The amyloid precursor protein (APP) and its proteolytic product amyloid beta (Aβ) are associated with both familial and sporadic forms of Alzheimer disease (AD). Aberrant expression and function of microRNAs has been observed in AD. Here, we show that in rat hippocampal neurons cultured in vitro, the down-regulation of Argonaute-2, a key component of the RNA-induced silencing complex, produced an increase in APP levels. Using site-directed mutagenesis, a microRNA responsive element (RE) for miR-101 was identified in the 3’-untranslated region (UTR) of APP. The inhibition of endogenous miR-101 increased APP levels, whereas lentiviral-mediated miR-101 overexpression significantly reduced APP and Aβ load in hippocampal neurons. In addition, miR-101 contributed to the regulation of APP in response to the proinflammatory cytokine interleukin-1β (IL-1β). Thus, miR-101 is a negative regulator of APP expression and affects the accumulation of Aβ, suggesting a possible role for miR-101 in neuro-pathological conditions.

Alzheimer disease (AD) is the most common form of dementia in aged individuals and is characterized by Aβ plaques, which contain Aβ aggregates and neurofibrillary tangles which consist primarily of aggregated forms of the microtubule-stabilizing protein tau (reviewed in Ref. 1). Aβ peptides are derived from processing of the type I transmembrane protein APP through sequential cleavages by β and γ secretase (2–3). The Aβ load during pathology leads to neurological dysfunction. APP is linked to AD; familial AD can be caused by increased expression of APP due to either genomic duplication (4–5) or regulatory sequence alterations (6). Among the physiological and pathological activators of APP expression (7–8) is the proinflammatory cytokine IL-1β (9). IL-1β is produced in the central nervous system (CNS) in response to damage and influences neuronal function by interacting with the type I IL-1 receptor expressed on neurons (10–11). IL-1β is overexpressed in AD (12) and has been implicated in initiation and progression of AD pathology (13). In addition, IL-1β promotes APP transcription (14) and translation (9) in various cell types. Transcriptional and post-transcriptional regulation of APP expression has been widely studied and correlated to AD pathogenesis (15–16). Both cell type-specific promoter elements (17) and regulatory elements in the 5’- and 3’-UTRs of APP mRNA have been identified (9, 18).

MicroRNAs are an intriguing class of small noncoding RNA molecules which, in mammals, regulate gene expression primarily by imperfect base pairing with the 3’-UTR of specific target mRNAs (19). MicroRNAs associate with Argonaute proteins (Ago1–4, in mammals) (20–21), which constitute the core of the RNA-induced silencing complex (RISC), and mediate post-transcriptional repression of target messenger RNAs (19). Ago2 is expressed at high levels in human (22) and mouse (23) brain and Argonaute expression profiling or depleting Ago1–4 has been used to identify potential microRNA targets (24–25). Several studies have indicated that microRNAs define the spatial and temporal expression profiles of genes involved in neuronal development and differentiation (26). In addition, microRNAs are recruited during the execution of neuronal signal transduction pathways (27). Emerging evidence suggests that changes in expression of microRNAs are associated with neurodegenerative diseases (28). Profiling microRNAs from selected human brain areas has revealed significant changes in AD patients (29–31). A few microRNAs, involved in the regulation of genes causally linked to Alzheimer’s disease, are dysregulated in human AD patients (30, 32–33) and AD mouse models (34).

The hippocampus is one of the main brain regions affected during the early stages of AD, and changes in the hippocampus coincide with the memory deficits observed in AD patients. Therefore, elucidation of the molecular mechanisms regulating APP expression in primary hippocampal neurons will be useful in understanding this disease.

In the present work, we focused on identifying a microRNA regulating APP expression in primary cultures of rat hippocampal neurons. In addition, we have analyzed the response of this microRNA to treatment of the cultures with IL-1β.
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EXPERIMENTAL PROCEDURES

Cell Cultures—Hippocampal neurons were prepared from embryonic day 17–18 (E17–18) embryos from timed pregnancy Wistar rats (Charles River). The hippocampus was dissected out in Heps-buffered Hanks’ balanced salt solution and dissociated via trypsin/EDTA treatment. Cells were plated at 1 × 10^6 cells on 3.5-cm dishes precoated with poly(o-l-lysine) and cultured in neurobasal medium supplemented with B-27 and glucose.

RNA Extraction and Analysis—Total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. For Northern analysis of miRNAs, total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. For Northern analysis of miRNAs, total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer’s instructions.

Protein Extraction and Immunoblot Analysis—Brain tissues were homogenized in radioimmune precipitation assay buffer (1% Triton, 0.1% SDS, 0.5% deoxycholic acid, 50 mM NaPO4, 150 mM NaCl, 2 mM EDTA) supplemented with protease inhibitor mixture (Sigma-Aldrich). The same buffer was used to extract protein from cultured cells by scraping the cells from the culture dishes. Equal amounts of total protein were fractionated by electrophoresis using an 8% SDS-polyacrylamide gel, electroblotted to nitrocellulose membrane (Hybond-ECL, GE Healthcare), and blotted using anti-APP (4G8, 1:500, Sigma-Aldrich) or anti-cyclooxygenase-2 (Cox-2, Cayman) overnight at 4°C. Incubation with secondary peroxidase-coupled anti-mouse was performed at room temperature for 1 h. Immunoreactivity was determined by chemiluminescence (GE Healthcare).

Neuronal αB Fibril Isolation and Immunoblot Analysis—αB fibrils were obtained from 15 ml of culture medium (10^7 cells) treated with a TURBO DNA-free™ kit (Ambion), reverse-transcribed with SuperScript® III Reverse Transcriptase (Invitrogen), and amplified with FastStart Universal Probe Master (Roche) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Probes detecting TATA-binding protein (TBP), APP, and Ago2 were chosen from the Roche Universal Probe Library and used as recommended by the manufacturer with the corresponding primers designed by the QuikChange® Primer Design Program. Probe H129 was used with rno-APP-659-678FW 5'-GGAGCGGACACAGACTATGCG-3' and rno-TBP-1081-1103 RW 5'-CAATTCTGGGTGGATCATTCTG-3'; probe 69 with rno-APP-659-678FW 5'-GGAGCGGACACAGACTATGCG-3' and rno-TBP-1081-1103 RW 5'-CAATTCTGGGTGGATCATTCTG-3'; probe 77 with rno-Ago21548-1559FW 5'-AACACATACGCTGGTCTCCA-3' and rno-1603 1621AGO2RW 5'-CTCCACACCGTCTTACC-3'. Relative changes in gene expression were quantified using the comparative threshold method (Ct) after determining the Ct values for reference (TBP) and target genes in each sample set according to the 2^-ΔΔCt method. TBP was used as an endogenous control. All reactions were performed in triplicate.

Quantitative RT-PCR for miR-101—The Taqman microRNA reverse transcription kit (Applied Biosystems) and the FastStart Universal Probe Master (Roche) were used. The U6 snRNA was used for normalization of samples. The quantitative PCR procedure was carried out according to the instructions provided with the TaqMan microRNA assay kit (Applied Biosystems).

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RNA Extraction and Analysis—Total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. For Northern analysis of miRNAs, total RNA was separated on Tris-borate/EDTA 10% polyacrylamide gel, electroblotted to a Hybond-XL membrane (GE Healthcare). Hybridization was performed by real-time quantitative PCR. RNA was normalized to those obtained from U6 RNA. Band intensities were calculated using the Quantity One (Bio-Rad) software and normalized to those obtained from U6 RNA. Band intensities were calculated using the Quantity One (Bio-Rad) software and normalized to those obtained from U6 RNA.
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conditioned for 96 h with hippocampal neurons. Neurobasal medium was centrifuged at 10,000 × g, for 30 min to remove cell debris and, subsequently, ultracentrifuged at 100,000 × g, for 4 h at 4 °C to achieve sedimentation of fibrils and protofibrils (35). The sedimented material was resuspended in 70% formic acid, sonicated, and dissolved in 2× Tricine buffer. Samples were analyzed by electrophoresis on 10–16% Tris–Tricine gels, electroblotted to a nitrocellulose membrane (Hybond–ECL, GE Healthcare), and assayed with anti-APP (4G8, 1:500) or 6E10 (Signet).

**Immunocytochemistry**—Hippocampal neurons were fixed for 15 min in phosphate-buffered saline containing 4% formaldehyde and 4% sucrose, permeabilized with 0.1% Triton X-100 for 8 min, and processed for labeling with mouse monoclonal anti-APP (4G8). Nuclei were stained with Hoechst 33258 (0.5 μg/ml, Sigma Aldrich). A secondary antibody coupled to Alexa 594 was obtained from Molecular Probes (Invitrogen). Digital images were obtained with an Olympus BX51 microscope (100× and 60× oil objectives) equipped with a Spot Diagnostic Instruments camera and collected with Spot image analysis software.

**Luciferase Reporter Gene Constructs and Luciferase Assay**—The SG-APP 3′-UTR was purchased from Switch-Gear Genomics (SG-Luc-APP; Amplicon start: chr21:26174703; Amplicon end: chr21:26175879). The APP luciferase mutant constructs were generated using the QuickChange® II Site-directed Mutagenesis kit (Stratagene) and synthetic oligonucleotides: MUT1 UP, 5′-TATACAC ATATATATTGGTTTTTTTTGTAGATGAATATTTTAGCTGTATCAACTAGTG-3′; MUT1 DOWN, 5′-CAGTTTTGAAGAAAAATCTCTT-3′. MUT2 UP, 5′-AGAGAGATTGTAGTCTGCTGTTTCTGATA-3′; MUT2 DOWN, 5′-GAAGACCAATGCTTACTCATTTGAGTTTCTGATA-3′. Cells were plated at a density of 2 × 10^5 per well in 24-well plates and transfected after 24 h with 20 pmol of miRNA duplexes (Dharmacon), 20 ng of firefly luciferase expression vector and 10 ng of Renilla luciferase expression vector (pRL-TK Promega) conjugated to 1.2 μl of Lipofectamine 2000 (Invitrogen). Cells were lysed 24 h after transfection, and luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. The experiments were carried out in triplicate.

**Transfection of MicroRNA Inhibitors and IL-1β Treatment**—Hippocampal neurons cultured for 3 days in vitro (3 DIV) in 24-well plates were transfected with 500 nm microRNA hairpin inhibitors (Dharmacon, Thermo Scientific) using 2 μl of Lipofectamine 2000. At 4 days post-transfection, cells were harvested and subjected to Western blot analysis. Hippocampal neurons (7 DIV) were treated with 10 ng/ml rat IL-1β (Sigma-Aldrich) for 4, 6, or 8 h.

**shRNA and miRNA Lentiviral Vectors and Viral Particle Preparations**—To induce Ago2 down-regulation, a lentiviral vector expressing an siRNA, which targets rat Ago2 mRNA (sh-Ago2 TRC
A combined data analysis using the computational programs TargetScan and Pictar for detecting microRNA responsive elements identified microRNA 101 as a putative regulator of APP in hippocampal neurons. This conclusion was supported by the finding that siRNA knockdown of Ago2, a component of the RNA-induced silencing complex (RISC), which promotes microRNA-mediated gene silencing, resulted in a significant increase in APP protein levels (Fig. 2B). In contrast, APP protein levels decreased when Ago2 levels were reduced (Fig. 2A). These results suggest that microRNA-101 is involved in the regulation of APP translation in hippocampal neurons.

**RESULTS**

Silencing of Ago2 in Hippocampal Neurons Increases APP Protein

To explore the role of miRNAs in regulating APP gene expression, we first analyzed the level of APP protein in rat hippocampal cells in which the expression of Ago2 was downregulated. Hippocampal neurons were transduced with either a lentiviral vector containing Ago2 shRNA under the control of the U6 promoter or a control lentivirus expressing a scrambled siRNA. Ago2 shRNA expression resulted in a significant reduction of Ago2 mRNA in comparison to the control siRNA (Fig. 1A). Western blot analysis showed that APP levels were significantly higher in neurons in which Ago2 levels were reduced (Fig. 1, B and C). However, no significant alteration of APP mRNA was observed (Fig. 1D), suggesting that in hippocampal neurons, APP translation may be regulated by an Ago2/microRNA pathway.

**miR-101 is a Putative APP Regulator in the Rat Hippocampus**

A combined data analysis using the computational programs TargetScan and Pictar for detecting microRNA responsive elements within the 3′-UTR of APP, which is conserved among the human, rat, and murine genes, was conducted. Among the selected microRNAs (Fig. 2A), miR-101 had two putative target sites within the APP gene (Fig. 2A). Previous data indicate that mouse miR-101 was initially cloned as a brain-enriched microRNA (39) and in situ hybridization data from the Allen Brain Atlas have shown that the non-coding RNA AK021368, which encompasses microRNA 101a, is expressed in the mouse brain, including the hippocampus (40). These observations further prompted us to analyze APP and miR-101 expression during in vitro development of rat primary hippocampal neurons. In this cellular model, miR-101 levels increased during development from 2 up to 17 days in vitro (2B). In contrast, APP protein levels decreased (Fig. 2C), showing an inverse correla-

**FIGURE 4.** The miR-101 target site at 242–248 bp of the APP 3′-UTR is necessary for APP post-transcriptional regulation. A, schematic representation of luciferase constructs used in the luciferase assay. The miR-101 REs in the APP 3′-UTR are underlined. In the mutant constructs, nucleotides at positions 4 and 5 of the miR-101 REs are mutated, as indicated (in bold). B, PC12 cells were transfected independently with SG control plasmid or each of the four SG-APP luciferase reporter genes together with either miR-101 or a control miRNA (cel-miR-67) (100 nM). At 24 h post-transfection, luciferase activity was determined and normalized to the Renilla control. The data are presented as the normalized activity of miR-101-transfected cells relative to cells transfected with cel-miR-67. Data represent the mean of three independent experiments ± S.D. *, p < 0.05; **, p < 0.01 (t test).

**FIGURE 5.** Inhibition of miR-101 increases endogenous full-length APP. A, hippocampal neuron cultures were transfected (three independent experiments) with either miR-101 inhibitor or the miR inhibitor negative control. Protein extracts from hippocampal neurons were analyzed 96 h after transfection by immunoblotting using an antibody specifically recognizing full-length APP (4G8) or an antibody recognizing GAPDH. Lanes 1 and 4 and 2 and 5, 3 and 6 correspond to the first, second, and third transfection experiment, respectively. B, intensities of the bands for each miR inhibitor were quantified by densitometry and the results obtained with the 4G8 antibody were normalized to the anti-GAPDH signal and expressed as arbitrary units of optical density (OD). Results are expressed as means ± S.D. *, p < 0.05 (t test).
tion between miR-101 and its putative target (Fig. 2D). A similar analysis was performed using rat hippocampal tissues from animals from postnatal day 8 up to 6 months of age. The inverse correlation between levels of miR-101 and APP was confirmed in hippocampal tissues from Aβ1–42 transgenic mice (31). We found that inhibition of endogenous miR-101 significantly up-regulated APP protein levels (Fig. 5, A and B), indicating that hippocampal APP expression may be regulated by miR-101.

The effect of overexpressing miR-101 in differentiated hippocampal neurons was evaluated using a bicistronic lentiviral vector expressing enhanced green fluorescent protein (EGFP) under the control of the CMV promoter, in which the miR-101 sequence was inserted in an artificial hairpin structure under the control of the U6 promoter.

To confirm the efficacy of miR-101 action we analyzed protein levels of a validated miR-101 target, cyclooxygenase-2 (Cox-2) (42). Cox-2 levels decreased with miR-101 overexpression. Immunofluorescence and Western blot analysis demonstrated that increases in miR-101 level strongly down-regulated Cox-2 levels, as shown in the legend to Fig. 5. Results are expressed as means ± S.D. *p < 0.01 (t test); E. qRT-PCR for APP mRNA using hippocampal cell total RNA. Expression relative to pLB-scramble transduced cells with the means from three independent experiments were shown. *p < 0.05. F. Culture medium conditioned from lentiviral transduced hippocampal neurons was determined (41). We found that inhibition of miR-101 significantly up-regulated APP protein levels (Fig. 6, A–D). A corresponding slight down-regulation of APP mRNA was observed in hippocampal neurons overexpressing miR-101 (Fig. 6A).

miR-101 Modulates APP Expression in Hippocampal Neurons

To address whether miR-101 may regulate endogenous APP expression in cultured hippocampal neurons, we performed loss of function experiments by transfecting cells with specific microRNA hairpin inhibitors (Dharmacon). These molecules contain double-stranded flanking regions, around the reverse complement microRNA sequence, which increases the inhibitor function (41). We found that inhibition of endogenous miR-101 significantly up-regulated APP protein levels (Fig. 5, A and B), indicating that hippocampal APP expression may be regulated by miR-101.

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IL-1β treatment (Fig. 7C). As shown in Fig. 7D, up-regulation of miR-101 after IL-1β stimulation exhibited kinetics distinct from up-regulation of APP. The fact that APP and its negative regulator miR-101 are both transiently up-regulated after IL-1β treatment suggests that APP expression cannot be explained solely by the action of miR-101 and that additional regulatory pathways must be involved. In order to evaluate whether IL-1β-induced miR-101 up-regulation may contribute to the reduction in APP expression in the late phase of IL-1β treatment, miR-101 loss of function experiments were performed. After 8 h of IL-1β treatment, APP was still up-regulated in neurons in which miR-101 was inhibited (Fig. 7E), suggesting that miR-101 up-regulation may contribute to modulate APP levels under stress conditions.

**DISCUSSION**

APP is one of the genes potentially regulated by the microRNA pathway in hippocampal neurons, since APP protein levels were up-regulated in neurons in which Ago2 was silenced. Reducing Ago2 levels did not significantly affect APP mRNA levels, suggesting that APP up-regulation is not the consequence of an indirect effect on APP transcription.

The experiments presented here suggest that APP mRNA may be loaded into the RISC complex and may be directly regulated by specific microRNAs. Among microRNAs potentially targeting the APP 3'-UTR, miR-101 is a microRNA with two putative REs within the APP 3'-UTR and is also expressed in adult hippocampal tissue. Expression of miR-101 and APP both in embryonic primary hippocampal cell cultures and in postnatal rat hippocampal tissues further support the hypothesis that miR-101 is a repressor of hippocampal APP expression. Indeed, using a luciferase assay, we demonstrated that miR-101 actively represses a reporter containing the APP 3'-UTR. In addition, using site-directed mutagenesis, a functional interaction between miR-101 and one of two microRNA REs within the APP 3'-UTR was identified. A number of cis regulatory elements which stabilize or destabilize APP mRNA have been described within the APP 3'-UTR (44, 45). Moreover, a recent report demonstrated that a RE for miRNAs belonging to the miR-20 family down-regulates APP expression...
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(32). Taken together, these findings indicate that the APP 3’-UTR is the target of multiple regulatory pathways, which may constitute an integrated network. Further work will be required to define the functional interactions between the different regulatory elements.

Gain and loss of function experiments identified miR-101 as a negative regulator of APP in rat hippocampal neurons. MiR-101 overexpression reduced Aβ load and Cox-2 levels in neuronal cultures. Aβ assembly into oligomers and fibrils is involved in AD pathogenesis (1) and elevated levels of Cox-2, which contribute to neuroinflammation, are associated with AD (46). Thus, our observations suggest that miR-101 could play a protective role in AD. In fact, the Cox-2 reduction induced by miR-101 overexpression may contribute to the regulation of APP metabolism. Cox-2 is essential for the synthesis of prostaglandin E2 (PGE2) and PGE2 has been shown to induce synthesis of APP mRNA and holoprotein (47) and to stimulate Aβ production (48). Thus, the robust reduction of intracellular APP and of Aβ fibrils in the extracellular space may suggest that miR-101 can act by down-regulating both APP and Cox-2, which also reduces PGE2 levels. Whether other putative miR-101 targets may be associated with APP and Aβ metabolism is an interesting possibility that we will be addressed in future work.

The influence of IL-1β on APP expression has been analyzed in various cell types and, in primary neuronal cell cultures, IL-1β was able to up-regulate the transcription of APP (43). Here, we demonstrated that treatment of hippocampal neuron cultures with lower IL-1β concentrations increased APP through regulation of translation, since the steady-state level of APP mRNA did not increase after IL-1β treatment but APP protein levels were transiently up-regulated. In addition, miR-101 may contribute to the reduction in APP expression after prolonged IL-1β treatment, suggesting a role for miR-101 in the control of APP expression in response to IL-1β. However, the overall regulation of APP translation may be the result of multiple mechanisms. For instance, previous findings have suggested that, in human astrocytes (9), IL-1β stimulation regulates translation of APP through the 5’-UTR of the APP mRNA. Thus, further dissection of the precise role of miR-101 in regulating APP and its potential interaction with other regulatory pathways will be necessary.

Altered expression of microRNAs has been described in AD patients. Two independent miRNA expression profiles (30, 49) have shown that miR-101 is down-regulated in the human AD cerebral cortex, suggesting that miR-101 down-regulation may play a role in the development of AD. This hypothesis is in agreement with our results that indicate a direct role of miR-101 in controlling APP translation and Aβ fibril accumulation. Additional studies in AD murine models will be necessary to define the significance of miR-101 in the development and progression of AD pathology.

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