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·Original Article·

MicroRNA-98 induces an Alzheimer's disease-like disturbance by targeting insulin-like growth factor 1

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by extracellular senile plaques and intracellular neurofibrillary tangles. Many microRNAs (miRs) participate in regulating amyloid β (Aβ) formation and the metabolism of tau protein in the process of AD, and some are up-regulated in AD patients or transgenic models of AD. However, the role of miR-98 in AD remains unclear. Here, we showed that the expression of miR-98 was negatively correlated with the insulin-like growth factor 1 (IGF-1) protein level in APP/PS1 mice. MiR-98 target sites in IGF-1 were confirmed by luciferase assay in HEK293 cells. Overexpression of miR-98 in N2a/APP cells down-regulated the IGF-1 protein level and promoted Aβ production, whereas inhibition of miR-98 in N2a/APP cells up-regulated the IGF-1 protein level and suppressed Aβ production. Furthermore, overexpression of miR-98 in N2a/WT cells increased the phosphorylation of tau, whereas inhibition of miR-98 reduced it. These results suggest that miR-98 increases Aβ formation and tau phosphorylation by inhibiting the translation of IGF-1, which might provide a therapeutic strategy for AD.

Keywords: Alzheimer's disease; microRNA; miR-98; IGF-1

INTRODUCTION

Alzheimer's disease (AD) is one of the most common chronic neurodegenerative disorders, and is characterized clinically by progressive memory loss and pathologically by the formation of extracellular senile plaques and intracellular neurofibrillary tangles (NFTs). Senile plaques are formed by accumulated amyloid β (Aβ) and NFTs are attributed to hyperphosphorylated tau protein[1].

Insulin-like growth factor 1 (IGF-1), the IGF-1 receptor, and IGF-1-binding proteins are widely expressed in the rodent and human brains[2]. Emerging evidence has revealed the essential roles of IGF-1 in the brain, including its contribution to metabolism, neuromodulation, neuroendocrine secretion, and cognitive functions[3]. Besides, an age-related decline of IGF-1[4] and an association between deranged IGF-1-mediated signaling and AD have been reported. Further, IGF-1 accelerates Aβ clearance from the brain and reduces tau phosphorylation by inhibiting glycogen synthase kinase 3[5,6].

MicroRNAs (miRs) are non-coding RNAs of ~22 nucleotides and are widely distributed in eukaryotic cells. miRs regulate gene expression *via* the repression of translation or the induction of mRNA degradation by base-pairing with the 3'-untranslated regions (3'-UTRs) of their target mRNAs[7]. Many brain-enriched or brain-specific miRs have been identified and shown to play important roles in the regulation of energy metabolism, synaptic plasticity, and memory formation[8]. *In silico* studies of miR

expression profiles in AD patients or transgenic AD models have been reported by several groups, and many miRs are known to be involved in Aβ production and clearance as well as in tauopathy[9].

APP/PS1 double-transgenic mice overexpressing the amyloid precursor protein (APP) with the Swedish mutation and presenilin-1 with deletion of exon 9 exhibit early Aβ deposition accompanied by cognitive decline, corresponding to a form of early-onset AD. Several miRs, including miR-34a and miR-98, are up-regulated in APP/PS1 mice compared to control mice[10].

MiR-98 regulates Fas and Fas-mediated apoptosis, as well as the production of interleukin 6 (IL-6) and IL-10[11]. MiR-98 dysfunction has been found in several carcinomas[12]. However, its role in AD is not clear. In this study, we explored the role of miR-98 in the processes of Aβ production and tau phosphorylation.

MATERIALS AND METHODS

Animals

APP/PS1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). They were kept at 22°C under a 12 h:12 h light/dark cycle. All animal experiments were performed using protocols approved by the Animal Care and Use Committee of our institution.

Cell Lines and Cell Culture

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). N2a/WT and N2a/APP cells were a gift from Dr. Yuheng Wang (School of Basic Medical Sciences, Zhejiang University, Hangzhou, China). N2a/WT cells were cultured in medium containing 50% DMEM, 50% Opti-MEM, and 5% FBS. Stably-transfected N2a/APP cells were selected using 0.2 g/L G418 (Invitrogen, Carlsbad, CA).

Plasmids

The miR-98 overexpression plasmid and the control vector were from GeneCopoeia (Rockville, MD). The IGF-1 short-hairpin RNA (shRNA) and control shRNA plasmids were from GenePharm (Shanghai, China).

RNA Extraction and Real-time Quantitative PCR (qPCR)

Total RNA was isolated from mouse hippocampus samples or cells using the TRIzol reagent (Invitrogen). RNA was reverse-transcribed into cDNA using reverse transcriptase (Tiangen, Beijing, China). MiRs were prepared using a microRNA extraction kit (Tiangen). The expression of miR-98 was normalized to that of U6 snRNA. The expression of IGF-1 was normalized to that of β-actin.

3'-UTR Luciferase Reporter Assays

The sequence encoding the precursor of miR-98 (pre-miR-98: MI0000586) was inserted into the pEZX-MR01 vector (GeneCopoeia). The wild-type or mutated mouse IGF 3'-UTR sequence containing the possible binding sites, nucleotides 1380–1402 of the IGF-1 3'-UTR, was amplified and cloned into the psiCHECK-2 vector (Promega, Madison, WI). The inhibitor of miR-98 was from RiboBio (Guangzhou, China). HEK293 cells were transfected with a mixture of 0.02 μg psiCHECK-2-IGF-1 and 0.08 μg pEZX-MR01-pre-miR-98. *Renilla* luciferase or the pEZX-MR01 vector was used as a negative control. *Renilla* and firefly luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega).

Western Blot Analysis

Total cells or tissue lysates were prepared and subjected to 15% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and blotted as described previously[8].

Enzyme-linked Immunosorbent (ELISA) Assay

Aβ42 levels in cell lysates were quantified using sandwich ELISA, as described previously[8] and according to the instructions of the Aβ42 mouse ELISA kit from Abcam (Cambridge, United Kingdom).

Statistical Analysis

Data are presented as mean ± SD. Statistical significance was determined using Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

IGF-1 Protein Is Significantly Down-regulated in 6-month-old APP/PS1 Mice

Consistent with a previous report[10], miR-98 expression was increased in 6-month-old APP/PS1 mice (Fig. 1A). MiR-7d/e, members of the miR-7 family, were also detected using qPCR (Fig. 1A). Since IGF-1 was predicted to be a target of miR-98 by bioinformatics, we also measured

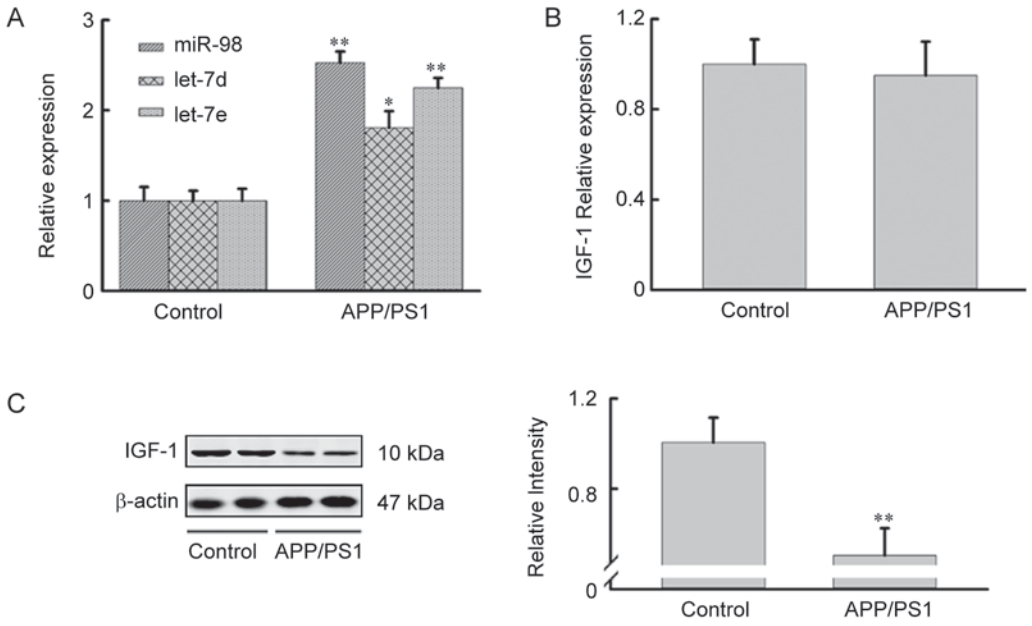

Fig. 1. IGF-1 is significantly down-regulated in 6-month-old APP/PS1 mice. A, B: Real-time quantitative PCR analysis of miR-98, let-7d, and let-7e (A) and IGF-1 (B) in 6-month-old control and APP/PS1 mice. C: Western blot analysis of the IGF-1 levels in hippocampal extracts from 6-month-old control and APP/PS1 mice. Data are from 3 separate experiments. The error bars represent standard deviations. ****P* **<0.05, *****P* **<0.01 ***versus*** **control mice, Student's** ***t*****-test.**

IGF-1 mRNA levels using qPCR, and found no significant difference between APP/PS1 and control mice (Fig. 1B). However, the protein level of IGF-1 was significantly lower in APP/PS1 mice than in control mice (Fig. 1C), suggesting the post-transcriptional regulation of IGF-1 expression in APP/PS1 mice.

IGF-1 Is A Direct Target of miR-98

To further confirm the connection between IGF-1 and its regulators in APP/PS1 mice compared with control mice, we combined Pictar, miRbase, and Targetscan to screen for potential microRNA-binding sequences. One binding site of miR-98 was found within the 3'-UTR of IGF-1 (Fig. 2A). A luciferase reporter construct containing the binding site (WT-3'-UTR) or the corresponding mutated site within the 3'-UTR of IGF-1 (Mut-3'-UTR) was generated and co-transfected with the miR-98 overexpression construct or its control vector into HEK293 cells. The luciferase activity was significantly inhibited in cells co-transfected with miR-98 and WT-3'-UTR compared with the control vector group, whereas Mut-3'-UTR luciferase activity changed only slightly (Fig. 2B). These results suggested that miR-98

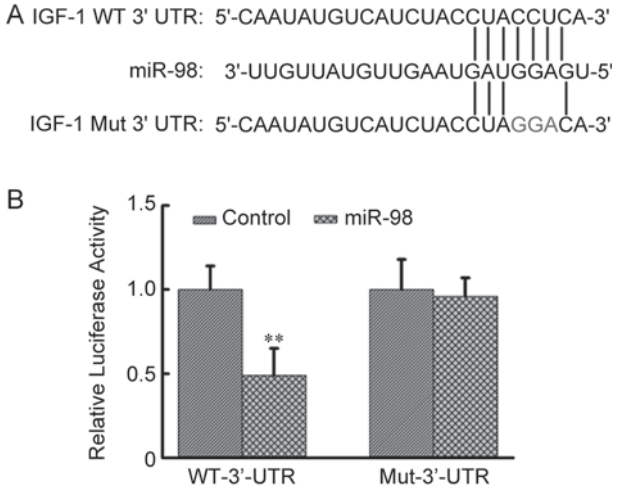

Fig. 2. IGF-1 is a direct target of miR-98. A: Schematic diagrams of binding of miR-98 with wide-type (WT) IGF-1 at the 3'-UTR, and the mutated (Mut) IGF-1 sequences. B: Dual-luciferase assays performed in HEK293 cells co-transfected with the reporter construct containing the 3'-UTR of the WT or mutated IGF-1 and the miR-98 overexpression plasmid or the control vector. Relative ***Renilla*** **luciferase activity was normalized to firefly luciferase activity. Data are from 3 separate experiments (Student's** ***t*****-test, *****P* **<0.01** ***versus*** **the IGF-1 WT 3'-UTR + control vector).**

inhibits IGF-1 expression posttranscriptionally.

MiR-98 Inhibits IGF-1 Translation

To directly validate whether miR-98 reduces the level of endogenous IGF-1, we transfected miR-98 into N2a/WT cells and found that the IGF-1 protein level was significantly down-regulated compared with that in N2a/WT cells transfected with control vector (Fig. 3A). In contrast, treatment with the inhibitor of miR-98 for 48 h led to a significant increase in the level of the endogenous IGF-1 protein (Fig. 3B). However, the levels of IGF-1 mRNA did not vary significantly among these experiments, suggesting that a post-transcriptional mechanism is involved in the inhibition of IGF-1 translation.

MiR-98 Increases Aβ Production and Tau Phosphorylation by Targeting IGF-1

To investigate the role of miR-98 in AD, we next used ELISA to measure the level of Aβ42 in lysates of N2a/APP cells that stably expressed human APP. The level of Aβ42 in N2a/APP cell lysates was increased significantly after transfection with miR-98, whereas 50 ng/mL IGF-1

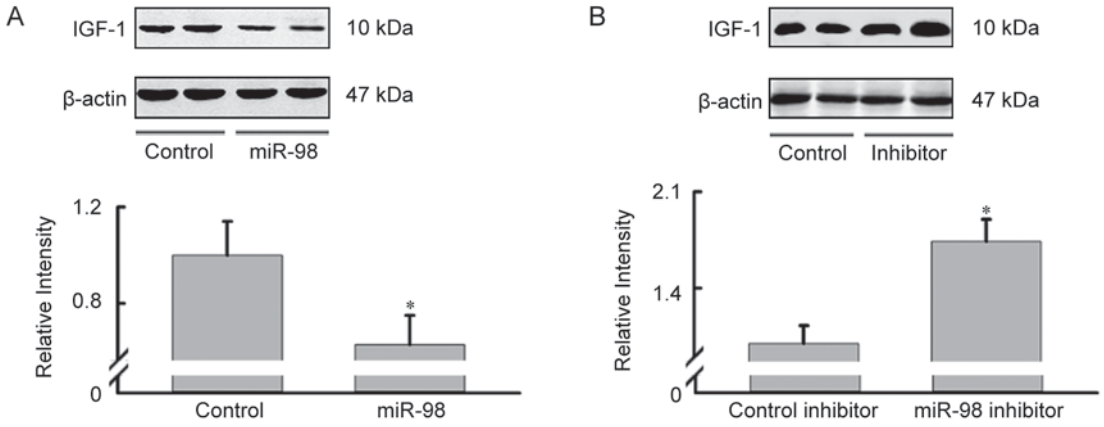

Fig. 3 MiR-98 inhibits IGF-1 translation. A: N2a/WT cells were transfected with miR-98 or the control vector for 48 h. The intensity of the IGF-1 bands was normalized to β-actin intensity. B: N2a/WT cells were transfected with a control inhibitor or the miR-98 inhibitor for 48 h. The intensity of the IGF-1 bands was normalized to the β-actin intensity (Student's *t*-test, **P* <0.05 *versus* the control group).

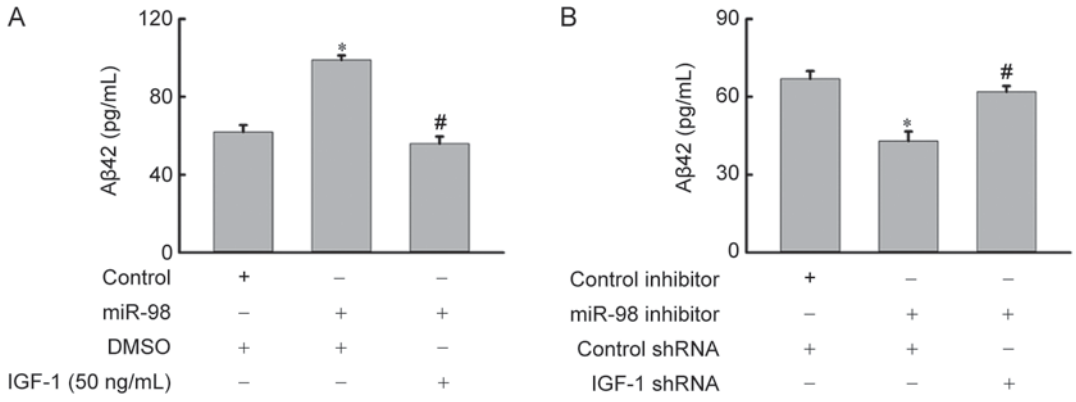

Fig. 4. MiR-98 increases Aβ production by targeting IGF-1. A: N2a/APP cells were transfected with miR-98 or the control vector and incubated with DMSO or 50 ng/mL IGF-1 for 48 h. Then the Aβ levels were measured using ELISA. B: N2a/APP cells were co-transfected with the miR-98 inhibitor or the control inhibitor and IGF-1 shRNA or the control shRNA for 48 h; the levels of Aβ were measured using ELISA. Data are from 3 separate experiments (Student's *t*-test, **P* <0.05 *versus* the first group, #*P* <0.05 *versus* the second group).

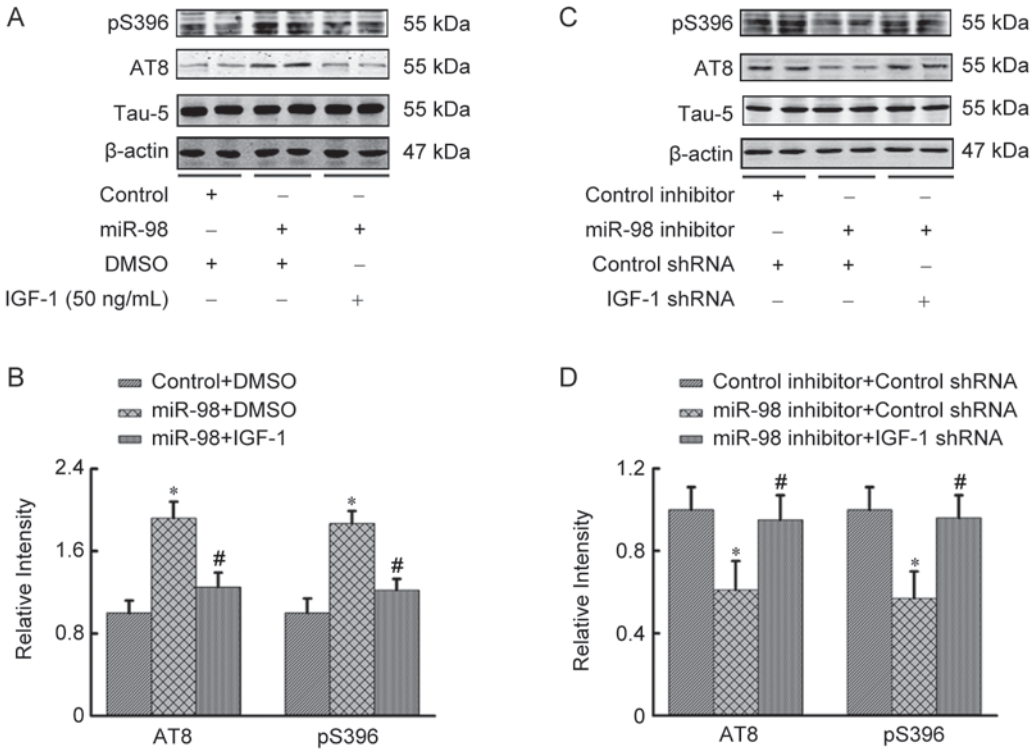

Fig. 5. MiR-98 increases tau phosphorylation by targeting IGF-1. A: N2a/WT cells were transfected with miR-98 or the control vector and incubated with DMSO or 50 ng/mL IGF-1 for 48 h. Tau phosphorylation was detected using pS396 and AT8 (which recognizes pSer202/Thr205). Tau-5 was used to detect the total levels of tau. B: N2a/WT cells were co-transfected with the miR-98 inhibitor or the control inhibitor and the IGF-1 shRNA or the control shRNA for 48 h; tau phosphorylation was detected. Data are from 3 separate experiments (Student's *t*-test, $^{*}P$ <0.05 *versus* the first group, $^{\#}P$ <0.05 *versus* the second group).

supplementation reversed this up-regulation (Fig. 4A). Conversely, inhibition of miR-98 reduced the level of Aβ42 in the lysates of N2a/APP compared with control transfected N2a/APP cells (Fig. 4B). Similarly, miR-98 vector transfection in N2a/WT cells increased tau phosphorylation at several sites, while 50 ng/mL IGF-1 application reversed these increases (Fig. 5A, B). Besides, inhibition of miR-98 reduced the tau phosphorylation (Fig. 5C, D), while IGF-1 shRNA reversed the decrease. Taken together, these data indicated that miR-98 negatively regulates IGF-1 translation and induces Aβ production and tau phosphorylation *in vitro*.

DISCUSSION

AD is the most common type of dementia in the elderly. It is characterized by impaired higher brain functions, including memory, thinking, and personality. AD is pathologically characterized by synaptic impairment, accumulation of NFTs, and Aβ deposition[13]. IGF-1 is decreased in AD patients and transgenic models of AD, and its therapeutic value has been described in several animal models[14,15].

Emerging evidence indicates that IGF-1 has a direct effect on Aβ metabolism and clearance[16]. Carro *et al.* demonstrated that the administration of IGF-1 to aged rats by chronic subcutaneous infusion reduces the level of Aβ in the brain parenchyma and increases the level of Aβ in the cerebrospinal fluid[17]. IGF-1 accelerates Aβ clearance from the cerebral parenchyma. Similarly, IGF-1 decreases the level of endogenous Aβ in Tg2576 transgenic mice, a model of AD amyloidosis. Treatment with IGF-1 remarkably reduces the immunoreactivity of Aβ in 1-year-old Tg2576 mice[18]. In another animal model of AD (APP/PS2 mice) in which impaired hippocampal-dependent cognition is accompanied by the early formation of Aβ plaques, systemic IGF-I administration for 3 months improves spatial

learning and memory and decreases the total brain Aβ load[17].

IGF-1 also influences the development of NFTs[19]. In AD, the hyperphosphorylation of tau impairs its function of promoting microtubule stabilization and assembly. Hyperphosphorylated tau dissociates from microtubules, forms abnormal filaments, and aggregates as NFTs. IGF-1 reduces the phosphorylation of tau at multiple sites. In addition, IGF-1 promotes the binding of tau to microtubules in cultured human neuronal cells[5]. In insulin receptor substrate 2-knockout mice, disruption of the IGF-1 signaling pathway increases tau phosphorylation in the brain[20]. Taken together, IGF-1 plays an essential role in the regulation of tau phosphorylation in neurons, suggesting a direct effect of IGF-1 on NFT pathology. However, IGF-1 might also reduce tau phosphorylation indirectly *via* its effect on Aβ, as described above, because Aβ may increase tau phosphorylation and the formation of filaments.

MiRs regulate gene expression by repressing mRNA translation or inducing mRNA degradation *via* the recognition of base-pairing sites located in the 3'-UTR of their target mRNAs. MiRs play important roles in various cellular processes, such as tissue morphogenesis, energy metabolism, neurogenesis, and major signaling pathways[21,22]. Compelling evidence indicates the presence of a direct link between miRs and AD[23,24]. Studies of miR expression profiles have identified numerous miRs that are involved in AD. Many of these miRs regulate Aβ formation by targeting enzymes involved in the Aβ production process or other molecules indirectly[8,25]. IGF-1 is negatively regulated by some miRs, including miR-1, miR-206, and miR-320, in many physiological processes[26,27].

MiR-98 is involved in the regulation of Fas and Fas-mediated apoptosis[28]. In addition, it inhibits the TP53 pathway and regulates cisplatin-induced cell death[29]. MiR-98 also plays essential roles in the immune system by targeting several interleukins[11]. However, its role in the central nervous system is not clear. Here, we demonstrated that miR-98 inhibited IGF-1 translation *in vitro* and increased Aβ formation and tau phosphorylation by down-regulating IGF-1. As miR-98 is widely involved in the regulation of synaptic plasticity, IL-6 and IL-10 production, and apoptosis, other molecules participating in the regulation of Aβ formation and tau phosphorylation might be regulated by miR-98,.

In conclusion, we showed that miR-98 negatively regulated the IGF-1 protein level *in vitro*. Because IGF-1 plays a critical role in Aβ formation and tau phosphorylation, increasing IGF-1 expression is suggested as a potential therapeutic strategy for AD. Our results suggest that miR-98 is an potential therapeutic target for the down-regulation of Aβ formation and tau phosphorylation. However, further studies *in vivo* are needed to address the delivery of the miR-98 inhibitor into the mouse hippocampus using adenoviruses to determine whether this can up-regulate IGF-1 protein levels and reduce Aβ formation.

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