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microRNA-34c is a novel target to treat dementias

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MicroRNAs are key regulators of transcriptome plasticity and have been implicated with the pathogenesis of brain diseases. Here, we employed massive parallel sequencing and provide, at an unprecedented depth, the complete and quantitative miRNAome of the mouse hippocampus, the prime target of neurodegenerative diseases such as Alzheimer's disease (AD). Using integrative genetics, we identify miR-34c as a negative constraint of memory consolidation and show that miR-34c levels are elevated in the hippocampus of AD patients and corresponding mouse models. In line with this, targeting miR-34 seed rescues learning ability in these mouse models. Our data suggest that miR-34c could be a marker for the onset of cognitive disturbances linked to AD and indicate that targeting miR-34c could be a suitable therapy.

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Introduction

As life expectancy increases age-associated dementias constitute a serious health problem for our societies. The hippocampus is a key brain structure for memory formation and among the first brain regions to be affected during aging and in neurodegenerative dementias such as Alzheimer's disease (AD), which arises on the pathological background of amyloid β plaques, neurofibrillary tangels, synaptic and neuronal loss (Mesulam, 1999; Kandel, 2001; Haass and Selkoe, 2007; Ittner and Götz, 2010) and cause severe memory impairment eventually leading to dementia. Efficient therapeutic strategies to treat memory decline are still missing, which is in part due to the fact that mechanisms underlying the pathogenesis of memory impairment in AD are only partially understood. Deregulated hippocampal gene expression has been observed during aging and brain diseases of animal models as well as in human patients (Lu *et al*, 2004; Berchtold *et al*, 2008; Selwood *et al*, 2009; Cao *et al*, 2010; Peleg *et al*, 2010; Ray and Zhang, 2010). To understand the mechanisms that are causative for this deregulated transcriptome plasticity is of utmost importance and may lead to the development of novel therapeutic strategies.

A possible regulatory mechanism that is only beginning to emerge is the regulation of gene expression via microRNAs (miRNAs). miRNAs play key roles in a wide range of biological processes and regulate gene expression mainly at the translational level (He and Hannon, 2004). Recent work implicated miRNAs with neuronal function (Schratt *et al*, 2006; Konopka *et al*, 2009; Siegel *et al*, 2009) and the pathogenesis of AD (Hébert *et al*, 2008; Lau and de Strooper, 2010). Until now, hippocampal miRNAs have been investigated using targeted or high-throughput approaches, but the latter have been limited to either microarrays or a relative small number of sequenced clone reads (Landgraf *et al*, 2007; Bak *et al*, 2008; Pena *et al*, 2009; Gao *et al*, 2010). Moreover, microarray analysis is prone to cross-hybridization between similar sequences, an attribute very common in case of miRNAs. In addition, competitive hybridization is a relative measure, limiting the dynamic range of high confidence data (Hurd and Nelson, 2009). Massive parallel sequencing of small RNAs offers remedies to the above problems and can provide an unprecedented insight into the small non-coding transcriptome of the cells (Hurd and Nelson, 2009).

Thus, we employed massive parallel sequencing and report at an unprecedented level of detail the complete and absolute digitized mouse hippocampal miRNAome. Our aim was to reveal miRNAs enriched in mouse hippocampus that until now had escaped identification as such by using other technologies and which might play an important role in hippocampal function and possibly age-associated memory impairment including AD pathogenesis. Using this approach, we find that miR-34c is implicated with the pathogenesis of cognitive decline. By combining molecular and behavioural experimental approaches, we show that miR-34c is required for memory function and provide data to suggest that targeting miR-34c could be a suitable approach to treat memory impairment in AD.

Results

Massive parallel sequencing reveals the hippocampal microRNAome at an unprecedented depth

Deep sequencing of mouse hippocampal small RNA libraries derived >24 million reads (Figure 1A–C; Supplementary

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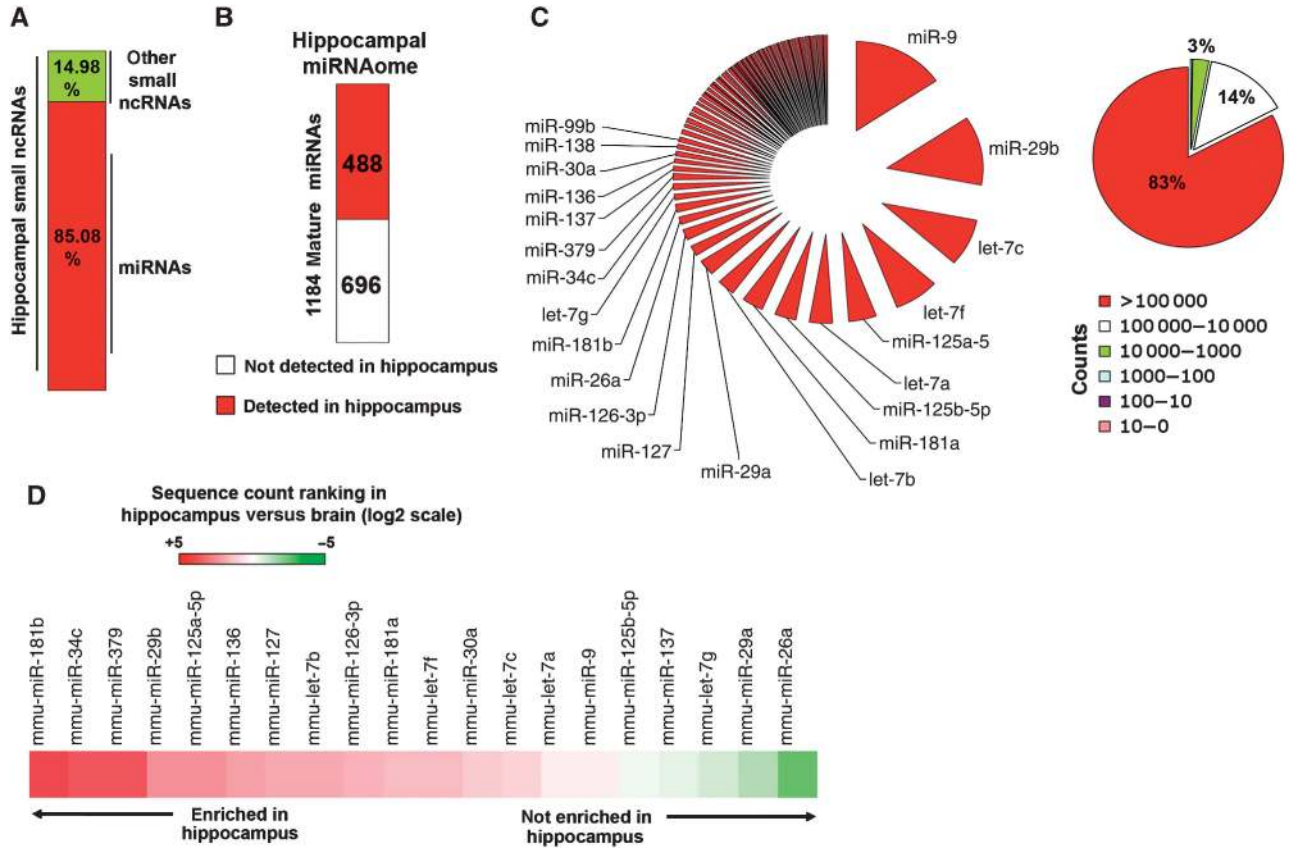


Figure 1 Massive parallel sequencing of small RNA libraries reveals the hippocampal miRNAome. (**A**) Contribution (in percent) of miRNAs to the total number of small non-coding RNAs in the range of 18–26 nt detected by sequencing. (**B**) Proportion of detected mature miRNAs, regarding the total number of known genes and miRNAs in miRBase. (**C**) Right panel: Distribution of miRNAs to different classes based to their sequence counts and contribution of each of these miRNA classes to the total miRNA sequence count. Left panel: Proportion of sequence counts per miRNA with respect to the total number of counts attributed to miRNAs in hippocampus. (**D**) Sequence counts of top ranking hippocampal miRNAs relative to the respective counts in whole brain −(log2 scale).

Figure S1; Supplementary Table S1). Previous sequencing approaches of this tissue did not exceed 100 000 sequenced clones (Pena *et al*, 2009) or did not include testing and quantification of a big number of known miRNAs that were identified and characterized after these studies (Landgraf *et al*, 2007). From the total 1184 known mature miRNAs encoded in the mouse genome, 488 were detected in the hippocampus (Figure 1B). Notably, out of the 488 hippocampal miRNAs, 23 miRNAs were highly expressed (beyond 100 000 counts). In fact these miRNAs accounted for almost 83% of all miRNA counts detected in hippocampal tissue (Figure 1C; Supplementary Table S1).

miR-34c is implicated with hippocampal function

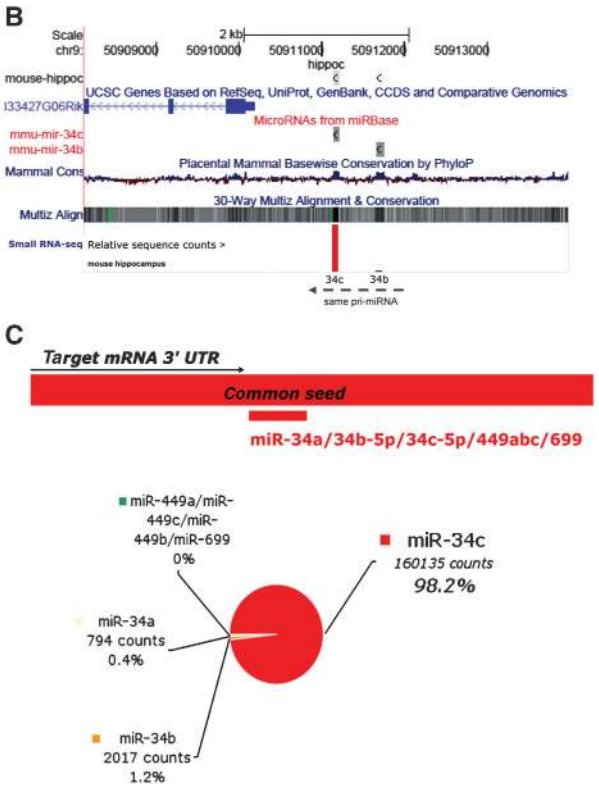
Different tissues have been shown to possess unique miRNA expression profiles in accordance with their specific structural and functional characteristics (Kim and Nam, 2006). As such we reasoned that a comparison of our data (Figure 1C) with the miRNA profile of the whole mouse brain (Supplementary Figure S2), for which comparable massive parallel sequencing data already exists (Chiang *et al*, 2010), would allow us to identify miRNAs that might have a unique role in hippocampal function and thus would be most suitable candidates for further analysis. The hippocampus (Figure 1C) and whole brain tissue (Supplementary Figure S2) display many similarities regarding the most highly expressed miRNAs, like miR-9 and members of the miR-29 and let-7 families, which as shown in Supplementary Figure S3A are implicated with the regulation of general cellular processes. However, a number of miRNAs that were minimally expressed in whole brain tissue, such as miR-181b, miR-34c or miR-379, were in contrast highly enriched in the hippocampus (Figure 1D). Among these three miRNAs, targets of miR-34c are highly predicted to be involved in neuronal processes and functions (Figure 2A) compared with other miRNAs tested (Supplementary Figure S3B–E), suggesting a specific role of miR-34c in the regulation of hippocampal functions. In addition, miR-34c is transcribed from the same cluster as miR-34b (Figure 2B). Nevertheless, in the hippocampus mature miR-34b is only found at very low levels (Figure 2C), suggesting a possible escape mechanism of miR-34c from the regular miRNA turnover further indicating a possible role of miR-34c in hippocampal function.

To further test the possibility that miR-34c may be important for hippocampal function, we applied an integrated genomic analysis. In a recent work, we identified 1362 hippocampal genes that are upregulated in mice 60 min after exposure to contextual fear conditioning, a commonly used paradigm for associative learning in rodents (Peleg *et al*, 2010; Supplementary Figure S4A and B). We questioned which of the highly expressed hippocampal miRNAs

A

miR-34c targets GO-term analysis

	Biological process	*P*-value
1	Cell morphogenesis involved in differentiation	3.80E−06
2	Axonogenesis	4.60E−06
3	Generation of neurons	4.70E−06
4	Cell projection morphogenesis	1.00E−05
5	Neuron projection morphogenesis	1.10E−05
	PANTHER database>biological process	
1	BP00224: Cell proliferation and differentiation	1.30E−05
2	BP00274: Cell communication	8.90E−05
3	BP00166: Neuronal activities	1.40E−03
4	BP00111: Intracellular signalling cascade	2.20E−03
5	BP00167: Synaptic transmission	2.40E−03
	Molecular function	
1	Gated channel activity	8.20E−04
2	Cation transmembrane transporter activity	3.00E−03
3	Ion channel activity	5.50E−03
4	Substrate-specific channel activity	7.00E−03
5	Transition metal ion binding	1.80E−02



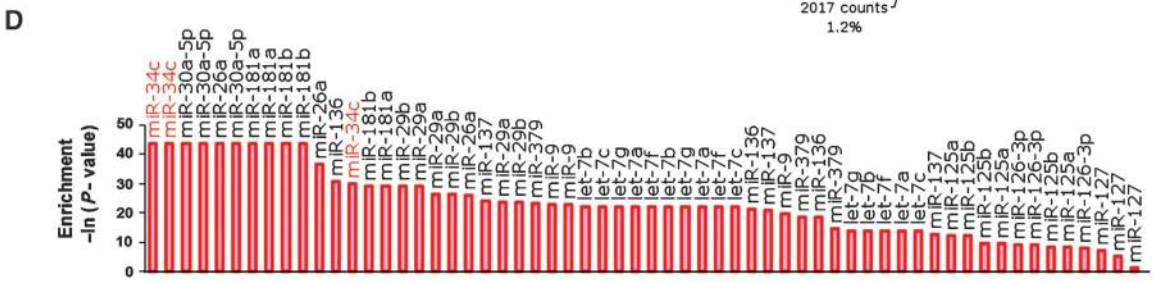


Figure 2 Genomic location of miR-34c and functional analysis of miR-34c targets. (**A**) Functional analysis of gene targets incorporating the miR-34c seed described in (**B**). A complete list of the genes that have been analysed is available in Supplementary Table SI (as defined in Targetscan Mouse, Release 5.1 12). Enrichment for Gene Ontology (GO) terms for biological processes and molecular functions at the fifth level as well as for the respective biological process terms from the PANTHER database was tested (top five terms for each GO term category are depicted). Analysis revealed a significant enrichment of miR-34c targets in terms strictly related to neuronal function, in contrast to other expressed or enriched in hippocampus miRNAs (see Supplementary Figure S3B–E). (**B**) UCSC browser (mm9) view of miR-34bc cluster. Genomic intervals of sequenced reads in mouse hippocampus are depicted in the first row in positions corresponding to miR-34c (left) and miR-34b (right) (third panel—UCSC miRNA track, as of January 2011). The next two panels depict conservation of the respective sequences among species (UCSC: placental mammal basewise conservation (up), multiz alignments of 30 vertebrates (down)). The last panel depicts the relative expression levels (according to sequence counts) of miR-34c and the other member of this cluster, miR-34b, which is minimally expressed. miR-34c presents high expression levels in mouse hippocampus and high evolutionary conservation, in line with an important role in cellular functions. (**C**) Percentage of each of miRNAs sharing the same with miR-34c seed and thus mRNA targets with regards to total amount of miRNAs detected in hippocampus targeting this seed. miR-34c represents >98% of miRNAs able to target this seed, revealing its almost exclusive control in regulation of mRNA targets of this group. miRNA seed as defined in Targetscan Mouse, Release 5.1 12. (**D**) miR-34c targets learning-associated genes. Enrichment of learning-associated genes upregulated 60 min after fear conditioning (see Supplementary Figure S4A) in seeds of miRNAs highly expressed in hippocampus.

identified by our deep sequencing analysis show a significant enrichment in targets within these 1362 learning-associated genes. Notably, miR-34c showed the highest grade of enrichment regarding predicted targets within this group of genes (Figure 2D) further suggesting a potential role for miR-34c in the regulation of memory function.

High miR-34c levels are linked to pathogenesis of learning impairment

Next, we tested whether miR-34c may play a role in memory impairment. As a first approach, we measured miR-34c levels in the hippocampus of 24-month-old mice, a model for age-associated memory impairment, and in APPPS1-21 mice, a model of amyloid pathology linked to AD. We found that hippocampal miR-34c was significantly upregulated in these two mouse models (Figure 3A), which correlated with impaired memory function (Figure 3B). Interestingly, among the already experimentally confirmed targets of the miR-34 family is the *Sirt1* mRNA (Yamakuchi *et al*, 2008), that has two binding sites of miR-34c (Supplementary Figure S4C). As shown by the respective luciferase assays in Supplementary Figure S4D, targeting of Sirt1 mRNA by miR-34c applies also to neuronal cells. Since hippocampal SIRT1 has been shown to be pivotal and necessary for memory function in

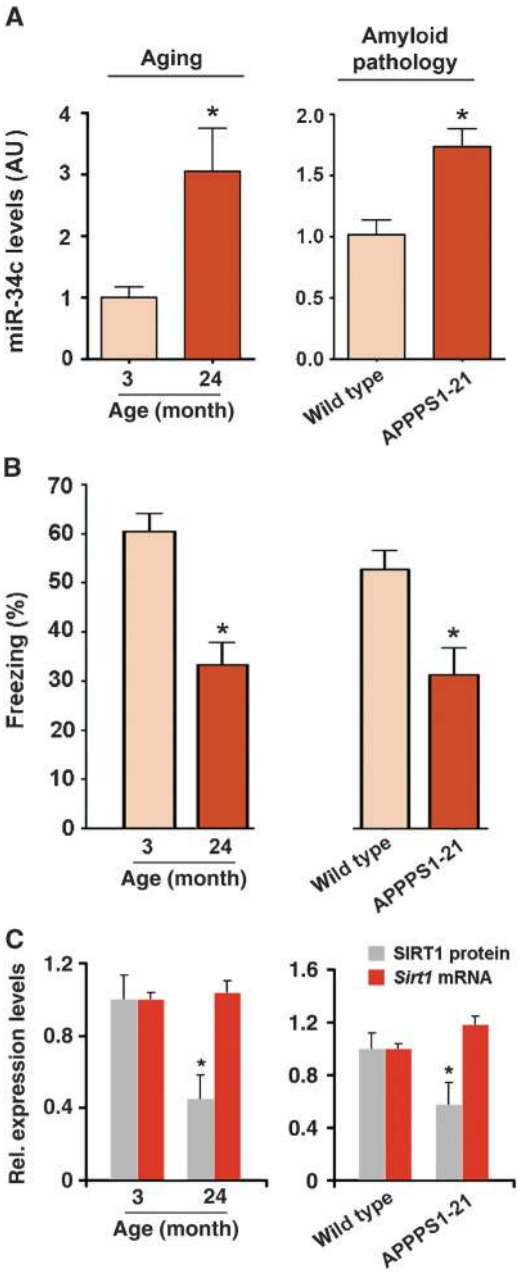


Figure 3 High levels of miR-34c in mouse models for learning impairment. (**A**) Hippocampal miR-34c expression levels determined by qPCR in two mouse models for memory impairment, (left) 24-month-old mice ($n=4$; $P=0.02$), (right) 12-month-old APPPS1-21 mice ($n=7$, 6; $P=0.01$). (**B**) Left panel: 3- and 24-month-old C57Bl6J mice were subjected to contextual fear conditioning and freezing behaviour was analysed 24 h later during the memory test. Freezing was significantly reduced in 24-month-old mice ($n=8$/group, $^*P<0.05$). Right panel: 12-month-old male APPPS1-21 mice were exposed to contextual fear conditioning. When compared with age-matched control littermates associative memory was impaired in APPPS1-21 mice. No difference in explorative behaviour or response to the foot-shock was observed among groups (data not shown). (**C**) SIRT1 protein levels are significantly reduced in the hippocampus of aged ($P=0.02$ versus young, $n=4$) and APPPS1-21 mice ($P=0.01$ versus wild type, $n=7$, 6). In line with a translational repression mode of action of miR-34c on SIRT1, *Sirt1* mRNA levels were not affected. Error bars indicate s.e.m.

mice (Gao *et al*, 2010; Michán *et al*, 2010), we reasoned that measuring SIRT1 levels would be a suitable read out of miR-34c activity *in vivo*. Indeed, learning-induced changes in SIRT1 protein levels showed an inverted correlation with the regulation of miR-34c 3 h after fear conditioning (Supplementary Figure S4E). Moreover, elevated hippocampal miR-34c levels in 24-month-old mice and in APPPS1-21 mice, correlated with decreased SIRT1 protein levels (Figure 3C). Notably, these findings in animal models translated into the human situation. To this end, we found that miR-34c levels were significantly increased in the hippocampus of human AD patients when compared with age-matched control (Figure 4). Within the same patients we observed downregulation of miR-124 that has been implicated with memory formation (Rajasethupathy, 2009) while the levels of miR-379 were unaffected (Supplementary Figure S5).

Thus, we decided to test directly, whether elevated hippocampal miR-34c levels would affect memory function. To this end, we established an *in vivo* small RNA transfection system (Figure 5A; Supplementary Figure S6) in which microinjections of a miR-34c mimic into the hippocampus resulted in significantly elevated miR-34c levels when measured 12 h after the last injection (Figure 5A). Next, mice were injected with miR-34c mimic and subjected to fear conditioning training when hippocampal miR-34c levels were significantly elevated, thus simulating a condition similar to that observed in 24-month-old mice, APPPS1-21 mice and in human AD patients (Figure 5A). Associative learning was significantly impaired in miR-34c mimic injected mice when compared with the scrambled miR-injected control group (Figure 5B). miR-34c induced impairment of associative learning corresponded with reduced SIRT1 levels (Figure 5C and D), confirming successful incorporation of miR-34c mimic into the RISC complex and specific translational repression of target mRNAs. Furthermore, specific blocking of the interaction between miR-34c and its SIRT1 3′UTR binding sites through miRNA target protectors (Figure 6A) was able to reverse the above effects. Administration of miR-34c *Sirt1* target protector was able to rescue miR-34c mimic-mediated learning impairment (Figure 6B) and resulted in increased SIRT1 protein levels (Figure 6C), thus confirming that action of miR-34c on SIRT1 protein *in vivo* is direct and based on miR-34c/mRNA interaction.

Impairment of memory consolidation upon injection of mir-34c mimic was also confirmed in two other behavioural tests, namely the novel object recognition paradigm (Supplementary Figure S7A) and the Morris water maze (Supplementary Figure S7B), further supporting a role of this miRNA as a negative constraint of memory formation.

Targeting miR-34c seed rescues learning impairment

Our data suggest that the miR-34c upregulation seen in AD brains contributes to clinical memory deficits. To test the therapeutic potential of targeting mir-34c signalling, we asked whether inhibition of miR-34c seed would affect memory function in a mouse model for neurodegeneration. Different miRNAs can share the same miRNA seed. In case of miR-34c, the corresponding seed is found in six additional miRs (miR-34a, miR34b, miR-449a/b/c and miR-699; Figure 2C). However, our sequencing analysis shows that these miRNAs are absent or present at very low levels in the hippocampus so that miR-34c accounts for >98% of all

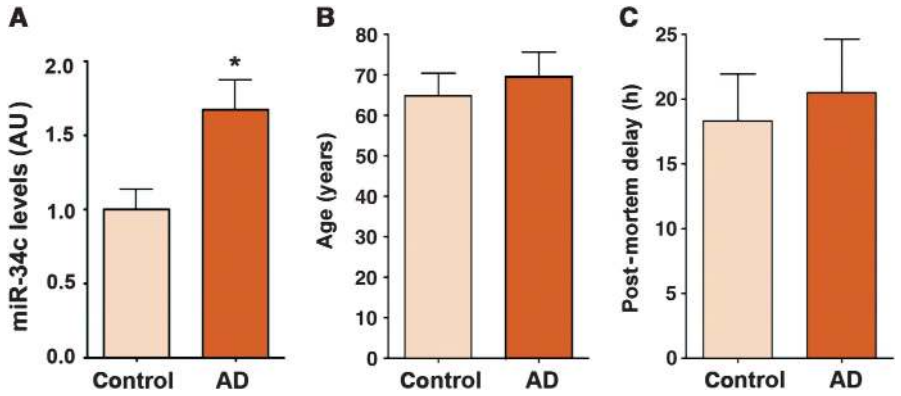


Figure 4 miR-34c is upregulated in AD patients. qPCR analysis was used to measure miR-34c levels in post-mortem tissue samples from AD patients and age-matched controls. (**A**) miR-34c levels (qPCR) are elevated in the hippocampus ($^*P = 0.05$) of human AD patients ($n = 6$; 4 females, 2 males) when compared with age-matched controls ($n = 8$, 2 females, 6 males). Five AD cases were diagnosed as Braack & Braack stage VI and one was diagnosed as Braack & Braack V. There was no significant difference for the age (**B**) or post-mortem delay (**C**) among groups. Error bars indicate s.e.m.

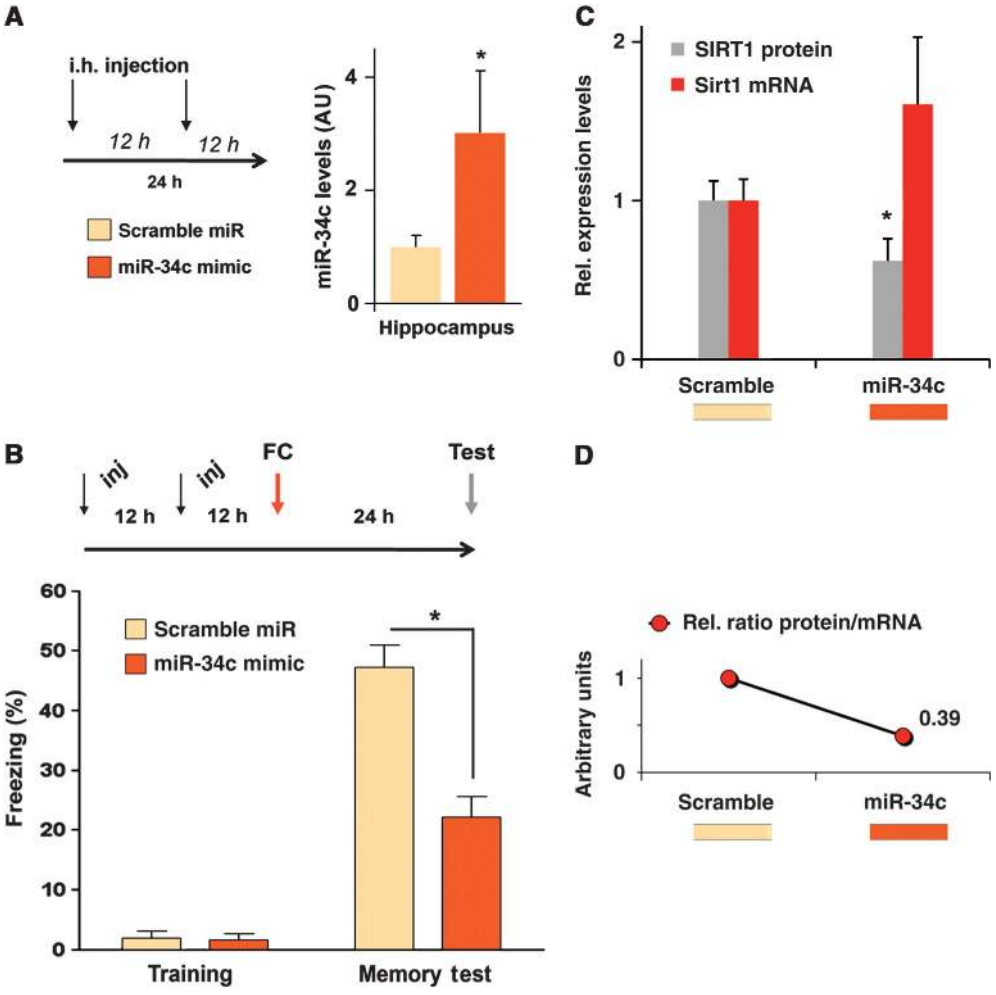


Figure 5 High levels of miR-34c are implicated with memory impairment. (**A**) Left panel: Experimental design for intrahippocampal administration of miR-34c mimic and the respective scramble miR. Right panel: Elevated hippocampal miR-34c levels after miR-34c mimic injection ($n = 5, 7$; $^*P = 0.05$). (**B**) Upper panel, experimental design. Impaired learning in 3-month-old wild-type mice with high levels of miR-34c after treatment with the miRNA mimic (miR-34c) ($n = 6$, $^*P < 0.001$). (**C**) Hippocampal SIRT1 levels correlate with miR-34c expression. miR-34c was injected into the hippocampus as shown in Figure 4A ($n = 4$; $^*P = 0.04$). Sirt1 mRNA and protein levels were measured 24 h after fear conditioning. While elevation of miR-34c did not significantly affected Sirt1 mRNA levels (there was even a trend for increased Sirt1 mRNA, possible through compensatory mechanisms) we observed a dramatic effect in translation of this mRNA, leading to a significant repression of Sirt-1 protein levels. Error bars indicate s.e.m. (**D**) Relative SIRT1 protein/mRNA ratio for the experiment shown in (**A**).

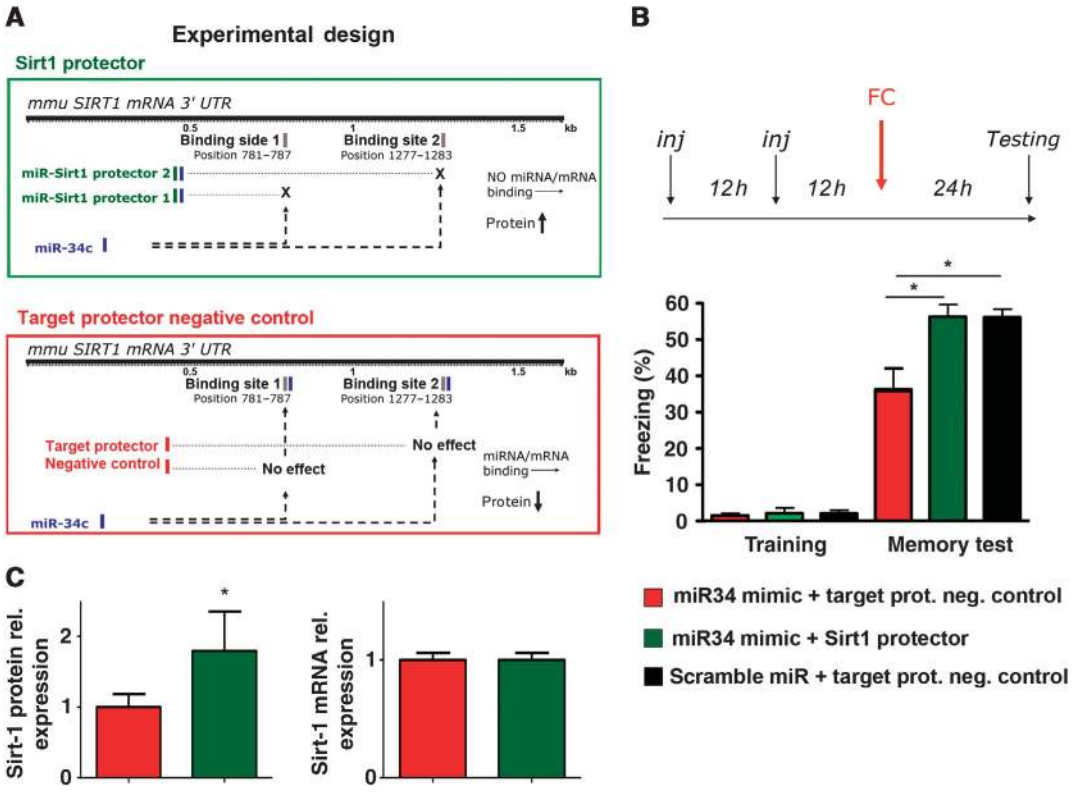


Figure 6 miR-34c induces reduction of SIRT1 protein levels via its binding to SIRT1 3′UTR. (**A**) Experimental design. Upper panel: We designed miR-34c-Sirt1 protectors that hybridize to both miR-34c binding sites within the 3′ UTR of the *Sirt1* mRNA. These protectors prevent miR34c mimic from binding to the *Sirt1* mRNA. Lower panel: A control target protector oligonucleotide was used to exclude non-specific effects. (**B**) Upper panel: Experimental design. Lower panel: While intrahippocampal injection of miR-34c mimic along with the target protector negative control impaired associative learning in the contextual fear conditioning paradigm, co-injection with the miR-34c-*Sirt1* protector rescues learning impairment ($n=8$, 8; $^*P=0.04$). Mice co-injection with a scramble miR and the target protector negative control served as an additional control ($n=8$, 6; $^*P=0.04$). (**C**) SIRT1 protein (left) and mRNA levels (right) in mice treated with the miR-34c mimic and miR-34c-*Sirt1* protector compared with mice injected with miR-34c mimic along with the target protector negative control ($^*P=0.07$; $n=7$, 5). Mmu, mus musculus. Error bars indicate s.e.m.

reads obtained for those six miRNAs (Figure 2C). To exclude any compensatory action of the remaining 2% of miR-34a and miR-34b, the inhibitors used in our experiments targeted also these miRNAs (miR-34 seed inhibitors).

We injected 12-month-old APPPS1-21 mice with our seed miR-34 inhibitors (Figure 7A). Notably, while APPPS1-21 mice injected with the scrambled oligo showed the expected impaired associative memory, miR-34 seed inhibitor-treated mice exhibited a memory performance that was similar to age-matched wild-type mice (Figure 7B). Rescued memory function correlated with restored levels of SIRT1 protein confirming functionality of miR-34 seed inhibitor *in vivo* (Figure 7C). Similar effects were observed when miR-34 seed inhibitors were injected in aged mice (Supplementary Figure S8). Moreover, inhibition of miR-34 seed was able to enhance memory also in 3-month-old wild-type animals (Supplementary Figure S9), an effect that might involve target genes additional to SIRT1.

Discussion

Our study provides a number of novel observations. First, we provide a complete and absolute digitized quantification of the mouse hippocampal miRNAome at an unprecedented depth that will be a suitable tool for further analysis. Of note is the fact that a very small number of miRNAs (23 miRNAs) accounts for almost the total of miRNA reads detected in hippocampus (>80%). This makes the detection of low-abundance sequences difficult when only a small number of reads is produced. In addition, it makes quantification of changes in highly represented sequences equally challenging. These differences between low- and high-abundance miRNAs emphasize the importance of using a high-throughput approach like deep sequencing, with an unlimited fully quantitative dynamic range of signal, in order to determine and compare hippocampal miRNAs expression profile. Thus, to our knowledge, the current study consists the most complete and detailed representation of mouse hippocampus microRNAome until now.

Second, on the basis of our sequencing approach and integrative genomics, we identify miR-34c as a miRNA linked to hippocampal function. miR-34c showed the highest grade of enrichment regarding predicted target genes within a group of hippocampal genes that are regulated during memory formation. A role for miR-34c in the adult brain is further supported by a recent study, in which miRNA expression across different inbred mouse strains was correlated with cognitive phenotype and miR34c was among those miRNAs suggested as potential regulators of cognitive function (Parsons *et al*, 2008). Importantly, our data strongly suggest that miR-34c is implicated with memory impairment in AD. To this end, miR-34c is upregulated in the aging mouse

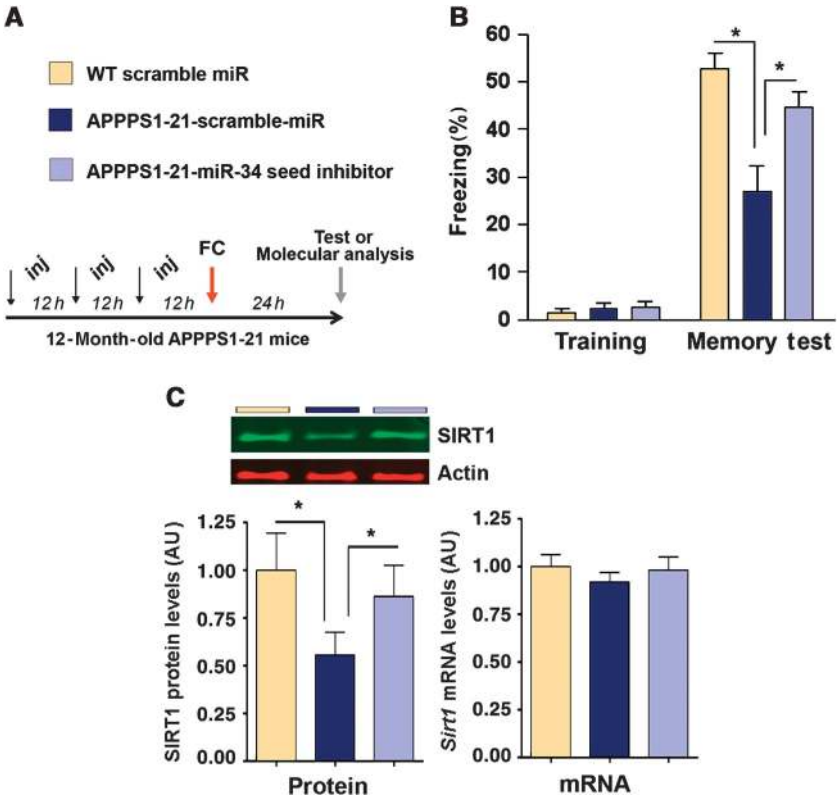


Figure 7 Targeting miR-34c seed rescues learning impairment in mouse model for AD. (**A**) Experimental design. (**B**) Impaired learning of 12-month-old APPPS1-21 mice (*$P = 0.001$) is partially rescued after inhibition of the miR-34c activity (*$P = 0.04$; $n = 7$–8/group). (**C**) SIRT1 protein (left) and mRNA levels (left) in miR34 seed inhibitor-treated mice (*$P < 0.05$; $n = 8$). Error bars indicate s.e.m.

hippocampus, in the hippocampus of APPPS1-21 mice and in the hippocampus of human AD patients. While the miRNA pathway involving miR-34 family has been previously studied in other biological contexts like cancer (Luan *et al*, 2010) its role in the adult brain has not been addressed previously. Our data show that elevation of hippocampal miR-34c levels impairs memory function in mice, suggesting that increased miR-34c expression observed in aged mice, APPPS1-21 mice and in human AD patients contributes to memory impairment. This view is further supported by the fact that targeting miR-34c in cognitively impaired APPPS1-21 and aged wild-type mice was able to improve associative learning. Of note, memory function was improved by only three consecutive injections of miR-34 seed inhibitors within 36 h, indicating that miR-34c directly contributes to AD and age-associated memory impairment. While the mechanisms that leads to the accumulation of miR-34c in the hippocampus of AD patients remain to be elucidated, it is interesting to note that amyloid-β peptides can induce rather rapid changes in miRNA expression in hippocampal neurons (Schonrock *et al*, 2010). The currently accepted view describes miRNAs as important regulators that help to maintain transcriptome homeostasis (Leung and Sharp, 2010). Given that miR-34c is regulated during hippocampal memory formation (see Supplementary Figure S4B), it is therefore also likely that aberrant plasticity, at least in part, contributes to pathologically high levels of miR-34c.

In our study, we used SIRT1 as a read out of miR-34c activity *in vivo*, because SIRT1 was a confirmed target of the miR-34 family in other biological systems (Yamakuchi and Lowenstein, 2009) and we could show that miR-34c also targets *Sirt1* mRNA in neurons. Since loss of SIRT1 has been implicated with impaired memory function (Michán *et al*, 2010) it can be speculated that miR-34c-mediated memory impairment is regulated, at least in part, via decreased hippocampal SIRT1 levels. This idea is supported by the fact that the treatment of APPPS1-21 mice with miR-34 seed inhibitor reinstated physiological SIRT1 levels. In addition, targeting elevated miR-34c levels in aged mice was able to reinstate learning behaviour and physiological SIRT1 levels. Thus, our data suggest that deregulation of the circuit involving miR-34c and SIRT1 contributes to age-associated cognitive decline. Although our data using miR-34c *Sirt1* target protectors indicate that miR-34c mimic regulates memory function via binding to *Sirt1* mRNA, it is unlikely that SIRT1 is the sole target by which miR-34c affects memory function. Further studies are needed to reveal the precise pathways through which this miRNA controls memory formation.

While high levels of miR-34c in disease models correlate with impaired memory function, miR-34c levels are transiently elevated 3 h after mice are exposed to contextual fear conditioning (Supplementary Figure S4B). Interestingly, the transient increase of miR-34c expression in response to fear conditioning follows the gene expression wave of its predicted targets (see Figure 2D and Supplementary Figure S4A and B) and supports a feedback regulation model similar to that already shown for this miRNA in other biological contexts (Yamakuchi and Lowenstein, 2009; see Supplementary Figure S4F). However, it should be noted that molecular

mechanisms underlying the observed improvement of memory function after inhibition of miR-34c activity may differ between young wild-type mice and disease models. In disease models, miR-34c levels are chronically elevated which results in decreased SIRT1 protein levels. Targeting the miR-34 seed in disease models reinstates physiological miR-34c activity and SIRT1 protein levels. Inhibition of miR-34c in young wild-type mice may act through other mechanisms, which may include the regulation of other proteins than SIRT1. Indeed, another experimentally confirmed target of miR-34 family is c-MYC (Cannell and Bushell, 2010; Christoffersen *et al*, 2010) which is also regulated via miR-34c during contextual fear conditioning (Supplementary Figure S10), suggesting a multi-dimensional action of miR-34c.

miRNAs control expression levels by degradation of target mRNA, translational repression or both (Chekulaeva and Filipowicz, 2008; Eulalio *et al*, 2008; Filipowicz *et al*, 2008). In the disease models tested here, mRNA levels of *Sirt1* remained unchanged despite the significant changes in protein levels. miRNA target protection of *Sirt1* mRNA by abnormally high levels of miR-34c also did not affect mRNA levels. Thus, in case of SIRT1, our data show that in the biological context of aging and AD, control by miR-34c is mainly realized through translational repression.

The use of virus-free methods to target gene expression through miRNAs is a promising approach for gene therapy and has been recently applied to the adult mouse brain (Gao *et al*, 2010). Our study reveals the therapeutic potential of such an approach especially for treatment of dementias. Although this technology has just evolved and many technical challenges and questions remain open, our data show that targeting miRNA function in hippocampus with seed inhibitors can have a remarkable effect on the regulation of the respective miRNA targets and cognitive function. Thus, the approach outlined in this study might be of great importance in treating diseases like Alzheimer characterized by abnormal levels of a miRNAs (Lau and de Strooper, 2010).

In conclusion, by using massive parallel sequencing we identify miR-34c as a candidate miRNA implicated with hippocampal and memory function. We furthermore show miR-34c is significantly upregulated in mouse models for AD and in AD patients. Notably, targeting miR-34c rescues memory function in a mouse model for AD-linked amyloid pathology suggesting that strategies to manipulate miR-34c could open a suitable novel virus-free therapeutic avenue to treat cognitive diseases.

Materials and methods

Animals

In all, 3–24-month-old male mice (C57Bl/6J) were housed with free access to food and water under standard light/dark conditions (12–12 h). As a model for AD, we employed the double transgenic APPPS1-21 mouse co-expressing the KM670/671NL 'Swedish' mutation of the amyloid precursor protein (APP) and the L166P mutation of the presenilin 1 (PS1) gene, on C57BL/6J strain background (Radde *et al*, 2006). This mouse exhibits aggressive β-amyloid pathology and cognitive deficits at an early age. All experiments were carried out in accordance with the animal protection law and were approved by the District Government of Germany. Behavioural tests are described in detail in Supplementary data.

Cannulae implants and hippocampal injections

Hippocampal injections were performed as described previously (Sananbenesi *et al*, 2007). In brief, mice were anaesthetized and microcannulae were stereotactically implanted to the hippocampus (1.0 mm posterior to the bregma; 1.0 mm lateral from midline; and 1.5 mm ventral). After recovery from surgery, mice received bilateral injections (1 μl, at a rate of 0.3 μl/min per side) of miRNA mimics and inhibitors at the indicated time points before FC (as indicated by arrows in the experimental schedules of the respective figures). For hippocampal injections, a 1.5-mm gauge needle that extended 0.5 mm beyond the tip of the guide cannula was used. Sequences and transfection conditions for miRNA mimics and inhibitors as well as target protectors used are available in Supplementary data.

Real-time quantitative RT–PCR and western blot analysis

Total RNA (including miRNAs) and protein were isolated from mice hippocampi (both hemispheres for each preparation) using the TRI-Reagent (Sigma-Aldrich, Steinheim, Germany) according to manufacturer's recommendations, but with the step of RNA precipitation in isopropanol extended to a total of 60 min and protein dissolved in 9 M Urea dissolved in 1% SDS. With the exception of experiments involving injection of mimics and inhibitors where the CA regions were specifically dissected under a stereomicroscope (Motic) as described previously (Hagihara *et al*, 2009), whole hippocampal lysates were used. Conversion of 1 μg RNA into cDNA and Real-time PCR detection of miRNAs were carried out using the miScript Reverse Transcription Kit and the miScript SYBR-Green PCR Kit (Qiagen), respectively, on a LC480 LightCycler (Roche Applied Science, Mannheim, Germany). Exact primer sequences and conditions are listed in Supplementary data. Proteins were resolved by SDS–PAGE and western blot analysis was performed as described previously (Kuczera *et al*, 2010), using the following antibodies: Rabbit Sirt-1 (dil. 1:10 000, Millipore), mouse β-actin (dil. 1:2500, sc-69870, Santa Cruz, CA, USA). Further details are available in Supplementary data.

Small RNA massive parallel sequencing

Sequencing was performed in an Illumina GAII Genome Analyzer at Fasteris SA (Plan-les-Ouates, Switzerland) using the Chrysalis 36 cycles v4.0 sequencing kit and RTA SCS v2.6 and GERALD 1.5.1 data analysis pipeline. The respective protocols are provided by Illumina (Illumina, San Diego, CA) at http://www.illumina.com/systems/genome_analyzer_iix.ilmn. Each of the four biological replicates representing a small RNA library from one mouse hippocampus was indexed separately. In total, 24 811 899 reads representing 1 339 842 546 bases were derived, with 97.8% of the reads being attributed to a sample in average. The sequences and insert sequence files, provided in solexa fast-q format, are available through the Galaxy data sharing platform (Taylor *et al*, 2007; Goecks *et al*, 2010) at (http://main.g2.bx.psu.edu/u/fischerlab/h/sm——zovoilis-et-al; published history SM–).

More than 89% of sequenced reads were found within a length range of 18–26 nt, a range covering all known miRNAs, and thus only reads within this range have been further used for mapping (Supplementary Figure S1). Mapping, annotation and quantification of sequenced reads are described in detail in Supplementary data.

Bioinformatics and statistical analysis

For computational prediction of miRNA targets, we used the TargetScan web platform (http://www.targetscan.com/release 5.1; Lewis *et al*, 2005). For functional annotation of miRNA targets, we used the data mining environment provided by the DAVID platform (Huang *et al*, 2009). The functional annotation module was applied for gene ontology terms and terms in PANTHER database using an EASE score of 0.1 and a minimum number of two counts. For testing enrichment in specific KEGG pathways of miRNA targets we used DIANA-mirPath (Papadopoulos *et al*, 2009). The software performs an enrichment analysis of multiple miRNA target genes comparing each set of miRNA targets with all known KEGG pathways. The combinatorial effect of co-expressed miRNAs in the modulation of a given pathway is taken into account by the simultaneous analysis of multiple miRNAs (Papadopoulos *et al*, 2009). For the analysis of the gene expression data regarding miRNA function and determination of their respective enrichment in miRNA targets we used DIANA-mirExTra (default running parameters), an algorithm that can identify miRNA effects to the expression levels of protein-coding transcripts, based on the frequency of six nucleotide long motifs (hexamers) in the 3′UTR sequences of genes (Alexiou *et al*, 2010). Data of qPCR, western

blotting and behavioural studies are expressed as mean ± s.e. (standard error), and unless stated otherwise significance was defined performing Student's *t*-test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: AZ coordinated the RNA sequencing, performed the analysis and contributed to all other experiments. HYA contributed to the experiments related to the *in vivo* manipulation of miR-34c. RCAB performed some of the immunoblot analysis, performed the isolation of hippocampal subregions and performed the experiments shown in Figure 6. RMS performed some of the immunoblot analysis and behavioural experiments. DE performed the experiments shown in Supplementary Figure S4D and contributed to the writing of the manuscript. PR performed some of the qPCR experiments. LF performed the RNA sequencing. ID, AS, PF, SBJ and FS contributed to the analysis of post-mortem brain tissue and SB contributed to some of the qPCR experiments. AZ, HYA and AF conceived the experimental design.

Conflict of interest

The authors declare the following conflict of interests. A patent application for the use of miR-34 to treat neurodegenerative diseases has been filed.

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