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Journal
Journal of Alzheimer's Disease, 42(4)

ISSN
1387-2877

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Publication Date
2014

DOI
10.3233/jad-140204

Peer reviewed

Published in final edited form as:

J Alzheimers Dis. 2014 ; 42(4): 1229–1238. doi:10.3233/JAD-140204.

Up-regulation of miR-181 decreases c-Fos and SIRT-1 in the hippocampus of 3xTg-AD mice

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Abstract

MicroRNAs are a group of small RNAs that regulate diverse cellular processes including neuronal function. Recent studies have shown that dysregulation of specific microRNAs is critically involved in the development of Alzheimer's disease (AD). Most of these reports have focused on microRNAs implicated in alterations of Aβ and tau. However, studies exploring the relation between microRNAs dysregulation in AD and synaptic plasticity are scarce despite the well-known involvement of microRNAs in synaptic plasticity. Since impairments in synaptic plasticity and neuronal loss are two important features displayed in AD patients, it is feasible to hypothesize that alterations in plasticity-related microRNAs underlie AD progression. Here, levels of a small number of microRNAs implicated in normal neuronal function and/or plasticity were examined in an AD model. Twelve-month old 3xTg-AD mice with plaques and tangles presented a significant up-regulation of miR-181 in the hippocampus compared to age-matched wild type mice. Increased miR-181 was not detected in pre-pathological 3xTg-AD mice. Analysis of predicted targets of miR-181 identified c-Fos and SIRT-1, proteins critically involved in memory formation. Both, c-Fos and SIRT-1 levels were significantly decreased in the ventral hippocampus of twelve-month old 3xTg-AD mice. Over-expression of miR-181 in SH-SY5Y cells significantly decreased c-Fos and SIRT-1, strongly suggesting that miR-181 directly regulates the expression of these two proteins. These findings indicate a connection between miR-181 and proteins involve in synaptic plasticity and memory processing in a transgenic mouse model of AD. Our results suggest that microRNAs involved in synaptic plasticity might be an important factor that contributes to AD neuropathology.

Keywords

Alzheimer's disease; sirtuin-1; translational regulation; miRNA; synaptic plasticity

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INTRODUCTION

Alzheimer's disease (AD) is a devastating age-related neurodegenerative disorder characterized by progressive loss of cognitive and psychiatric functions, particularly memory decline. The postmortem AD brain display several pathological hallmarks including buildup of amyloid-beta (Aβ) species, intraneuronal neurofibrillary tau tangles and severe synaptic and neuronal loss [1]. The exact molecular interplay between these pathological hallmarks and cognitive decline is not well defined yet; although genetic evidence strongly suggests that the aberrant buildup of Aβ species lays upstream of tau neurofibrillary tangles formation, neurodegeneration and cognitive decline [2, 3]. The critical pathogenic role of Aβ has been proven in many AD animal models harboring mutations that increase the production or aggregation of Aβ in the brain (reviewed in [4, 5]). Among them, the triple transgenic mouse (3xTg-AD) carries the human mutant genes PS1 (PS1M146V), tau (tauP301L) and APP (SWE). This model mimics important features of the disease as described above such as, cumulative Aβ and tau pathologies in the cortex, hippocampus and amygdala [6]. In addition, 3xTg-AD mice present progressive memory loss that correlates well with the development of these neuropathological hallmarks in the brain [6, 7]. These features replicate the evolution of the disease in humans, and make the 3xTg-AD a useful model to investigate cellular and molecular mechanisms of the disease.

MicroRNAs are around 22 nucleotide-long RNAs that regulate gene expression at the post-transcriptional level for a wide range of cellular processes [8]. MicroRNAs bind to mRNAs together with a large complex of proteins to form the RNA-induced silencing complex (RISC). In this way, microRNAs modulate the stability and translation of mRNAs [8]. Recent research has shown microRNAs involvement in a large collection of neurobiology processes including neurogenesis, synaptic plasticity, brain aging and dementia [9–13]. Particularly in AD, microRNAs alterations have been observed in the brain and cerebrospinal fluid of AD patients [14–18]. These studies showed that dysregulated brain microRNAs are implicated in modification of Aβ and tau processing [14, 15], and inflammation [17, 18]. In this regard, miR-107 and miR-29 were both observed to be down-regulated in AD samples and showed to control BACE1 transcript [19, 20]. In the case of inflammation, increased levels of miR-125b and miR-146a have been found in the AD brain. These two microRNAs are under the control of the proinflammatory factor NF-κB, suggesting dysregulation of the microRNA-mediated immunological response [17, 18]. Furthermore, microRNAs that mediate synaptic plasticity have also been postulated to play a role in neurodegenerative disorders [21]. In this regard, miR-206 is increased in AD patients and in the Tg2576 mouse model of AD [22]. In Tg2576, an inverse correlation was observed between the levels of miR-206 and brain-derived neurotrophic factor (BDNF). Furthermore, restoration of BDNF levels by inhibition of miR-206 alleviated memory impairments and increased synaptic plasticity [22]. These findings indicate that microRNAs alterations may play a critical role in memory deficits observed in AD patients.

The aim of the present study was to identify novel microRNAs that play a critical role in in synaptic plasticity and are dysregulated in the 3xTg-AD mouse model. Using quantitative PCR, we analyzed the levels of a small number of microRNAs that are enriched in the mammalian brain or synaptoneurosomes and modulate synaptic functioning and/or plasticity

[23–31]. Here, we separately analyzed the dorsal and ventral regions of the hippocampus since pathology is first evident in the ventral part of the hippocampus and in later stages it migrates to the dorsal region in both AD patients and 3xTg-AD mice [32–34]. We found that miR-181 was significantly up-regulated in the dorsal and ventral hippocampus of twelve-month old 3xTg-AD mice. miR-181 was predicted to regulate c-Fos and SIRT-1, proteins critically involved in memory consolidation [35, 36]. The steady-state levels of c-Fos and SIRT-1 were significantly decreased in the ventral hippocampus of twelve-month old 3xTg-AD animals without modifications in their mRNAs levels. Furthermore, miR-181 over-expression in SH-SY5Y cells reduced SIRT-1 and c-Fos protein expression. These results support the view that alteration of miR-181 levels contributes to the cognitive neuropathological development of 3xTg-AD mice.

MATERIALS AND METHODS

Mice

All animal procedures were performed in accordance with National Institutes of Health and University of California guidelines and Use Committee at the University of California, Merced. The characterization of 3xTg-AD mice has been described previously [6]. In this study, 3.5, 6 & 12 month-old female wild type and 3xTg-AD mice (5 to 6 per group) were used.

Tissue preparation

After deep anaesthesia with sodium pentobarbital, mice were perfused transcardially with 0.1M phosphate-buffered saline, pH 7.4 (PBS). Hippocampi were dissected on wet ice and dorsal and ventral regions separated under a stereotaxic microscope (2.2–2.4 posterior to Bregma) [37]. Hippocampi from both hemispheres were pooled together and frozen in dry ice for biochemical analysis. Protein extracts were prepared by homogenizing brain regions in T-Per extraction buffer (150mg/mL, Thermo Fisher Scientific, Rockford, IL, USA), complemented with proteases (Complete Mini Protease Inhibitor Tablets, Roche Diagnostics GmbH, Germany) and phosphatases inhibitors (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 14,000 RPM for 10 min at 4°C. Protein concentration was determined for the supernatants using the Bradford assay following the manufacturer's protocol (Bio-Rad, Hercules, CA, USA).

Quantitative RT-PCR

RNA was purified from T-Per or M-Per lysates using the miReasy kit (Life Technologies, Grand Island, NY, USA) or Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. Quality of RNA was determined in a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) (all RINs ≥8.5). cDNA was produced from 500ng RNA using the NCODE Vilo cDNA systhesis kit (microRNAs) or SuperScript III First-Strand Synthesis kit (mRNAs) following the manufacturer's instructions (Life Technologies).

For qPCR, cDNA (1/10 dilution) was amplified on a MyIQ thermocycler (Biorad) using the SensiMix SYBR & Fluorescein kit (Bioline, Taunton, MA, USA) on the following

conditions. microRNAs: 95°C 10min; 40x (95°C 15 sec, 60°C 30 sec); dissociation curve 55°C-95°C with 0.5°C increments every 10 sec. mRNAs: 95°C 10min; 35x (95°C 30 sec, 60°C 30 sec, 72°C 30 sec); dissociation curve 55°C-95°C with 0.5°C increments every 10 sec. Data was corrected by efficiency [38] using the LinRegPCR software [39] and normalized to RNU6 (microRNAs) or GAPDH (mRNAs) levels. Primer sequences were selected using the Primer3 software [40] or from the Invitrogen website. A complete list of the primer sequences used in the present study is shown in table 1.

Immunoblotting

Equal amounts of protein were separated on 4–15% Bis-Tris gel and transferred to PVDF membranes. Membranes were blocked for 1 hour in TBS + 5% Non-fat Milk or Odyssey blocking solution (Li-cor). After blocking, membranes were incubated overnight with one or two of the following primary antibodies: cFos (1:1000, 4, Santa Cruz Biotechnology, Santa Cruz, CA, USA), SIRT-1 (1:5,000–10,000, ab12193, Abcam, Cambridge, MA, USA), TDP-43 (1:1000, 12892–1-AP, ProteinTech, Chicago, IL, USA), CAMKIIgamma (1:500, ab37999, Abcam, Cambridge, MA, USA), GAPDH (1:5,000, FL-335, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and tubulin (1:25,000, B-5-1-2, Sigma-Aldrich) in TBS + 5% Non-fat Milk or Odyssey blocking solution + 0.2% tween-20 at 4°C. After washes with TBS + 0.1% tween-20, membranes were incubated for 1 h with the specific secondary antibody(ies) at a dilution of 1:5000 (HRP-conjugated, Pierce Biotechnology, Rockford, IL, USA) or 1:20,000 (IRDye, Li-cor) in TBS + 5% Non-fat Milk + 0.2% tween-20 + 0.01% SDS. Blots were developed using Super Signal (Pierce Biotechnology) or scanned in an Odyssey infrared imager (Li-cor). Image Studio software (Li-cor) was used for protein quantification. Protein levels were normalized to GAPDH or tubulin.

SH-SY5Y cells transfection and samples preparation

Cells were seeded at ~70% confluency in 6-well plates and, 24 h later, transfected with 100nM hsa-miR-181a or human negative control 2 (MISSION microRNA Mimic, Sigma-Aldrich) using turbofect according to manufacturer's instructions (Thermo Scientific). 18 h later, media was replaced. After 48 h of total incubation time, cells were washed once with PBS, lysed with M-Per extraction buffer (200uL, Thermo Fisher Scientific) complemented with proteases and phosphatases inhibitors (Sigma-Aldrich), incubated 5 min at RT with gently agitation and centrifuged at 14,000 RPM for 10 min at 4°C. Immunoblot or RT-qPCR was performed as described above.

Statistical analysis

Student's t-test was used for comparisons between genotypes or transfection conditions. p-value ≤0.05 was considered significant.

RESULTS

Increased levels of miR-181 were detected in the hippocampus of 12-month old 3xTg-AD mice.

Since synaptic dysfunction is an early pathological event in AD and strongly correlates with cognitive impairments [41, 42], we focused on a small subset of microRNAs enriched in the

brain with a role in synaptic function [23–31]. None of the microRNAs analyzed in this study showed statistical differences between genotypes in 3.5-month old animals (Suppl. Fig. 1A, D; supplementary data 1). However, in 6-month old 3xTg-AD, miR-181 was significantly increased in the ventral hippocampus ($t(9) = 2.25$, $P < 0.05$) (Fig.1A). Augmented levels of miR-181 were also observed at 12 months in both dorsal ($t(9) = 3.99$, $P < 0.01$) and ventral ($t(9) = 2.41$, $P < 0.05$) regions of the hippocampus (Fig.1A–B).

The predicted targets of miR-181 SIRT-1 and c-Fos are down-regulated in the ventral hippocampus of 12-month old 3xTg-AD mice.

The TargetScan prediction software was used to identify potential targets of miR-181 paying particular attention to proteins that are relevant to plasticity [43] (Supplementary Data 1–3). Protein levels of potential targets were assessed by immunoblot. Among other mRNAs, miR-181 was predicted to interact with the human and mouse mRNAs of the desacetylase sirtuin-1 (SIRT-1) and the transcription factor c-Fos (Supplementary data 1–3). Figure 2 panels 2B and 2E show that steady-state levels of both these proteins were down-regulated in the ventral hippocampus of 3xTg-AD mice at 12 months of age ($t(9) = 2.48$, $p<0.05$ for SIRT-1, and $t(9) = 3.38$, $p<0.05$ for c-Fos). A significant reduction of c-Fos was also detected in the ventral hippocampus of 6-month old 3xTg-AD mice ($t(9) = 2.64$, $P < 0.05$), which correlated with the increased levels of miR-181 (Fig. 2E). However, down-regulation of SIRT-1 was not detected in the ventral hippocampus of 3xTg-AD mice at 6 months (Fig. 2B). In the dorsal hippocampus, SIRT-1 and c-Fos protein levels were similar between genotypes in the different ages (Fig. 2A, 2D). Analysis of mRNAs levels in 12-month old samples showed no differences in SIRT-1 and c-Fos between genotypes in the ventral hippocampus (Fig. 2C and 2F), pointing to translational regulation of c-Fos and SIRT-1 by miR-181. Among the other potential targets of miR-181, we investigated TDP-43 and CAMKIIgamma and observed similar protein levels in wild type and 3xTg-AD mice in both hippocampal regions in the different age groups (Fig S2).

Over-expression of miR-181 decreased protein levels of SIRT-1 and c-Fos in SH-SY5Y cells.

In order to provide evidence that up-regulation of miR-181 decreases the translation of c-Fos and SIRT-1 *in vitro*, we transiently over-expressed a double-stranded RNA mimic of miR-181 in the human neuroblastoma cell line SH-SY5Y. Like any other cell line, SH-SY5Y is not a bona fide neuron, but it propagates via mitosis, a feature not normally seen in adult neurons. However, SH-SY5Y cells have proven useful when exploring microRNAs role in neurological diseases [44, 45]. Fourty-eight hours after transfection of miR-181 or a non-related sequence as a negative control, the steady-state levels of SIRT-1 and c-Fos were determined. Figure 3A–B shows that c-Fos and SIRT-1 were significantly reduced in cells with miR-181 augmented levels ($t(12) = 2.2$, $p<0.05$ for SIRT-1, and $t(13) = 2.83$, $p<0.05$ for c-Fos), supporting our findings in 3xTg-AD hippocampal samples.

DISCUSSION

Several studies have shown microRNAs dysregulation in AD patients and animal models [12, 14–18, 46–48]. MicroRNAs altered in AD have been implicated in the regulation of Aβ and tau processing [14, 15], inflammation [17, 18] and synaptic plasticity [22]. Here, we

observed increased miR-181 expression in both the dorsal and ventral hippocampus of 3xTg-AD mice. Up-regulation of miR-181 was observed earlier in the ventral region, consistent with the fact that pathology is first evident in the ventral part of the hippocampus and in later stages it migrates to the dorsal area in the 3xTg-AD mouse and AD patients [32–34]. Up-regulation of miR-181 in 3xTg-AD mice was associated with significant decreased levels of SIRT-1 and c-Fos proteins. In addition, we found that miR-181 directly regulates c-Fos and SIRT-1 basal levels in SH-SY5Y cells. Therefore, our study suggests that dysregulation of microRNAs that controls synaptic plasticity is an important component for the development of cognitive deficits in a transgenic mouse model of AD.

Although miR-181 has consistently been reported to be altered in AD, the trend of the change is still matter of controversy. Most groups have reported that miR-181 is down-regulated [14, 20, 49, 50], while others have observed the opposite result [51]. As noted in previous reports, discrepancies may be related to differences in a number of factors that include accuracy of the diagnosis, gender, medication history, samples acquisition and processing, brain region analyzed, methodologies used in the analysis and intrinsic human individuality [17]. In addition, miR-181 is a microRNA family composed of four members, miR-181a-d. In humans and mice, miR-181a and miR-181b are in the same genetic cluster, while miR-181c and miR-181d are part of a different cluster [52]. Clustered miRNAs are commonly transcribed as polycistrons and have similar expression pattern. Discrepancies in miR-181 expression levels in AD patients can be explained, in part, by the fact that transcription of miR-181a and b is regulated in a different and independent way to miR-181c and d. In this regard, studies that found miR-181 down-regulation focused in miR-181c, while the only group who reported incremented miR-181 in AD patients targeted miR-181b sequence. In the present work, we observed a raise in the expression of miR-181 in 3xTg-AD mice using the mature sequence of miR-181a. This finding is consistent with the observation that miR-181b is augmented in samples of AD patients [51]. Our results are also in line with one other study where a connection between miR-181a and plasticity was established [30]. In that report, miR-181a was observed to negatively regulate the GluA2 subunit of AMPA receptors in neuronal primary cultures. Furthermore, miR-181a over-expression leaded to a reduction of spine formation and diminished basal synaptic transmission [30]. Conversely, over-expression of miR-181a in the nucleus accumbens was observed to enhance conditioned place preference memory, indicating that miR-181a is not a negative regulator of plasticity and memory in all instances, but its role depends on the brain region and specific available targets [53].

A major question to be resolved is whether miR-181 dysregulation is a cause or a consequence of AD. In this regard, it was reported that Aβ treatment down-regulates miR181c in primary hippocampal neurons at times as short as one hour and, a similar reduction was observed in the AD mouse model APP23 [54]. On a follow up study, this group used the luciferase reporter assay to explore regulation of different mRNAs 3’UTR by miR-181c. They found that miR-181c negatively regulates SIRT-1 expression among other targets, *i.e.*, TRIM2 and BTBD3. The authors concluded that SIRT-1 up-regulation, due to miR-181c reduction, is a compensatory mechanism to protect cells from the characteristic toxicity present in AD [55]. In contrast to the Schonrock *et al.* reports, it was found that loss of miR-181c, among other microRNAs, increases serine palmitoyltransferase (SPT) and

consequently, Aβ levels [50]. SPT is the first rate-limiting enzyme in the *de novo* ceramides synthesis. Membrane ceramides favor mislocation of BACE1 and gamma-secretase to lipid rafts, and thereby promote Aβ formation. Therefore, ceramides have been proposed as a risk factor in familial AD [56]. In the study by Geekiyanage *et al.*, it was observed that some AD brain samples presented SPT and Aβ up-regulation and decreased levels of a group of microRNAs, including mir-181c, which potentially bind SPT mRNAs. Furthermore, a luciferase reporter construct confirmed that miR-137 and miR-181c decreased SPT mRNAs and Aβ expression levels and, anti-miR-137 or anti-miR-181c had the opposite effect [50].

In SH-SY5Y cells, we found that miR-181 directly regulates c-Fos and SIRT-1 basal levels. Deacetylase SIRT-1 and the transcription factor c-Fos are both proteins required for activity-dependent synaptic plasticity induced during memory formation [35, 36, 57], and our observations indicate that this process is mediated by miR-181. Gao *et al.*, demonstrated that SIRT-1 activation increases memory and dendritic spines formation. Their results showed that SIRT-1 negatively regulates miR-134 expression that in turn represses CREB translation leading to impairments in memory and plasticity [35]. In the cortex of AD patients, SIRT-1 protein levels are reduced and, a negative correlation between SIRT-1 levels and cognition was found [58]. Similarly, a decline in SIRT-1 concentration was observed in patients with AD compared to healthy elderly individuals [59]. Overall, these studies correlate with our findings and support that SIRT-1 is one of the proteins with an important role in the regulation of synaptic plasticity that is disrupted in AD.

Evidence also points to that SIRT-1 diminution favors the development of Aβ and tau pathologies. In an AD mouse model bearing the APP (APPswe) and presenilin-1 (PSEN1 deltaE9) transgenes, it was observed a reduction in Aβ levels by over-expressing SIRT-1 and the opposite effect when SIRT-1 was knocked-out. Together with pathology reduction, SIRT-1 up-regulation was reported to alleviate memory deficits [60]. Further experiments indicated that SIRT-1 suppresses Aβ production by increasing the levels of the alpha-secretase, ADAM10 and, therefore, stimulating non-amyloidegic cleavage of APP [60]. In regard to tau pathology, Min *et al.* found that acetylation and deacetylation of tau protein is mediated by the proteins p300 and SIRT-1, respectively. The authors also showed that tau acetylation prevents proteasome-mediated degradation of pathogenic forms of phosphorylated tau, supporting that SIRT-1 reduction promotes taupathology [61]. In addition, primary neurons treated with Aβ oligomers presented augmented levels of acetylated tau, indicating that Aβ accumulation induces tau acetylation [61].

c-Fos, together with c-Jun and ATF, compose the AP-1 (Activator protein 1) family of transcription factors. AP-1 is active when homo- or heterodimers of these proteins are formed, leading to regulation of a variety of cellular processes, including cellular events required in memory consolidation [36]. Previous work has shown reduced levels of c-Fos in a mouse model of AD [62]. In that study, basal levels and exploration-induced levels of Arc and c-Fos proteins were diminished in several regions of transgenic mice compared to wild type, suggesting that hyporeactivity to stimulation may explain to some extend the cognitive deficits observed in AD [62]. Our results contribute that c-Fos basal levels are also decreased in the hippocampus of 3xTg-AD mice and that c-Fos reduction is due to miR-181 up-

regulation. Consistently, Wu *et al.* reported that miR-181a repressed the expression of c-Fos in immune dendritic cells [63].

AD is a complex disorder for which the molecular mechanisms involved in its onset and early neuropathology remain to be elucidated. A plethora of factors have been shown to participate in different aspects of the disease and microRNAs have been found to be involved in many of these pathological events [14, 15, 17, 18, 22]. Our results, together with other report [22], support that microRNAs involved in synaptic plasticity regulation are pivotal in the development of AD; making this group of microRNAs suitable for further study in the search of AD molecular markers and therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This study was funded by the Alzheimer's Association NIRG-12-242598 (MK), NIA # 2R01 AG027544-06 (FML) and by The Larry Hillblom Foundation #2013-A-016-FEL (DBV). We thank Carlos F. Flores-Jasso for helpful comments on the manuscript.

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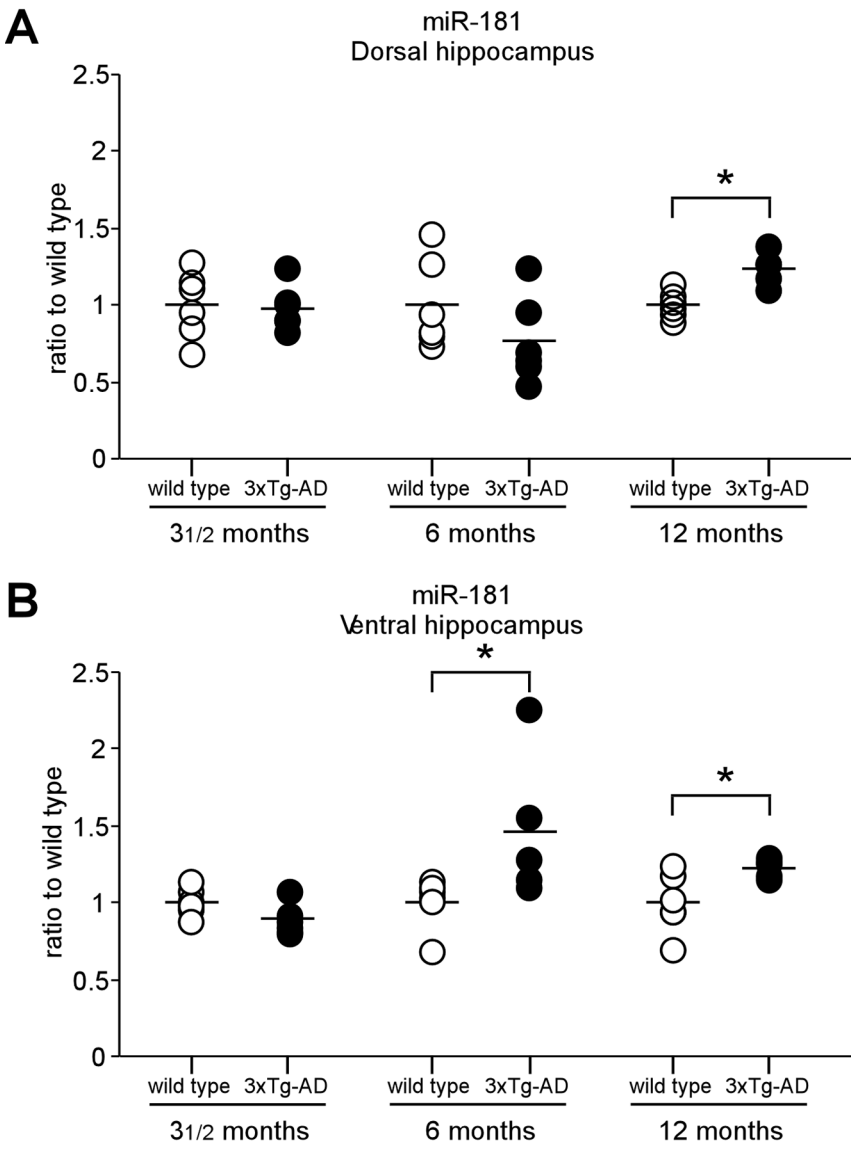

Figure 1. miR-181 is up-regulated in the hippocampus of 12 month-old 3xTg-AD mice.

miR-181 levels are shown for the dorsal **(A)** and ventral hippocampus **(B)** at 3.5, 6 and 12 months of age. Significant differences were detected in 12 month-old dorsal and ventral hippocampus, and at 6 months in the ventral hippocampus. * = $p < 0.05$.

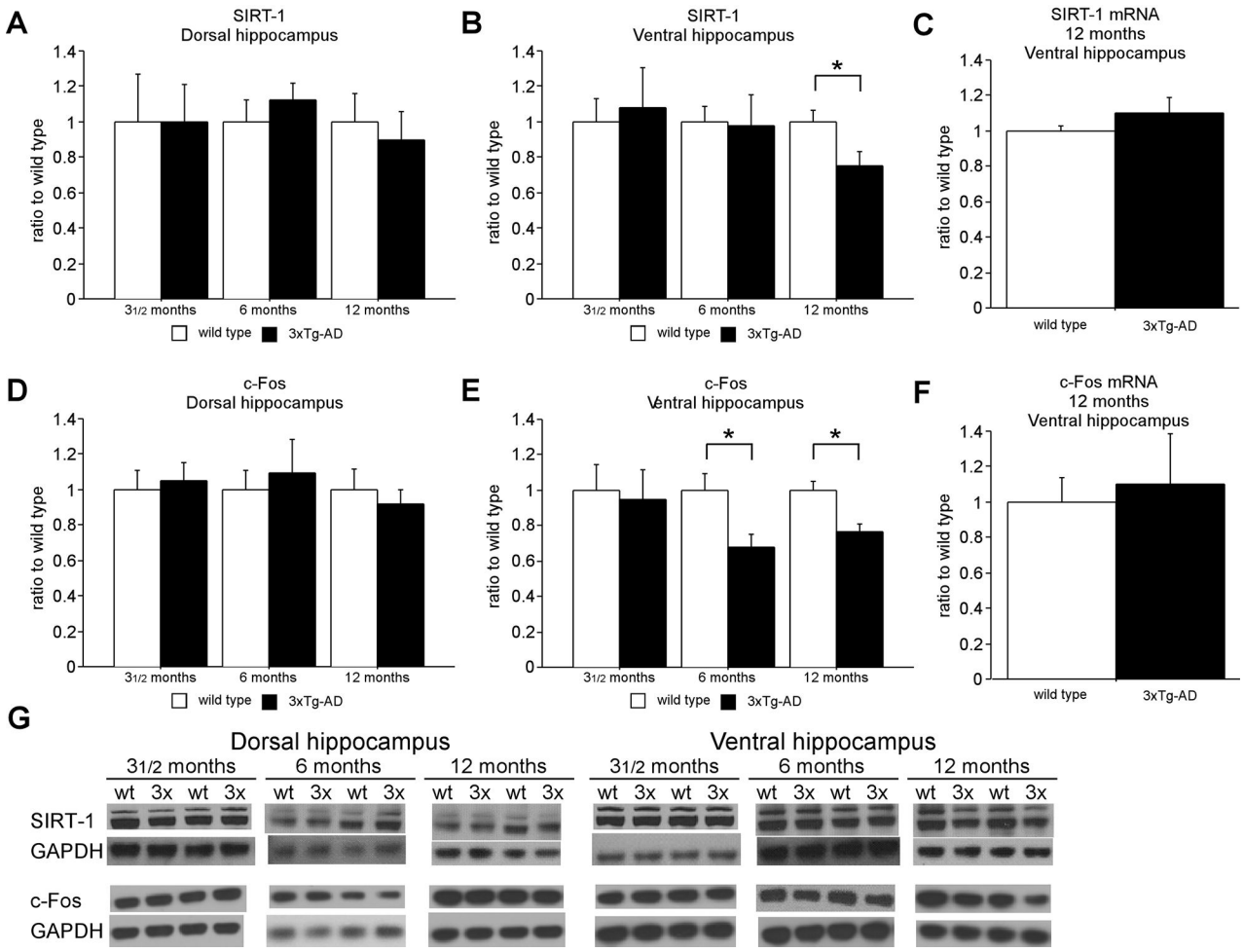

Figure 2. c-Fos and SIRT-1 protein levels are reduced in the ventral hippocampus of 12 month-old 3xTg-AD mice.

A. Immunoblots showed similar SIRT-1 steady-state levels in the dorsal hippocampus at different ages. **B.** Diminished SIRT-1 protein levels were found at 12 months in the ventral hippocampus. **C.** However, SIRT-1 mRNA levels were similar between genotypes at 12 months in the ventral hippocampus. **D.** Similar c-Fos steady-state levels were observed in the dorsal hippocampus at different ages. **E.** In the ventral hippocampus, reduced c-Fos protein levels were found at 6 and 12 months. **F.** However, c-Fos mRNA levels were similar between genotypes at 12 months in the ventral hippocampus. **G.** Representative blots of the data presented in A-B, D-E. * = $p < 0.05$.

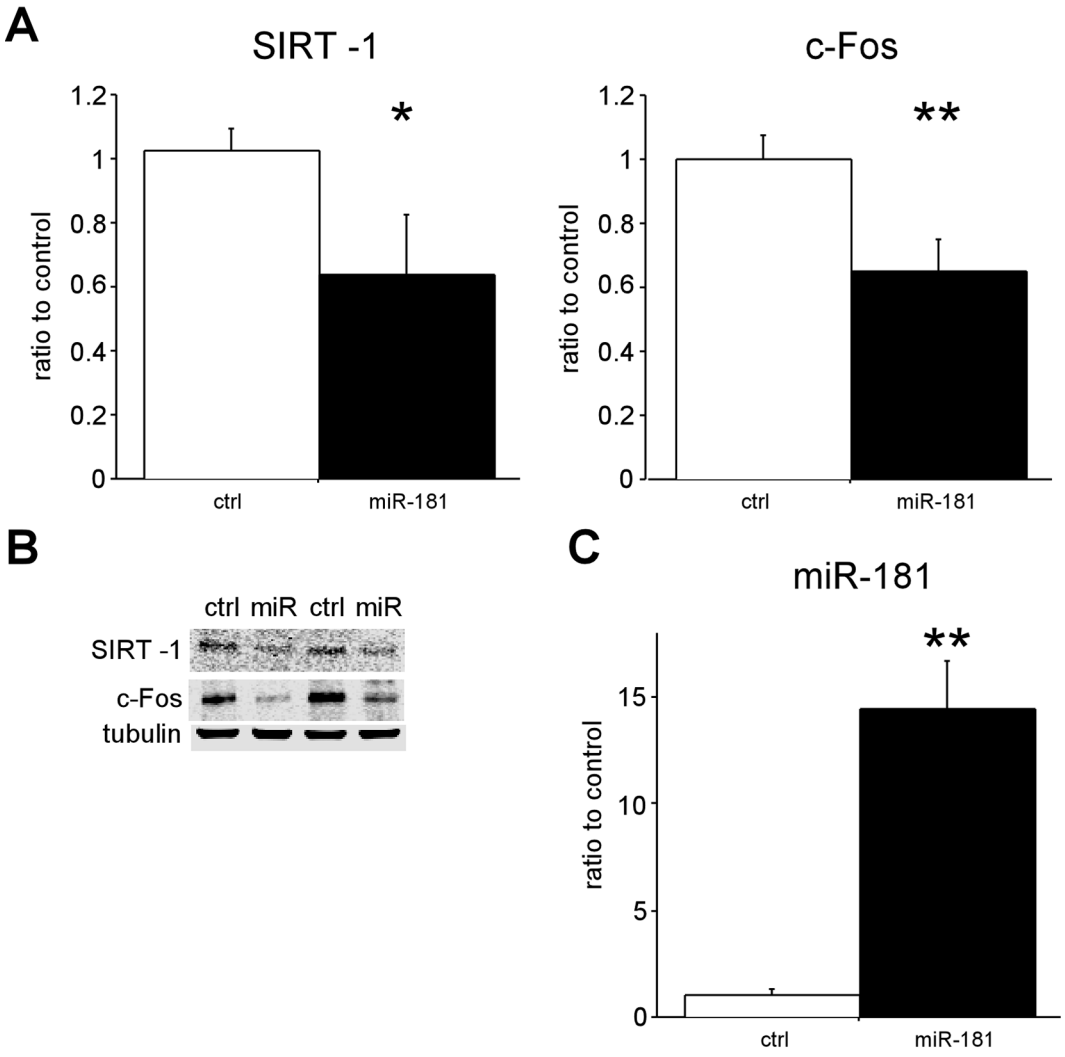

Figure 3. Human neuroblastoma SH-SY5Y cells present reduced SIRT-1 and c-Fos protein levels when miR181 is over-expressed.

A. SIRT-1 and c-Fos protein levels were diminished in cells transfected with miR-181a mimics **B**. Representative blots of the data presented in A. **C**. Detection by qPCR of miR-181 showed significant increased levels in cells transfected with the microRNA mimic. * = $p < 0.05$, ** = $p < 0.01$.

Table 1.

List of primer sequences used in the present study.

primer	sequence
miR-181	5’-GGTAAGGTGCATCTAGTGCAGATAG-3’
miR-219	5’-TGATTGTCCAAACGCAATTCT-3’
miR-218	5’-CGTTGTGCTTGATCTAACCATGT-3’
miR-708	5’-AAGGAGCTTACAATCTAGCTGGG-3’
miR-29	5’-TAGCACCATCTGAAATCGGTTA-3’
miR-107	5’-CAGCATTGTACAGGGCTATCA-3’
miR-125	5’-TCCCTGAGACCCTAACTTGTGA-3’
miR-25	5’-ATTGCACTTGTCTCGGTCTGA-3’
miR-26	5’-GATTTCAAGTAATCCAGGATAGGCT-3’
miR-27	5’-TCACAGTGGCTAAGTTCCGC-3’
miR-30	5’-TGTAAACATCCTCGACTGGAAG-3’
RNU6	5’-AAATTCGTGAAGCGTTCCAT-3’
sirt-1 forward	5’-AGTTCCAGCCGTCTCTGTGT-3’
sirt-1 reverse	5’-CTCCACGAACAGCTTCACAA-3’
c-fos forward	5’-CTCCCGTGGTCACCTGTACT-3’
c-fos reverse	5’-TTGCCTTCTCTGACTGCTCA-3’
gapdh forward	5’-AACTTTGGCATTGTGGAAGG-3’
gapdh reverse	5’-ACACATTGGGGGTAGGAACA-3’