Neurobiology of Disease 33 (2009) 422–428

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

MicroRNA regulation of Alzheimer's Amyloid precursor protein expression

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ARTICLE INFO

Article history:
Received 22 August 2008
Revised 14 November 2008
Accepted 25 November 2008
Available online 9 December 2008

Keywords:
MicroRNA
Non-coding RNA
Amyloid precursor protein
Alzheimer's disease
Gene transcription
Secretase

ABSTRACT

Gene dosage effects of *Amyloid precursor protein* (*APP*) can cause familial AD. Recent evidence suggest that microRNA (miRNA) pathways, implicated in gene transcriptional control, could be involved in the development of sporadic Alzheimer's disease (AD). We therefore investigated whether miRNAs could participate in the regulation of *APP* gene expression. We show that miRNAs belonging to the miR-20a family (that is, miR-20a, miR-17-5p and miR-106b) could regulate APP expression *in vitro* and at the endogenous level in neuronal cell lines. A tight correlation between these miRNAs and APP was found during brain development and in differentiating neurons. We thus identify miRNAs as novel endogenous regulators of APP expression, suggesting that variations in miRNA expression could contribute to changes in APP expression in the brain during development and disease. This possibility is further corroborated by the observation that a statistically significant decrease in miR-106b expression was found in sporadic AD patients.

Introduction

The Aβ peptides accumulating in the brain of AD patients result from proteolytic cleavage of APP by β- and γ-secretases (De Strooper et al., 1998; Vassar et al., 1999; Haass, 2004). Increased expression of APP is associated with the pathogenesis of Alzheimer's disease. Indeed, gene duplications of the *app* locus on chromosome 21 is believed to cause AD pathology (Podlisny et al., 1987; Rovelet-Lecrux et al., 2006). Similarly in Down's syndrome (trisomy 21) it is believed that the associated extra copy of the *app* gene is the cause of the large incidence of AD in these patients (Podlisny et al., 1987). Moreover, polymorphisms in the APP promotor that increase transcription have been associated with AD (Theuns et al., 2006). Thus, understanding the mechanisms that regulate APP expression is relevant for our understanding of the pathogenesis of AD.

The small non-coding microRNAs (miRNAs) control gene expression networks at the posttranscriptional level (Ambros, 2004; Bartel, 2004) *via* imperfect complementary binding to the 3′ untranslated region (3′UTR) of target mRNAs leading to their translational repression and sometimes degradation. Abundantly expressed in the central nervous system, several miRNAs show a high degree of temporal and spatial specificity (Barad et al., 2004; Miska et al., 2004; Sempere et al., 2004; Smirnova et al., 2005) and are thought to play a role in neuronal cell specification, differentiation and synaptic plasticity (Smirnova et al., 2005; Kosik, 2006; Schratt et al., 2006).

The possibility that miRNA pathways could contribute to neurodegenerative disorders in human is gaining popularity (Hebert and De Strooper, 2007). Indeed, potential roles for miRNA dysfunction in the development of sporadic Parkinson's disease have been proposed (Kim et al., 2007; Wang et al., 2008a). In addition, we (Hebert et al., 2008) and others (Wang et al., 2008b) have shown that miRNAs might contribute to increased BACE1/β-secretase expression in sporadic AD. Here, we extended this investigation and asked whether miRNAs could be involved in the gene expression regulation of APP.

Materials and methods

Patient information

The non-dementia ($n=11$) and AD dementia ($n=19$) patients were from the Geriatric Department of E. Roux Hospital at Limeil-Brevannes and the Lille CH&U Hospital, France (ADERMA network). Clinical, neuropathologic, biochemical and genetic data for these patients were presented elsewhere (Hebert et al., 2008). Blocks from the anterior temporal cortex or cerebellum were dissected from each case and snap frozen in liquid nitrogen.

Abbreviations: ORF, Open reading frame; qRT-PCR, Quantitative RT-PCR; 3′UTR, 3′ untranslated region.

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Available online on ScienceDirect (www.sciencedirect.com).

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doi:10.1016/j.nbd.2008.11.009

Cell lines

HeLa, Neuro2A and SK-N-SH cells were cultured DMEM/F12 medium supplemented with 10% FCS as described (Hebert et al., 2006). Primary cultures of cortical neurons were prepared from embryonic day 17 C57/BL6 mice as described (Camacho et al., 2004). Mouse ES cells were differentiated into pure (~90–95%) glutamatergic neurons as described (Bibel et al., 2004). Specific technical details are available on demand.

Antibodies

Polyclonal APP B63 (previously named B10) (Hebert et al., 2006), monoclonal E2F1 (#KH95, Santa Cruz Biotechnology, Inc.), monoclonal N-RAS (#F155, Santa Cruz Biotechnology, Inc.) and monoclonal β-Actin (Sigma-Aldrich) were used.

Protein extraction and Western blot analysis

Cells were rinsed with cold PBS and lysed in buffer: 1% Triton X-100, 50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA and complete protease inhibitors (Roche). Protein from brain (human and mouse) and neurons was extracted using the miRVana PARIS kit (Ambion). Immunoblot analysis was performed as described (Hebert et al., 2006).

RNA extraction and quantitative RT-PCR

2–6 total brains from wild-type BL/6 mice or 2–3 sister cultures from neurons (primary or ES cell-derived) were pooled per time point. Total RNA was extracted using the miRVana PARIS kit (Ambion) according to the manufacturer's instructions. RT-PCR as well as quantitative PCR procedures was carried out as described (Hebert et al., 2006). Primer sequences to quantify mRNA are: human APP forward 5′ AAAAC-GAAGTTGAGCCTGTTGAT 3′, reverse 5′ GAACCTGGTCGAGTGGTCAGT 3′; human β-Actin forward 5′ CACCCTGAAGTACCCCATGG 3′, reverse 5′ TGCCAGATTTTCTCCATGTCG 3′. For miRNA quantifications, probe-specific Taqman miRNA assays (Applied Biosystems) were used according to the manufacturer's instructions. Relative expression was calculated by using the comparative CT method.

Northern blotting

A pre-made 'mouse brain aging blot' (See-gene) was used which contained ~20 μg total RNA per lane isolated from mouse brain at

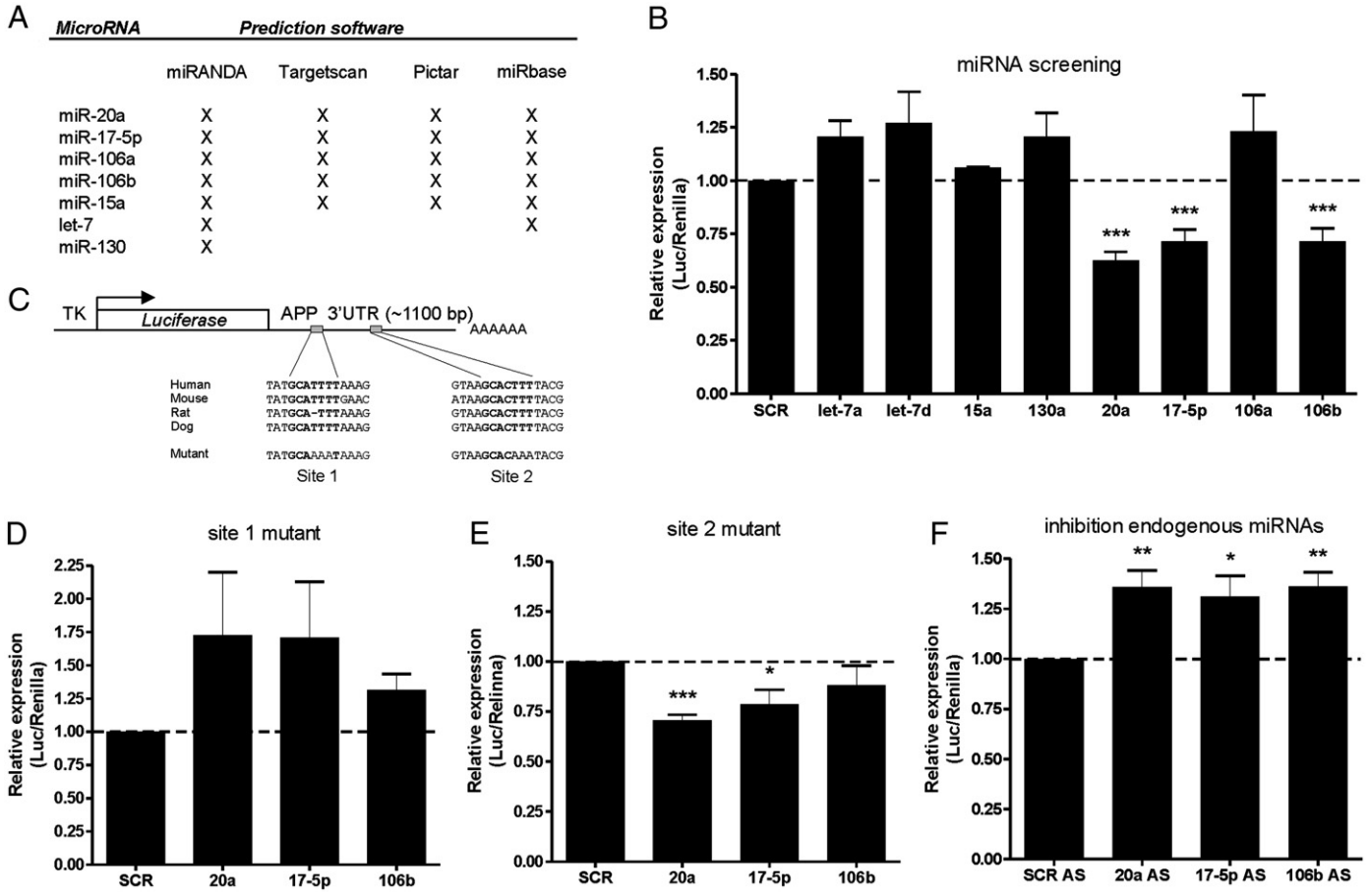

Fig. 1. Validation of candidate miRNAs. (A) List of candidate miRNAs used in this study identified by various algorithms: miRanda (microrna.org), Targetscan (targetscan.org), Pictar (pictar.bio.nyu.edu) and miRbase (microrna.sanger.ac.uk). "X" indicates positive hit in the algorithm. (B) APP 3′UTR wildtype luciferase and *Renilla* luciferase constructs were co-transfected into HeLa cells with the indicated pre-miRNA oligonucleotides (scrambled sequence, let-7a, let-7d, miR-15a, miR-130a, miR-106a, miR-106b, miR-17-5p and miR-20a) at a final concentration of 75 nM. (C) Schematic representation (not to scale) of the APP 3′UTR luciferase construct used in this study. TK, thymidine kinase promotor. The sequence as well as the "top score" putative binding sites for miR-20a, miR-17-5p, miR-106a and miR-106b is shown. The miRNA seed sequences are in bold. In the APP 3′UTR mutant constructs (site 1 or site 2), the binding sites for miRs are mutated as indicated. (D) (E) APP 3′UTR mutant constructs were co-transfected with the indicated pre-miRs at a final concentration of 75 nM. (F) APP 3′UTR wildtype luciferase construct was co-transfected into HeLa cells with the indicated anti-(complementary) miRNA oligonucleotides at a final concentration of 75 nM. In all experiments, normalized sensor luciferase activity is shown as a fold difference of the scrambled oligonucleotide control. Error bars represent standard deviations derived from three or more independent experiments performed in duplicate. Statistical significance between control (scrambled miR-treated) and candidate pre-miR-treated HeLa cells was determined by a Student's paired *t* test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

different developmental stages. The blot was subsequently hybridized with probes specific for mouse GAPDH and APP mRNAs. The APP probe (407 bp) was PCR amplified from mouse APP cDNA. The GAPDH probe (543 bp) was provided by the company.

Transfections, DNA cloning and luciferase assays

150,000 (HeLa), 250,000 (Neuro2A) or 500,000 (SK-N-SH) cells were plated in 6 well plates. The next day, cells were transfected with 75 nM of pre-miRs (see list in text) (Pre-miR™ miRNA Precursor Molecules, Ambion), anti-miRs (miRCURY LNA™ microRNA knock-down probes, Exiqon) or scrambled sequence sense or antisense (Ambion or Exiqon) using LipofectAMINE 2000 following the manufacturer's instructions. 48 h post-transfection, cells were processed for immunoblot analysis. The 3′UTRs of APP (~1100 bp) and the TK promoter were amplified from human chromosomal DNA and cloned into the pGL3-luciferase basic vector (Promega). Inserts were confirmed by sequencing. For the luciferase assays, 75 nM of pre-miRs (Ambion) was co-transfected with the sensor vector (wt or mutant) and the *Renilla* control vector (Promega). 26–28 h post-transfection, the measurements were performed using the Dual luciferase reporter assay kit (Promega). The APP luciferase mutant construct was generated using the QuickChange II K-XL Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Statistical methodologies

APP and β-Actin densitometric quantifications were performed using *ImageQuant* software (Amersham Biosciences). Statistical significance was determined using a Student's paired *t* or Mann Whitney *U* test as indicated in the text. Calculations were made using *GraphPad Prism 4* software.

Results

APP is a miRNA target gene

We searched for potential miRNA target sites within the 3′UTR of APP mRNA using available bioinformatics logarithms on-line (miRANDA, TargetScan, miRBase and Pictar). Based on this screen, we selected miRNAs miR-20a, miR-17-5p, miR-106a, miR-106b, miR-15a, let-7a, let-7d and miR-130 for further validation (Figs. 1A, B). Please note that miR-20a, miR-17-5p, miR-106a and miR-106b belong to the same miRNA family (microrna.sanger.ac.uk). Of note, these predicted miRNA target sites are highly conserved in 4 different mammals, including human, mouse, rat and dog (Fig. 1A).

We generated a reporter construct containing the full-length (~1100 bp) human APP 3′UTR downstream of *luciferase* (Fig. 1C) and co-transfected this with synthetic miRNA precursors (pre-miRs) in HeLa cells. We found that miR-20a, miR-17-5p, and miRNA106b

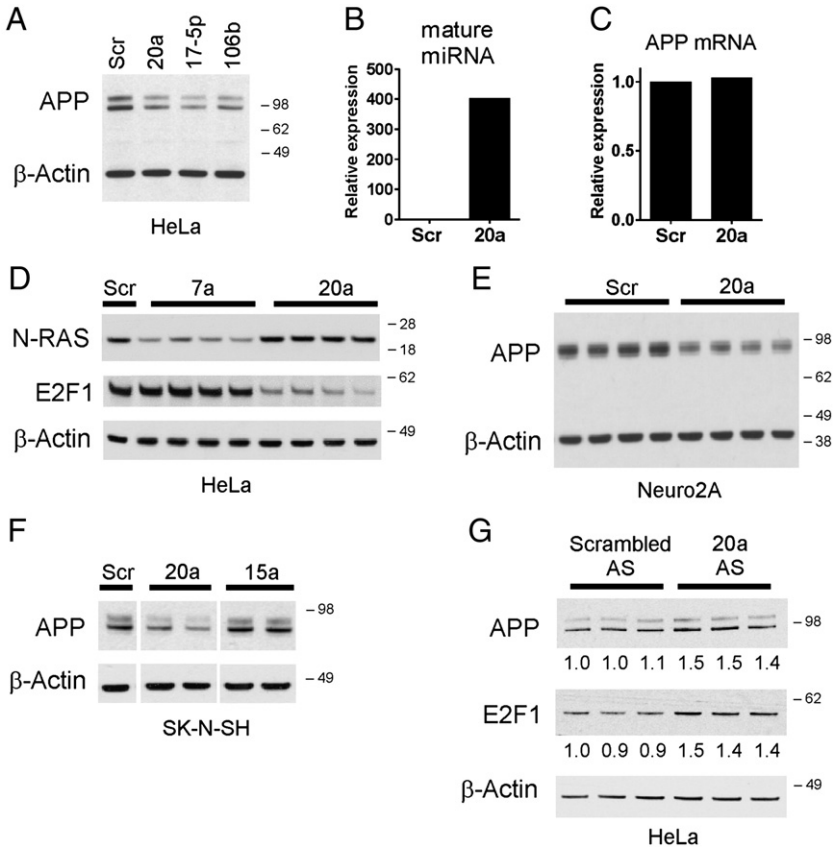

Fig. 2. Regulation of APP by miRNAs. (A) Western blot analysis of endogenous APP and β-Actin in HeLa cells treated with the indicated pre-miRs. A scrambled oligonucleotide sequence (Scr) was used as control. (B) qRT-PCR of control or pre-miR-20a-transfected HeLa cells (75 nM). Increase (in fold, compared to scrambled sequence) in mature miR-20a levels is indicated. (C) qRT-PCR of APP mRNA levels (in fold) of HeLa cells treated with 75 nM of pre-miR-20a or a control scrambled sequence. The average value of two independent experiments is shown. GAPDH was used as qRT-PCR normalization control. Similar results were obtained using HPRT as qRT-PCR normalization control (data not shown). (D) Western blot analysis of N-Ras and E2F1 in HeLa cells treated with pre-miR-20a or pre-let-7a. Quadruplicate samples are shown. (E) Endogenous APP in Neuro2A cells treated with 75 nM of pre-miR-20a or oligonucleotide control. Quadruplicate samples are shown. (F) Endogenous APP in SK-N-SH neuroblastoma cells treated with 75 nM of pre-miR-20a. Here, a scrambled oligonucleotide and pre-miR-15a were used as controls. Duplicate samples are shown. (G) Endogenous APP and E2F1 in cells treated with 75 nM of antisense anti-miR-20a oligonucleotides. An antisense scrambled oligonucleotide was used as control. β-Actin was used as loading and normalization control. E2F1 is shown as a positive control. Triplicate samples are displayed. Numbers indicate fold changes when compared to β-Actin.

affected significantly luciferase expression (Fig. 1B). In contrast, a scrambled miRNA sequence, as well as the other candidate miRNAs (miR-15a, miR-130a, let-7d and let-7a) showed no effect in this assay. Curiously, miR-106a, which is closely related to miR-106b, had in this assay an inconsistent effect. This might allude at the importance of non-seed sequences in the recognition of mRNA target sites. We confirmed the effect on the luciferase reporter of miR-20a, miR-17-5p and miR-106b in HEK293 and in COS1 cells ruling out cell-type specific effects (data not shown).

We also excluded off-target effects, by mutating the two most likely ("top score") miRNA binding sequences within the 3′UTR of APP mRNA (site 1 and site 2, see Fig. 1C). Mutation of the first putative miRNA binding site indeed abolished the suppressing effects of miR-20a, miR-17-5p and miR-106b (Fig. 1D), while mutation of site 2 had no significant effects on luciferase activity (Fig. 1E). As miR-20a, miR-17-5p and miR-106b are expressed at endogenous levels in HeLa cells (O'Donnell et al., 2005), we checked finally whether these contributed also to the regulation of the luciferase reporter. We transfected complementary anti-miR oligonucleotides and these caused a significant increase in luciferase expression (Fig. 1F). Thus we identify three miRNAs that bind directly and specifically to the 3′UTR of APP.

Regulation of endogenous APP by miRNAs

We next investigated whether the identified miRNAs could also affect endogenous APP expression. Transient transfection of miR-20a, miR-17-5p and miR-106b affected the expression of APP (Fig. 2A). In contrast, a scrambled control (Fig. 2A, lane 1) as well as miR-130a, miR-15a and let-7a (data not shown) did not affect APP expression, consistent with the luciferase assays. Of mention, the APP family member APLP2, which is functionally related to APP but does not contain a putative target site for miR-20a, miR-17-5p and miR-106b, was not affected by the overexpression of these miRNAs (data not shown), thus providing an additional internal control. By qRT-PCR, we confirmed the increase in expression of mature (functional) miR-20a in the transfected cells (Fig. 2B). Of note, APP mRNA expression was not affected by miR-20a overexpression (Fig. 2C), indicating that this miRNA blocks protein translation and does not induce (direct) degradation of the target mRNA. The effects of miRNA transfection on APP expression are of similar extents to what is seen with previously characterized miRNA target genes, E2F1 (O'Donnell et al., 2005) and N-Ras (Johnson et al., 2005), which were included as positive controls (Fig. 2D). We confirmed the effects of miR-20a in

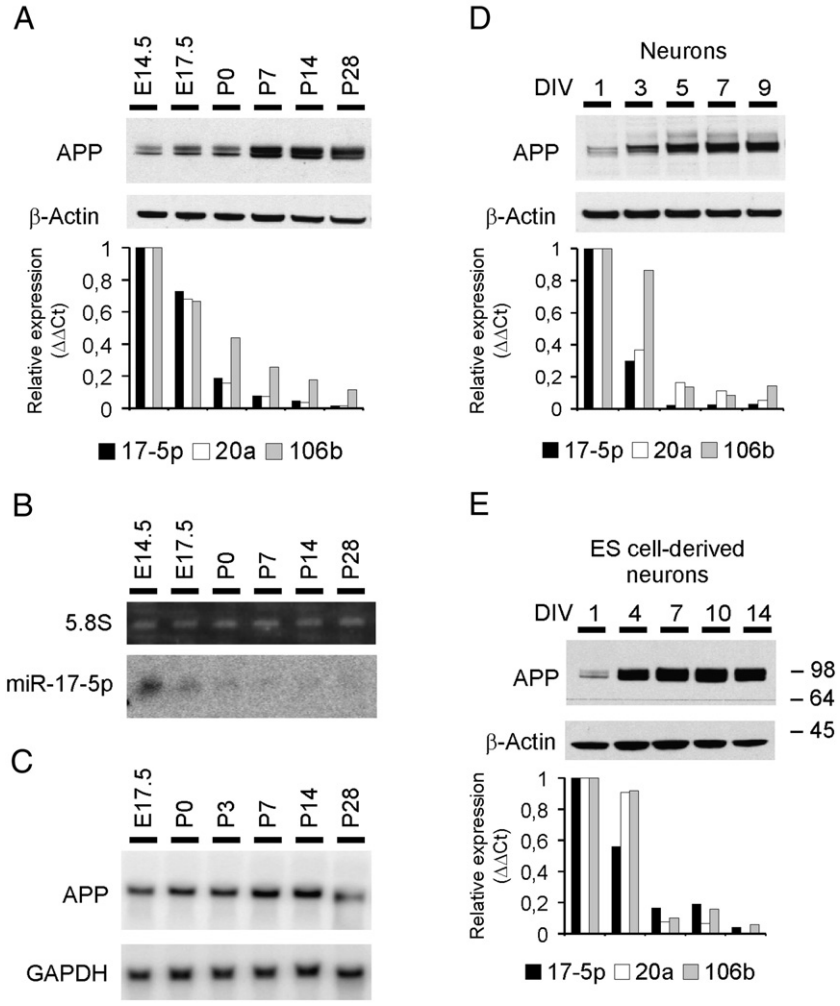

Fig. 3. APP and miRNA co-expression in the developing brain and in primary neurons. (A) Western blot analysis of APP from pre- and postnatal mouse brain. β-Actin was used as loading control. Mature miR-17-5p, miR-20a and miR-106b expression levels were measured by qRT-PCR from total mouse brain RNA. RNU19 was used as normalization control. The relative expression (in fold) of miRNAs was calculated using the relative quantification method (using E14.5 as 1 fold). Note that miR-20a and miR-17-5p are downregulated approximately 100 fold during this time period. miR-106b is downregulated approximately 10 fold. Each time point represents the average of 2 to 6 brains. (B) Northern blot analysis for miR-17-5p from total RNA. Ribosomal 5.8S was used as loading control. (C) Northern blot analysis of murine APP mRNA levels. GAPDH was used as loading control. (D) APP and miRNA co-expression in primary cultured neurons or (E) ES cell-derived glutamatergic neurons. Two to three culture dishes of neurons were collected at each time point (days *in vitro*, DIV), pooled, and analyzed by Western blot for APP and β-Actin. qRT-PCR of miR-20a, miR-17-5p and miR-106b is shown.

neuroblastoma mouse Neuro2A (Fig. 2E) and human SK-N-SH (Fig. 2F) cells.

We verified also that blocking endogenously expressed miR-20 with an antisense oligonucleotide increased endogenous APP protein levels by ~50% (Fig. 2G). By qRT-PCR, we observed approximately 50–75% downregulation of endogenous miRNAs in these experimental conditions (data not shown). As this effect on protein levels was rather weak, we benchmarked this observation using E2F1 as a previously characterized internal control. Thus, the identified miRNAs regulate endogenous APP levels. Moreover, the antisense experiments demonstrate that these miRNAs, when expressed endogenously, are involved in the fine-tuning of APP expression, further validating the biological relevance of our observations.

Developmental co-regulation of APP and miRNAs in neurons

APP expression is tightly regulated during brain development (O'Hara et al., 1989). We therefore measured the developmental regulation of specific miRNAs during brain development using qRT-PCR. As shown in Fig. 3A, a dramatic reduction of miR-20a, miR-17-5p and miR-106b during brain development in mouse is remarkably well correlated with the upregulation of APP protein levels, whereas other candidate miRNAs are not correlated at all (data not shown). We confirmed in an independent approach these changes for miR-17-5p in Northern blot experiments (Fig. 3B).

While APP expression at the protein level changes dramatically during development (Fig. 3A), the APP mRNA levels remain remarkably stable over the analyzed time period (Fig. 3C). This is entirely consistent with the hypothesis that APP regulation during development is, at least partially, dependent on regulation of translation by miRNAs.

To confirm that the identified miRNAs could regulate APP in a cell autonomous fashion, we analyzed APP expression in primary mouse cortical neurons (Fig. 3D) and in glutamatergic neurons derived from mouse embryonic stem (ES) cells (Fig. 3E), and confirmed the tight correlation in expression between APP and miR-20a, miR-17-5p and miR-106b in these cells. Taken together, these results agree with the hypothesis that miRNAs could be involved in the regulation of APP during neuronal development.

Misregulation of miR-106b in sporadic AD brain

We finally asked whether changes in miRNAs could be associated with APP expression and AD. Using qRT-PCR, we found that miR-106b is expressed more abundantly in human brain (controls, $n=5$, average 65 years old) than miR-20a and miR-17-5p. Note here that we discuss relative levels of expression of miRNAs. These results are consistent with Northern blot experiments for miR-106b (as well as miR-20a and miR-17-5p) expression in mouse brain (Ventura et al., 2008).

We found a statistically significant reduction in expression of miR-106b (Fig. 4B), but not miR-20a (Fig. 4C), in AD brain ($n=19$) when compared to controls ($n=11$). Here, relative expression (crude ΔCt values are shown) was calculated using ubiquitously expressed miR-16 as normalization control, which was shown to be less variable in human brain samples (Hebert et al., 2008). The reduction in expression of miR-106b in AD brain is in line with previous miRNA

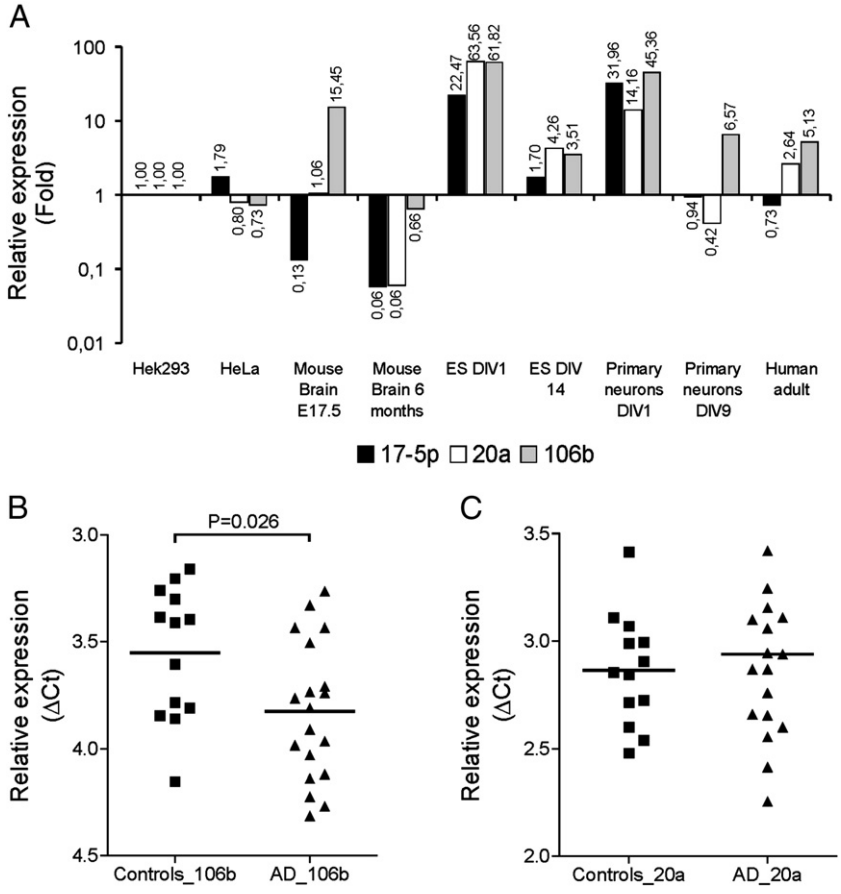

Fig. 4. Decreased miR-106b levels in sporadic AD brain. (A) miR-17-5p, miR-20a and miR-106b levels were quantified by qRT-PCR in various tissues/cell lines used in this study. U19 small nucleolar RNA (RNU19) was used as normalizing control. The relative expression was calculated using the ΔΔCt method (using Hek293 cells as 1 fold). (B) miR-106b and (C) miR-20a levels were quantified by qRT-PCR in the brain of controls ($n=11$) and AD patients ($n=19$). The ubiquitously expressed miR-16 was used as normalization control. Relative expression was calculated using the ΔCt method. Note that crude Ct values for miR-16, miR-106b and miR-20a all range from 20 to 25. Statistical significance was determined by a Mann–Whitney test.

profiling experiments (Hebert et al., 2008). Although the change in miR-106b expression level in AD patients is significant, we could not further establish a correlation between this change and APP expression as APP levels varied enormously, even in control brains [data not shown and Hebert et al. (2008)], which suggests that multiple parameters affect APP expression in the brain. This is not entirely unexpected as a variety of parameters, including brain trauma, can cause APP upregulation (Leyssen et al., 2005).

Discussion

The current work provides strong evidence that the expression of APP is regulated by miRNAs. This conclusion is based on: (1) *in vitro* validation experiments showing that luciferase expression under the control of the APP 3′UTR sequence can be regulated by selected miRNAs (such as miR-20a). Downregulation is mediated by direct binding of these miRNAs to an identified target site in the APP 3′UTR as mutation of this region abrogates this effect. (2) The direct demonstration that overexpression of miR-20a (and miR-17-5p/106b) can regulate the expression of endogenous APP protein in cell lines *in vitro*, and that inhibition of endogenous miR-20a with antisense nucleotides can inversely affect endogenous APP expression. Importantly, APP expression regulation by miRNAs is observed in different cell types, including mouse and human neuroblastoma cell lines. (3) The remarkable correlation in expression of specific miRNAs, miR-20a, miR-17-5p and miR-106b, with APP during brain development and in differentiating neurons *in vitro*. Importantly, APP mRNA levels did not significantly change during the investigated developmental window, which is typical for miRNA-regulated transcription. Furthermore, these miRNAs are expressed together with APP protein in primary neurons and in glutamatergic neurons derived from ES cells, in agreement with a presumed cell autonomous mode of action. We have tried to modulate miRNA expression in primary neurons directly, but transfection efficiency with pre-miRs and anti-miRs was too low to make reliable conclusions from these assays. Our data nevertheless suggest strongly that miR-20a, miR-17-5p and miR-106b may contribute, at least in part, to the developmental regulation of APP expression in brain and in neurons differentiating *in vitro* (O'Hara et al., 1989).

The involvement of other miRNAs or posttranscriptional mechanisms (such as protein turnover, transport, etc.) in APP expression regulation should not be ignored, although transcriptional regulation of APP expression (Theuns and Van Broeckhoven, 2000) seems in the developmental setting less important than miRNA regulation, as APP mRNA expression was not significantly changed during the developmental time window we investigated.

Other reports have associated miR-20a (and 17-5p), which belongs to the miR-17-92 cluster on chromosome 13 in mouse, in the regulation of cellular proliferation (Hayashita et al., 2005; Hossain et al., 2006; Fontana et al., 2007; Venturini et al., 2007). Misregulation of this cluster has been linked to cancer in mouse and human (He et al., 2005; O'Donnell et al., 2005). Gene knockout of the miR-17-92 cluster in mouse leads to neonatal lethality with defects in the development of heart, lungs, and B cells (Ventura et al., 2008). It is estimated that each miRNA can regulate hundreds of different target genes, controlling important biological pathways (Alvarez-Garcia and Miska, 2005; Baek et al., 2008; Selbach et al., 2008). Beside APP, miRNAs of the miR-20a family target E2F1 (O'Donnell et al., 2005) and p21/CDKN1A (Ivanovska et al., 2008) involved in cell cycle control. Whether APP and these proteins function synergically in cellular proliferation and in neuronal development is an interesting possibility that we will address in further work.

Recent genetic evidence suggest that misregulation of expression of the *APP* gene can cause AD. *APP* gene duplications (Rovelet-Lecrux et al., 2006) or trisomy 21 (Podlisny et al., 1987), and *APP* gene promoter mutations (Theuns et al., 2006) that increase its expression, have been implicated in the disease process. Our work therefore raises the interesting question whether changes in miRNA expression could contribute to AD pathogenesis. Some evidence for this hypothesis was obtained in our previous study that showed a clear correlation between increased BACE1 levels and decreased miR-29a/b-1 expression (Hebert et al., 2008). While we find in the current study a significant decrease in miR-106b expression in the brain of AD patients, due to the large variation in APP expression levels in control and diseased brain, the correlation between miR-106b and APP expression in AD brain was not significant (data not shown). Further work is therefore needed to establish this link with certainty.

Finally, modulators of APP expression are very attractive therapeutic targets for AD. The results provided herein suggest that small RNA oligonucleotides, such as antagomiRs (Krutzfeldt et al., 2005; Krutzfeldt et al., 2007) or pre-miRs can be used to modulate endogenous miRNA and APP levels in the brain.

In conclusion, our work strongly indicates that APP expression is partially regulated by a series of specific miRNAs in physiological conditions. This sets the stage for further investigations to explore the role of this regulatory mechanism in the physiology and pathophysiology of this intriguing protein.

Acknowledgments

We would like to thank Diana Dominguez for performing the APP Northern blots. This work was supported by an excellence grant from the Belgian “Stichting voor Alzheimer onderzoek”, the Fund for Scientific Research, Flanders, K.U.Leuven (GOA and Methusalem grant), the Federal Office for Scientific Affairs (IUAP P6/43), Belgium, and AIRMA (Association Internationale pour la Recherche sur la Maladie d'Alzheimer) for the grant funding to support the brain bank of AD. SSH was a Marie Curie fellow.

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