Differential Regulation of Interleukin-1 Receptor-associated Kinase-1 (IRAK-1) and IRAK-2 by MicroRNA-146a and NF-κB in Stressed Human Astroglial Cells and in Alzheimer Disease*

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Specific microRNAs (miRNAs), small non-coding RNAs that support homeostatic gene expression, are significantly altered in abundance in human neurological disorders. In monocytes, increased expression of an NF-κB-regulated miRNA-146a down-regulates expression of the interleukin-1 receptor-associated kinase-1 (IRAK-1), an essential component of Toll-like/IL-1 receptor signaling. Here we extend those observations to the hippocampus and neocortex of Alzheimer disease (AD) brain and to stressed human astroglial (HAG) cells in primary culture. In 66 control and AD samples we note a significant up-regulation of miRNA-146a coupled to down-regulation of IRAK-1 and a compensatory up-regulation of IRAK-2. Using miRNA-146a–, IRAK-1–, or IRAK-2 promoter–luciferase reporter constructs, we observe decreases in IRAK-1 and increases in miRNA-146a and IRAK-2 expression in interleukin-1β (IL-1β) and amyloid-β 42 (Aβ42) peptide-stressed HAG cells. NF-κB–mediated transcriptional control of human IRAK-2 was localized to between −119 and +12 bp of the immediate IRAK-2 promoter. The NF-κB inhibitors curcumin, pyrrolidine dithiocarbamate or CAY10512 abrogated both IRAK-2 and miRNA-146a expression, whereas IRAK-1 was up-regulated. Incubation of a protected antisense miRNA-146a was found to inhibit miRNA-146a and restore IRAK-1, whereas IRAK-2 remained unaffected. These data suggest a significantly independent regulation of IRAK-1 and IRAK-2 in AD and in IL-1β + Aβ42 peptide-stressed HAG cells and that an inducible, NF-κB–sensitive, miRNA-146a–mediated down-regulation of IRAK-1 coupled to an NF-κB–induced up-regulation of IRAK-2 expression drives an extensively sustained inflammatory response. The interactive signaling of NF-κB and miRNA-146a further illustrate interplay between inducible transcription factors and pro-inflammatory miRNAs that regulate brain IRAK expression. The combinatorial use of NF-κB inhibitors with miRNA-146a or antisense miRNA-146a may have potential as a bi-pronged therapeutic strategy directed against IRAK-2–driven pathogenic signaling.

The innate immune response and inflammatory signaling play determinant roles in brain homeostasis, neuroprotection, and repair; however, altered or excessive signaling in these injury defense systems contributes to the irreversible degeneration of brain cells, as typified in the common, age-related neurodegenerative disorder Alzheimer disease (AD).2 In innate immune signaling members of the Toll-like receptor (TLR) or IL-1 receptor (IL-1R) superfamily, via their common Toll and IL-1R (TIR) domains, act as extracellular sensors to detect pathogens and cytotoxic molecules. This enables cells to respond to toxins and inflammatory cytokines by mounting effective neuroprotective immune responses (1–5). A family of interleukin-1 receptor-associated kinases (IRAKs) in the human genome, including IRAK-1, IRAK-2, IRAK-4, and IRAK-M, are key mediators in the immune pathways utilized by TLR/IL-1R (TIR) signaling (3–7). By means of their integral kinase and multiple adaptor functions, IRAKs initiate diverse downstream signaling processes and a cascade of events that can eventually lead to the induction of pro-inflammatory transcription factors such as NF-κB. Further recruitment of NF-κB essential modulator (NEMO/IKKα/β) and adaptor proteins either enhance or mis-regulate both the innate immune response and inflammatory gene expression (5–8).

Abundant DNA array, Northern, RT-PCR, and Western gene expression analysis of AD brains have repeatedly shown a significant disruption in the homeostatic expression of essential brain genes and a progressive up-regulation of inflammatory gene expression, driven in part by overactivation of transcription factor NF-κB. This supports both the development and progression of neurodegenerative disease processes (9–16). Indeed the TLR/IL-1R-IRAK-NF-κB signaling axis is substantially over-stimulated in AD brain (6, 9–12). Components of this innate immunity and inflammatory pathway are known to play a central role in driving neuropathology, in part via overexpression of interleukin-1 β (IL-1β) and up-regulating the generation of the 42-amino acid amyloid β 42 (Aβ42) peptide. These in turn induce transcription from the

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2 The abbreviations used are: AD, Alzheimer disease; Aβ42, amyloid β 42 amino acid peptide; HAG, human astroglial; IL-1R, IL-1 receptor; IRAK, interleukin-1 receptor-associated kinase; miRNA, microRNA; PDTC, pyrrolidine dithiocarbamate; TIR, Toll and IL-1R; TLR, Toll-like receptor; HNG, human neuronal glial; PMI, post-mortem interval; ANOVA, analysis of variance; TRAF6, TNF, receptor-associated factor 6.
miRNA-146a and IRAK-2 Signaling in AD Brain

proinflammatory prostaglandin synthase cyclooxygenase-2 (COX-2) gene and stimulate apoptotic brain cell death and neural tissue degeneration (5, 9–12, 17–19).

MicroRNAs (miRNAs) have emerged as important epigenetic, post-transcriptional, regulators of brain gene expression and the immune response and have been recently implicated in a surprisingly wide variety of human brain disorders including AD (20–27). A mouse and human brain abundant miRNA-146a has been specifically associated with up-regulated inflammatory signaling in temporal lobe epilepsy (21), in prion-induced neurodegeneration (22), in down-regulating IRAK-1 in endotoxin- and cytokine-challenged human monocytes (28), and in the down-regulation of complement factor H, an important repressor of inflammatory signaling in AD brain (29). The miRNA-146a has been found to be further up-regulated in cytokine-, A2103, anti-pack (BulletKit CC-3185) containing growth factors were and HAG primary cells, maintenance medium, and bullet packs (BulletKit CC-3185) containing growth factors were obtained from Lonza (Walkersville, MD). Human-specific anti-actin (A2103), anti-β-tubulin III (neuron-specific; T8660), anti-glial fibrillary acidic protein (glial specific; G9269), anti-IRAK-1 (B5, sc-55530, and C-19; sc-1894), anti-IRAK-2 (B-22, sc-130788, and G-20, sc-23652), anti-IRAK-4 (H-100, sc-99154), anti-IRAK-M (C-20, sc-23656), anti-MyD88 (N-19, sc-8196), and anti-TRAF6 (C-16, sc-33897) primary antibodies were obtained from Sigma or Santa Cruz Biotechnology (Santa Cruz, CA) and used according to the manufacturer’s instructions. Hoechst 33258 (H-1398; Invitrogen) and all other reagents were of the highest grades commercially available and were used without further purification.

Human Brain Tissues—With the emergence of sophisticated techniques for gene expression analysis, the quality of tissues being studied becomes increasingly critical. Brain tissues used in these studies were carefully selected from several hundred potential specimens obtained from archived tissues or extracts at the LSU Neuroscience Center, New Orleans LA, the University of California at Irvine, California, and the Oregon Health Sciences Center, Portland, OR. Human brain tissues were used in accordance with the institutional review board at the LSU Health Sciences Center and donor institutions (6, 14, 15). Table 1 summarizes the selection of brain tissues used in this study. All AD brain tissues were sporadic; as post-mortem interval (PMI; death to brain-freezing interval) is a factor that can affect RNA quality (2, 7, 9–14, 22), all RNAs were derived from control or AD tissues having a mean PMI of 3.1 h or less. Consortium to Establish a Registry for Alzheimer’s Disease/NIH criteria were used to categorize AD tissues in accordance with established guidelines; AD tissues used in these studies had a clinical dementia rating (an index of cognitive decline) ranging from a clinical dementia rating of 0.5 to 3.0, indicating mild to a severe stage of this neurologic disorder (29, 36).

miRNA Isolation from Human Brain Tissues and Cells—In brain tissue studies 10-mg wet weight samples were isolated from the superior temporal lobe neocortex (Brodmann area A22), hippocampus, brain stem, thalamus, or cerebellum of AD brain or age-matched controls. Tissues were gently homogenized using a mini-pstle homogenizer in tissue isolation buffer (Kontes; Fisher; Refs. 10, 11, 29). In HAG cell studies treated or untreated cells from 3 to 5, 70% confluent 3.5-cm diameter 6-well CoStar plates were scraped and pooled, taken up into a 20-ml syringe, RNase- and DNase-free, diethyl pyrocarbonate plastic-ware-treated, and gently packed using centrifugation (10, 30). For both tissues and cells a guanidine isothiocyanate/silica gel-based membrane total RNA purification system was utilized to isolate total miRNA RNA from each sample (PureLink™, Invitrogen). Total RNA concentrations were quantified using RNA 6000 Nano LabChips and a 2100 Bioanalyzer (Caliper Technologies, Mountainview, CA; Agilent Technologies, Palo Alto, CA). Total RNA yield was typically about 1.3 µg of total RNA per mg wet weight of tissue. No significant differences between the spectral purity or molecular size of small RNA between AD and control tissue samples were noted (Table 1). Total small RNA samples were typically run out on 15% Tris borate-EDTA-urea polyacrylamide denaturing gels (TBE-urea; Invitrogen) and after ethidium bromide staining total miRNA species (~25 nucleotides) were excised and end-labeled using [γ-32P]αATP (6000 Ci/mmol) according to the manufacturer’s protocols (Invitrogen) and as previously described (11, 29, 30, 32).

DNA Arrays and Brain-enriched miRNAs—As a preliminary screen and to obtain general trends for miRNA abundance, total miRNA was pooled and analyzed as an AD group (n = 36) and an age-matched control group (n = 30) (Fig. 1) using commercially available miRNA arrays (LC Sciences, Houston TX; Ref. 29 and data not shown). Specific controls and miRNAs showing strong hybridization signals in disease or controls were studied further using robotically generated miRNA panels (11, 29, 30, 32). Briefly, DNA targets for human 5 S ribosomal RNA (5 S RNA), miRNA-132, and miRNA-146a (Table 2) were spotted onto GeneScreen Plus nylon membranes either by hand pipetting or by using a Biomek® 2000 laboratory automation work station (Beckmann, Fullerton, CA). These mini-miRNA array panels were then
cross-linked, baked, hybridized, and probed according to the manufacturer’s protocol (NEN® Research Products, Boston MA) (11, 29, 30, 32). Every second mini-miRNA array panel generated was normalized by probing with purified single radiolabeled miRNAs (5 S RNA, miRNA-132 and/or miRNA-146a) to ascertain equivalent 5 S RNA and individual miRNA loadings (29). Mini-miRNA panels were subsequently probed with total labeled miRNAs isolated from various AD brain regions or stressed HNG or HAG cells and controls; AD, HAG cell, or control extracts (20 μg) containing miRNA or 5 S RNA (5 μg) were run out on 15% TBE-urea denaturing gels, transferred to GeneScreen membranes, cross-linked, baked, hybridized, and probed with specific DNA oligomers corresponding to specific miRNAs (Table 2), radiolabeled using [γ-32P]ATP (6000 Ci/mmole) and a T4 polynuclotide kinase labeling system (Invitrogen) (11, 29, 30, 32).

**HNG and HAG Cells in Primary Culture—**HNG cells were cultured as previously described (11, 15, 29) (Fig. 2). Human astroglial (HAG) cells (CC-2565; Lonza) were grown using an astrocyte growth medium consisting of astrocyte basal medium (Lonza CC-3187) supplemented with astrocyte growth medium SingleQuots (Lonza). Astrocyte basal medium was changed at 2-day intervals; after 1 week of culture HAG cells received at each astrocyte growth medium change IL-1β (10 ng/ml; I4019, Sigma) plus Aβ42 peptide (5 μM; Sigma); control HAG cells received cell culture grade human serum albumin (Sigma; containing no biological activity) at the same concentrations as a control. After the additions, HAG cells were cultured for 0.5 additional week after which total RNA and protein fractions were prepared (14–16).

**Promoter Construction and HAG Cell Transfection—**To further study activity of the human IRAK-1 and IRAK-2 immediate promoter region, including their transcription start sites, 144 oligonucleotide ultramers were synthesized de novo (Integrated DNA Technologies, Coralville, IA; Sigma). These oligomers were annealed yielding overhanging 5’ and 3’ XhoI and BgIII restriction sites and polyacrylamide gel-purified for subsequent cloning into pGL3-based luciferase reporter vectors (Promega Corp.) to yield pGL3-promoter-IRAK-1 and pGL3-promoter-IRAK-2 (Fig. 3). A pre-miRNA-146a promoter-luciferase reporter vector (A547) and Renilla control expression control vectors were obtained from commercial sources (Addgene, Cambridge, MA; Promega). One-week-old HAG cells were transfected with pGL3-promoter-IRAK-1, pGL3-promoter IRAK −2, pre-miRNA-146a promoter-luciferase reporter vector, or Renilla control vectors using FuGENE 6 following the manufacturer’s instructions (Roche Diagnostics). At 29 h post-transfection, under various treatment conditions (Figs. 4 and 5), cells were processed for luciferase assay using a luciferase reporter assay kit (Dual Luciferase System, Promega) (29).

**Electrophoretic Mobility Shift Assay—**Gel shift assay for NF-κB and Sp1, consensus sequence oligonucleotide radiolabeling, normalization, and quantification were performed as previously described (19, 29) (Fig. 4).

**Anti-miRNA 146a and NF-κB Inhibitors—**A 22-oligonucleotide anti-miRNA-146a (AM-146a; 5’-AACCCATG-GAATTCAGTTCTCTCA-3’) or an anti-miRNA-146a control (AM-146ac) 5’-CACATACAAGGGACTTCTACT-3’ was used at 25 nm ambient concentration in HAG cells that were replenished at every change of astrocyte basal medium (see above; Lonza) for a total treatment time of 0.5 week after IL-1β + Aβ42 peptide induction. As required, 0.5-week-old HAG cells were treated with (a) curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-hepta-diene-3,5-dione diferuloylmethane; purity >98.5%, Axxora, San Diego, CA), dissolved in dimethyl sulfoxide as a 100 mm stock solution, and used at 5 μM ambient concentration in the astrocyte basal medium cell medium, (b) the metal chelator, anti-oxidant, and NF-κB translocation inhibitor pyrroldine dithiocarbamate (PTDC; P8765; Sigma) at 25 μM PTDC, or with (c) the polyphenolic trans-stilbene resveratrol analog CAY10512 (10009536; Cayman Chemical, Ann Arbor, MI) as required (Fig. 5).

**Signal Quantitation, Data Analysis, and Interpretation—**5 S ribosomal RNA (5 S RNA), an abundant 107 nucleotide structural RNA, and the 22 nucleotide miRNA-132 are two human brain-abundant small RNAs used as internal controls for miRNA-146a signal determinations in each brain tissue or cell sample. Relative miRNA-132, miRNA-146a, IRAK-1, and IRAK-2 mRNA signal strengths were quantified against 5 S RNA in each sample using data-acquisition software provided with a GS250 molecular imager (Bio-Rad). IRAK-1 and IRAK-2 promoters were searched for transcription factor binding sites using the PROMO 3 (University of Catalonia, Barcelona, Spain) or the TRANSFAC (BioBase Biological Databases, Wolfenbuttel, Germany) DNA sequence search algorithms. Graphic presentations of resultant data were analyzed using Excel algorithms (Microsoft, Seattle, WA), and figures were generated using Adobe Photoshop 6.0 and Adobe Illustrator CS3 (Adobe Systems, San Jose CA). Statistical significance was analyzed using a two-way factorial analysis of variance (p, ANOVA; SAS Institute, Cary, NC). A p < 0.05 was deemed as statistically significant; experimental values are expressed as the means ± 1 S.D.

**RESULTS**

**Selection of Human Brain Tissues and Messenger RNA Quality—**Table 1 shows the number, age, age range, PMI, RNA optical quality (A260/280), RNA 28 S/18 S ratios, and RNA yields of adult control (n = 30) and Alzheimer (n = 36) brain tissues, many of which were previously analyzed for global miRNA and mRNA gene expression patterns (9–12, 29, 30, 32, 39) (Fig. 1). PMIs for age-matched control or AD human brain tissues were all ≤3.1 h; in focused studies involving IRAK-1, IRAK-2, and IRAK-4 abundance, shorter PMIs were selected from this larger group (Fig. 2). The entire study group tissues exhibited no significant differences in age (71.5 ± 6.1 versus 72.2 ± 7.6 yr, p < 0.85), PMI (mean 2.3 ± 2.1 versus 2.5 ± 1.8 h, p < 0.95), RNA A260/280 indices (2.10 ± 0.4 versus 2.09 ± 0.4, p < 0.95), or RNA 28 S/18 S (1.5 versus 1.45, p < 0.91) age-matched control and AD, respectively. We further noted no differences in total RNA yields between the control and AD groups, although there was a trend in some...
younger adult brains and the cerebellum for a slightly higher total RNA yield.

**miRNA-146a Up-regulation in AD Brain**—miRNA-146a was found to be consistently up-regulated in AD brain (Fig. 1, A and B) as a function of disease progression (Fig. 1C), and this change was focused in specific brain regions that exhibit AD neuropathological change (Fig. 1D).

**IRAK Signaling in AD Neocortex and in Stressed HAG Cells**—In relation to control β-actin levels, we observe in control brain tissues higher relative levels of IRAK-2 within the same tissue sample, while in AD-affected brain tissues lower relative levels of IRAK-1 (Fig. 2).

**TABLE 1**
Summary of tissues from control and Alzheimer groups used in this study

| Group     | n  | Age ± S.D. | Age range | Mean PMI* | RNA A$_{260/280}$ | RNA 28 S/18 S | RNA yieldb |
|-----------|----|------------|-----------|-----------|-------------------|--------------|------------|
| Control   | 30 | 71.5 ± 6.1 | 64–77a    | 3.0       | 2.10              | 1.5          | 1.3        |
| Alzheimer | 36 | 72.2 ± 7.6 | 66–79a    | 3.1       | 2.09              | 1.45         | 1.3        |

FIGURE 1. miRNA-146a up-regulation in AD superior temporal lobe neocortex compared with short PMI age-matched control brains. A, using fluorescent miRNA array and Northern dot-blot analysis, miRNA-146a showed consistent increases in short PMI AD superior temporal lobe neocortex (n = 36) when compared with age- and area-matched controls (n = 30). A related brain-enriched miRNA-132 showed no such increases. All values shown are relative to a control 5 S RNA within the same sample. All AD cases were approximately mid-stage AD (clinical dementia rating 1.5; see Fig. 1C). Clinical data of control and AD brains are shown in Table 1. For ease of comparison, a horizontal dashed line at 1.0 indicates the mean of miRNA-146a in control neocortex. B, miRNA-146a averaged a greater than 2.6-fold increase in the neocortical samples examined. Previous studies have shown comparable increases in AD affected hippocampal CA1 (29). For ease of comparison a horizontal dashed line at 1.0 indicates control miRNA-132 levels; *, p < 0.01 (ANOVA). C, miRNA-146a showed a graded increase in AD-affected superior temporal lobe neocortex as the clinical dementia rating (clinical dementia rating; an index of disease severity (37) advanced). D, these changes were confined to the hippocampus (HIPP) and neocortex (NCTX) of AD brains, areas targeted by AD neuropathology; control areas such as the brain stem (BST), cerebellum (CBM), and thalamus (THA) within the same brains showed no such elevations; a horizontal dashed line is included at 1.0 for ease of comparison. For D, n = 3–5 determinations from n = 16 brains; significance over control: *, p < 0.05; **, p < 0.01 (ANOVA).
miRNA-146a and IRAK-2 Signaling in AD Brain

**TABLE 2**
DNA sequences of 5 S RNA, miRNA-132, miRNA-146a, and anti-miRNA-146a (AM-146A) and AM-146a control probes used in this study

The 5 S RNA probe was derived from the first 22 nucleotides of the 107 nucleotide human 5 S ribosomal RNA (5 S RNA) (29). AM-146a control (AM-146ac), containing the same nucleotide composition as anti-miRNA-146a, is a scrambled anti-miRNA-146a oligonucleotide used as a gene expression control (Fig. 5).

| Small RNA species | GenBank™ accession number or designation | DNA sequence (5’–3’) |
|-------------------|----------------------------------------|---------------------|
| 5 SRNA            | X51545                                 | ATACCTCTGTTTCTCTTTGAGAT |
| miRNA-132         | NR_029674                              | TAAAGCTCTACGCCTGTTGCTG |
| miRNA-146a        | EU147785                               | TGGGGCTGAATTCTCGATGTT |
| anti-miRNA-146a   | AM-146a                                | AAAAAAAGGATCTGTTGCTA |
| AM-146a control   | AM-146ac                               | CACATACAGGGACTTTCTACT |

2, A and B). Notably we found no change in the relative levels of IRAK-4 or IRAK-M between either control or AD-affected brain. Because levels of miRNA-146a were significantly higher in HAG cells versus HNG cells when either stressed with IL-1β and Aβ42 peptides and because HAG cells are an integral part of the brain neurovascular unit and inflammatory response (33–36), stressed and control HAG cells were used in all subsequent experiments (Fig. 2C).

Because the proinflammatory cytokine IL-1β and Aβ42 peptide production are significantly up-regulated in AD brain and play a central role in the regulation of inflammatory responses and increase in abundance as the disease progresses (12–19), we chose next to examine the effects of these inducible pathogenic peptides on HNG and HAG cells in primary culture. Compared with HNG cells, HAG cells exhibit a significantly higher miRNA-146a up-regulation (Fig. 2C). Either IL-1β, Aβ42 peptide, or IL-1β + Aβ42 peptide together induced IRAK-2 and miRNA-146a but not IRAK-1 in this particular cell type (Fig. 2D). Because IL-1β + Aβ42 peptide together had a significantly stronger induction of IRAK-2 and miRNA-146a, this combination was used in all subsequent experiments.

**DNA Sequence Characteristics of Oligonucleotides Used**—Table 2 and Fig. 3 show the sequences of synthetic oligonucleotides used in these studies. DNA sequence characteristics and stabilities for 5 S RNA, miRNA-132, miRNA-146a, AM-146a, and AM-146ac have been previously described in detail (29, 39). Figs. 3, A and B, further describe the human IRAK-1 immediate promoter located at human chromosome Xq28 containing 7 Sp1 GC-rich DNA binding sites and the human IRAK-2 immediate promoter located at human chromosome 3p25.3. Fig. 3C shows the miRNA-mRNA complementarity sequence alignment between miRNA-146a and the IRAK-1 mRNA 3’-UTR containing 15/22 matches, or 68% complementarity, and a calculated free energy of association of −29.1 kcal/mol (MIRBASE algorithm; Cambridge UK). Notably, this feature of the miRNA-146a-IRAK-1 mRNA 3’-UTR recognition is conspicuously absent from the IRAK-2 mRNA 3’-UTR, although it is present in the 3’-UTRs of other inflammation-related mRNAs such as those encoding complement factor H and the tumor necrosis factor receptor-associated factor 6 (TRAF6 (11, 28–30, 32)).
miRNA-146a and IRAK-2 Signaling in AD Brain

Relative Abundance of Sp1 and NF-κB in IL-1β + Aβ42-stressed HAG Cells—Using a gel shift assay, stressed-HAG cells exhibited a significant 3.6-fold up-regulation of relative signal strength for NF-κB with no change in relative signal strength for Sp1. This NF-κB activation was quenched using three different NF-κB inhibitors (Fig. 4, A–C).

miRNA-146a Is Transcribed from a NF-κB-regulated Gene—miRNA-146a is transcribed from a pre-miRNA-146a gene, and the 5’ regulatory region of that gene has been sequenced, revealing 3 upstream NF-κB-DNA binding sites in the immediate promoter (NT_023133 (28, 29)). The current data suggest that a significant part of the inflammatory signaling pathway in IL-1β- and Aβ42-triggered HAG cells involves an up-regulation of NF-κB, an important transcription factor responsible for driving transcription of not only miRNA-146a but also of related pro-inflammatory genes and their interrelated pathogenic signaling cascades (Fig. 4B) (10, 14).

Effects of NF-κB Inhibitors on miRNA-146a, IRAK-1 and IRAK-2 Promoter-luciferase Reporters—Up-regulation of NF-κB was associated with a direct increase in the IRAK-2 but not the IRAK-1 promoter reporter (Fig. 4C). The inclusion of NF-κB inhibitors was associated with a significant down-regulation in IRAK-1 promoter activity and a stimulated expression of the IRAK-1 luciferase reporter (Fig. 4C). A coordinated interplay of miRNA-146a and NF-κB signaling thereby appears to regulate IRAK-1 and IRAK-2 expression in IL-1β + Aβ42-stressed HAG cells; miRNA-146a was induced about 3.5-fold over control, and miRNA-146a activation was quenched by three different NF-κB inhibitors. As monitored by luciferase reporter, IRAK-1 was induced 1.2-fold over control, and IRAK-2 was induced 3.5-fold over controls. In the

![DNA sequence structure of the human IRAK-1, human IRAK-2 gene immediate promoters, and a highly stable miRNA-146a-IRAK-1 mRNA-3'–UTR interaction. A and B, the human IRAK-2 promoter contains a single NF-κB binding consensus sequence from −111 to −102 bp of the IRAK-2 promoter. This feature is missing from the immediate IRAK-1 promoter. Further studies showed that the IRAK-2, but not the IRAK-1 gene, is under NF-κB transcriptional control (Fig. 4). XhoI and BglII restriction sites (underlined) were used for ligation into pGL3 vectors (see text). The bent arrow at + 1 indicates the start of transcription. AP2α, NF-κB, and Sp1 binding sites are bold and highlighted in yellow. C, the sequence of the 22 nucleotide miRNA-146a (highlighted in red) shows highly specific complementarity to the human IRAK-1 mRNA 3’–UTR (the target sequence is highlighted in yellow). 14 of 22 base pairs of the 5’ end of miRNA-146a align. The structural stability of the 22 nucleotide oligomer is −29.1 kcal/mol. This feature is absent from the IRAK-2 mRNA 3’–UTR (data not shown). Expression data suggest that the IRAK-1 mRNA 3’–UTR, but not the IRAK-2 mRNA, is regulated by miRNA-146a (28).

![Differential activation NF-κB and Sp1 and miRNA-146a, IRAK-1, or IRAK-2 gene promoter-luciferase reporter activities in IL-1β + Aβ42-stressed HAG primary cells. A, the NF-κB p50/p65 complex is up-regulated in IL-1β + Aβ42-stressed HAG primary cells: p65 (upper arrowhead), p50 (middle arrowhead). Sp1 activation is unchanged after any treatment condition (lower arrowhead). B, activation of miRNA-146a luciferase reporter expression by IL-1β + Aβ42 and inhibition by CAY10512, curcumin, or PDTC is shown. C, differential activation of IRAK-1 and IRAK-2 gene promoter-luciferase reporters (pGL3-promoter-IRAK-1, pGL3-promoter-IRAK-2 constructs) by IL-1β + Aβ42 and inhibition by CAY10512, curcumin, or PDTC is shown. n = 4–5; significance over control: * p < 0.05 (ANOVA).]
Similarly, when the NF-κB inhibitor PDTC was included in the assay, which down-regulated miRNA-146a (Fig. 4A), IRAK-1 increased in expression, and IRAK-2 decreased in expression. The inclusion of both AM-146a and PDTC significantly up-regulated IRAK-1 expression and down-regulated both IRAK-2 and miRNA-146a. These effects are reiterated in the expression profiles of IRAK-1 and IRAK-2 protein shown in Fig. 5B.

**DISCUSSION**

As techniques in quantitative analytical gene expression in human neurobiology advance, the quality of the brain tissue samples being studied becomes increasingly critical (38–41). This is especially important in the study of regional gene expression patterns in human brain where the PMI is a key determinant of total RNA quality (38, 39, 41). As a measure of human superior temporal lobe messenger RNA integrity, previous studies utilizing total RNA isolation or run-on gene transcription and Northern dot-blot hybridization of newly synthesized RNA indicated that human brain extracts of up to about 4 h PMI were efficient in incorporating [α-32P]UTP radiolabel into new DNA transcription products highly representative of the physiological status of the cell, after which there was a precipitous decline in de novo mRNA biosynthetic capacity and RNA quality (38). Other more recent studies have reiterated these observations and concerns (39–41). In the current studies, control and AD-affected human brain tissues (n = 66) were carefully selected from hundreds of potential specimens in domestic brain tissue banks, and tissues were carefully selected with a mean PMI averaging 3.1 h or less (Table 1). In these same tissues we show a consistent, significant increase in the AD brain of miRNA-146a compared with the internal controls 5S RNA and an unchanging brain abundant miRNA-132 (Fig. 1, A and B), a significant increase in miRNA-146a as the severity of AD progresses (Fig. 1C), and a significant compartmentalization of these effects to the hippocampus and temporal lobe neocortex of the brain, but not in the brain stem, cerebellum, or thalamus of anatomical regions unaffected by the AD process and within the same brain (Fig. 1D). Importantly, the temporal lobe neocortex, a brain area of higher-order cognitive function, is specifically targeted by AD neuropathology and is a primary region of interest that is used to indentify early events in Alzheimer-type neurodegenerative change (37, 38, 42).

Interestingly, we observe IRAK-1 to be absent in young human brains but relatively abundant in aged human neocortex (Fig. 2A and Ref. (6)). In aged human neocortex we further observe a highly significant increase in the expression of IRAK-1 in control over AD and, conversely, an up-regulation of IRAK-2 in AD over control. We further note no significant change in either IRAK-4 or IRAK-M expression in comparison to β-actin controls within the same brain tissue samples (Fig. 2, A and B; data not shown).

Because the proinflammatory cytokine IL-1β and Aβ42 peptide production are strongly up-regulated and accumulate in AD brain and also play determinant roles in driving immune and inflammatory responses, we next chose to examine the effects of these inducible pathogenic peptides in primary
miRNA-146a and IRAK-2 Signaling in AD Brain

cultures of HNG and HAG primary cells. HAG cells were found to exhibit a significantly up-regulated expression of the inflammation-associated miRNA-146a in comparison to HNG cells (Fig. 2C). HAG cells are of further interest as they are an important cell type of the human neurovascular unit and a major modulator of the brain innate immune and inflammatory response (33–36). Either IL-1β alone or Aβ42 peptide alone and especially IL-1β + Aβ42 peptide together induced IRAK-2 and miRNA-146a but not IRAK-1 in this particular cell type (Fig. 2D). Because IL-1β + Aβ42 peptide together had a significantly stronger induction of IRAK-2 and miRNA-146a, this combination was used in all subsequent experimentation. We further examined the mechanism of this up-regulation by examining the promoter structure of the human IRAK-1 and IRAK-2 genes, which are located on two different human chromosomes (chr Xq28 and chr 3p25.3 respectively; Fig. 3, A and B). The human IRAK-1 immediate promoter is extremely G+C-rich (83%) and contains 11, sometimes overlapping, binding sites for the GC box element 5’-(C/G)CC(C/G)N(C/G)N(C/G)-3’ recognized by the zinc finger transcription factor Sp1 (Fig. 3A). We also note an AP-2a binding site (5’-AGAGGC-3’) centered at −97 bp from the start of transcription of the IRAK-1 gene; however, as analyzed by gel shift assay, the levels of AP-2a were found not to significantly change after IL-1β + Aβ42 peptide-induced stress in HAG cells (data not shown). On the other hand the human IRAK-2 immediate promoter is far less G+C-rich (41%) and contains putative 2 Sp1 binding sites and a single NF-κB binding site 82% homologous to the canonical NF-κB binding site of 5’-GGGGRNNNYY(C/G)(C/G)-3’ (19, 28–30) located from −111 to −101 bp upstream of the IRAK-2 start of transcription. We note that the NF-κB binding site partially overlaps with a relatively nonspecific zinc finger-rich CTCF binding site (consensus 5’-CNNNNNNCCCTC-3’), which has been previously shown to be important in transcriptional control of the IRAK-2 promoter in non-neural cell types (45). We further found that DNA sequences upstream of −130 bp (−270 to −130 bp) in the human IRAK-2 promoter had insignificant effects on luciferase reporter activities in stressed HAG cell studies (Fig. 3; data not shown).

To further assess the importance of these transcription factors in an IL-1β + Aβ42-induced HAG cell response, we next examined Sp1- and NF-κB-DNA binding using a gel shift assay (5, 19, 28). We observed a significant up-regulation of NF-κB-DNA p50/p65 binding in stressed HAG cells. Although Sp1-DNA was not significantly induced during this treatment, NF-κB-DNA binding was increased 3.6-fold over controls. The NF-κB inhibitors CAY10512, curcumin, and PDTC sharply quenched this induction (Fig. 4A). Previous studies have shown NF-κB-DNA binding to be significantly up-regulated in AD brain and in IL-1β, Aβ42 peptide, or oxidation-stressed human brain cells (19, 29).

We next studied relative luciferase signal yield in promoter constructs of the human IRAK-1 and IRAK-2 gene promoter transfected into control and stressed HAG primary cultures. Because miRNA-146a is an NF-κB-regulated gene, we also examined the activity of the A547 construct containing the miRNA-146a promoter with luciferase reporter (29). miRNA-146a was found to be up-regulated about 3.5-fold by IL-1β + Aβ42 peptide, but this induction was significantly attenuated in the presence of three different classes of NF-κB inhibitors (Fig. 4B). In related studies the IRAK-2 promoter construct was found to be induced 3.6-fold over controls in the presence of IL-1β + Aβ42 peptide; however, this induction was quenched to non-significant levels in the presence of 3 different NF-κB inhibitors (Fig. 2C). This suggests that IRAK-2 is under NF-κB regulatory control, as would be suggested by an NF-κB binding site in the IRAK-2, but not the IRAK-1 immediate promoter. In these experiments IRAK-1 was found not to be up-regulated in the presence of IL-1β + Aβ42 peptide and an up-regulated NF-κB but instead was moderately up-regulated to about 2.3-fold over controls when 3 NF-κB inhibitors were present (Fig. 2C). Given the known decrease of miRNA-146a in the presence of NF-κB inhibitors, we reasoned that increases in IRAK-1 were the result of decreases in miRNA-146a, a negative regulator of IRAK-1, due to its strong interaction with the IRAK-1 mRNA 3’-UTR (Fig. 3C) (28, 29).

To further sort out this apparent NF-κB- and miRNA-146a-mediated control of IRAK-1 and IRAK-2 expression, we next used differential combinations of a specific miRNA-146a antisense sequence (anti-miRNA-146a; AM-146a) and the three different NF-κB inhibitors to dissect the activation of IRAK-1, IRAK-2, and miRNA-146a in control and stressed HAG cells. Although AM-146a had significant inducing effects on IRAK-1 mRNA, no significant effects were observed on the abundance of IRAK-2 mRNA (Fig. 5A). In the presence of the highly efficient NF-κB inhibitor PDTC, IRAK-2 and miRNA-146a expression was inhibited to less than control levels, suggesting that the regulation of these two elements by NF-κB and the de-repression of IRAK-1 via miRNA-146a expression-quenching by PDTC (Figs. 4A and 5A). Interestingly, the inclusion of both AM-146a and PDTC very significantly quenched both IRAK-2 and miRNA-146a expression (Fig. 5A). In this instance IRAK-1 levels increased significantly again due to the inhibition of miRNA-146a in the system (Fig. 5A). Expression of IRAK-1 and IRAK-2 at the protein level was found to be similarly affected (Fig. 5B). Although IRAK expression has shown cell-type specificity (Fig. 2C) (45), these results suggest that a significant part of IRAK-1 or IRAK-2 transcriptional control lies within the immediate promoter region in stressed HAG cells. We cannot exclude that other transcription factors or DNA sequences further upstream from the human IRAK-1 or IRAK-2 promoter sequences studied here have ancillary regulatory controls on IRAK-1 or IRAK-2 gene expression in different brain cell types.

A normally functioning TIR and innate immune response is required to maintain healthy immune defense; both acute and chronic inflammatory pathological conditions arise if these systems are induced either too strongly or for too long. IRAK-1 was originally thought to be central to the TLR/IL-1R-NF-κB signaling axis; however, recent data show that it is dispensable for NF-κB activation via some TLRs and that IRAK-2 is the more critical element for a more sustained TLR-IL-1R-mediated NF-κB activation (4–8, 44). In AD,
emerging evidence supports the hypothesis that the TLR/IL-1R-IRAK-NF-κB innate immunity pathway plays a regulatory role in mediating the neuropathological effects of the Aβ42 peptide (3, 10). In AD this signaling pathway appears to provide a critical link between immune stimulants, such as Aβ42 peptides, and the initiation of host defense, as Aβ42-mediated TLR-IL-1R activation further modulates the release of inflammatory cytokines and COX-2 via downstream effects (2, 3, 43). Interestingly, Aβ42 peptide stimulation of TLR3, TLR5, TLR8, TLR9, and TLR10 transcription has been found to be severely depressed in AD mononuclear cells, and down-regulation of these TLRs may impair microglia-mediated clearance of Aβ42-deposits in the AD brain (43, 44). Interestingly, IL-1β + Aβ42 peptides are both increased in AD brain, and IL-1β + Aβ42 peptide-stressed brain cells emulate many of the neurochemical, pathological, and gene expression changes characteristic of AD (10, 11, 14–16, 29, 30, 32). These studies, therefore, provide evidence in an immune-responsive brain cell type that a stress-induced NF-κB-activated, miRNA-146a-mediated down-regulation IRAK-1 coupled to an NF-κB-driven up-regulation of IRAK-2 provides an important basis for a self-perpetuating inflammatory signaling loop. Notably, in primary macrophages, IRAK-2 activation has been shown to be essential to mount an optimal response to TLR/IL-1R (8). The presence of an alternately regulated IRAK-1/IRAK-2 signaling system adds a further degree of complexity to the variety of mechanisms known to regulate the brain innate immune and inflammatory response both in health and disease and in primary cell models used to study AD-type neurodegenerative mechanisms (11, 26, 27, 29, 30). Importantly, in these studies no significant changes were observed in IRAK-4, IRAK-M, MyD88, or TRAF6 abundance either in AD or in stressed HAG cells (Fig. 2 and data not shown). It will be interesting to study other adaptor components of the TLR/IL-1R-NF-κB cascade and in particular NEMO/IKKα/β signaling to learn how they further modulate pathogenic activities.

In summary, miRNA-146a is emerging as a key small RNA regulator of the innate immune response and pro-inflammatory signaling in several human neurodegenerative diseases associated with a strong inflammatory component (21, 22, 29, 39, 46, 47). The current work describes an NF-κB- and miRNA-146a-mediated IRAK-1 and IRAK-2 expression network in IL-1β + Aβ42-treated HAG cells stressed for up to one-third of their in vitro lifespan. Analogous features are observed in specifically affected anatomical regions of AD brain. Notably, this TLR/IL-1R-IRAK-2-NF-κB self-reinforcing pathogenic loop occupies a key central position within the TLR/IL-1R-NF-κB signaling axis (Fig. 6). AD and stressed brain cells also associate with a significant down-regulation in another miRNA-146a target encoding complement factor H, a key repressor of inflammatory signaling in the complement cascade (11, 29, 30). NF-κB and miRNA-146a are also sharply up-regulated in human brain cells in response to an HSV-1 challenge and to other neurotrophic viral infections as well as in other human neuroinflammatory diseases (11, 21, 22, 46, 47). These findings further illustrate an important instance where a specific pro-inflammatory transcription factor and an immune system-related miRNA cooperate to orchestrate pathogenic responses in brain disease and further suggest that up-regulation of miRNA-146a is an important contributor to inflammatory neuropathology (11, 21, 22, 26–30). The use of directed anti-miRNA strategies to repress the effects of up-regulated miRNAs may represent an effective therapeutic approach; antisense-miRNA strategies may be preferred over antisense-mRNA strategies because of the potential of a single antisense-miRNA to affect the regulation of multiple disease-related genes. Indeed the combinatorial use of AM-146a with selective NF-κB inhibitors may have potential as an effective bi-pronged therapeutic attack against chronically mis-regulated, pathogenic IRAK-2-mediated signaling pathways that drive neurodegenerative disease processes (Fig. 6).
miRNA-146a and IRAK-2 Signaling in AD Brain

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