mueller, C. Recombinant AAV as a platform for translating the therapeutic potential of RNA interference. Mol. Ther. 22, 692–701 (2014).
63. Liu, C. G. et al. MicroRNA-135a and -200b, potential Biomarkers for Alzheimer’s disease, regulate β secretase and amyloid precursor protein. Brain Res. 1583, 55–64 (2014).
64. Mannironi, C. et al. miR-135a regulates synaptic transmission and anxiety-like behavior in amygdala. Mol. Neurobiol. 55, 3301–3315 (2018).
65. Ryan, B., Logan, B. J., Abraham, W. C. & Williams, J. M. MicroRNAs, miR-23a-3p and miR-151-3p, are regulated in dentate gyrus neuropil following induction of long-term potentiation in vivo. PLoS ONE 12, e0170407 (2017).
66. Ryan, B. & Williams, J. M. Novel microRNA revealed by systematic analysis of the microRNA transcriptome in dentate gyrus granule cells. Neurosci. Lett. 707, 132280 (2019).
67. Ryan, B., Joachim, G. & Williams, J. M. Plasticity-related miRNA and their potential contribution to the maintenance of long-term potentiation. Front. Mol. Neurosci. 8, 4 (2015).
68. Chen, C. L., Liu, H. & Guan, X. Changes in microRNA expression profile in hippocampus during the acquisition and extinction of cocaine-induced conditioned place preference in rats. J. Biomed. Sci. 20, 96 (2013).
69. Swanger, S. A., Mattheyes, A. L., Gentry, E. G. & Herskowitz, I. JH. ROCK1 and ROCK2 inhibition alters dendritic spine morphology in hippocampal neurons. Cell. Logist. 5, e113266 (2015).
70. Kroiss, A. et al. Androgen-regulated microRNA-135a decreases prostate cancer cell migration and invasion through downregulating ROCK1 and ROCK2. Oncogene 34, 2846–2855 (2015).
71. Wishart, T. M. et al. Combining comparative proteomics and molecular genetics uncovers regulators of synaptic and axonal stability and degeneration in vivo. PLoS Genet. 8, e1002936 (2012).
72. Zhou, Z., Meng, Y., Asrar, S., Todorovski, Z. & Jia, Z. A critical role of Rho kinase ROCK2 in the regulation of spine and synaptic function. Neuropharmacology 56, 81–89 (2009).
73. Feng, Y., LoGrasso, P. V., Defert, O. & Li, R. Rho Kinase (ROCK) inhibitors and their therapeutic potential. J. Med. Chem. 59, 2269–2300 (2016).
74. Shaw, A. E. & Bamburg, J. R. Peptide regulation of coflin activity in the CNS: a novel therapeutic approach for treatment of multiple neurological disorders. Pharm. Ther. 175, 17–27 (2017).
75. Ji, S. P. et al. Disruption of PTEN coupling with 5-HT2C receptors suppresses behavioral responses induced by drugs of abuse. Nat. Med. 12, 324–329 (2006).
76. Ma, M. et al. A novel pathway regulates social hierarchy via lncRNA AtiLAS and postsynaptic synapsin IIIb. Cell Res. 30, 105–118 (2020).