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Original Article

microRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF)

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Abstract: Human cerebrospinal fluid (CSF), produced by the choroid plexus and secreted into the brain ventricles and subarachnoid space, plays critical roles in intra-cerebral transport and the biophysical and immune protection of the brain. CSF composition provides valuable insight into soluble pathogenic bio-markers that may be diagnostic for brain disease. In these experiments we analyzed amyloid beta (Aβ) peptide and micro RNA (miRNA) abundance in CSF and in short post-mortem interval (PMI <2.1 hr) brain tissue-derived extracellular fluid (ECF) from Alzheimer's disease (AD) and age-matched control neocortex. There was a trend for decreased abundance of Aβ42 in the CSF and ECF in AD but it did not reach statistical significance (mean age ~72 yr; N=12; p~0.06, ANOVA). The most abundant nucleic acids in AD CSF and ECF were miRNAs, and their speciation and inducibility were studied further. Fluorescent miRNA-array-based analysis indicated significant increases in miRNA-9, miRNA-125b, miRNA-146a, miRNA-155 in AD CSF and ECF (N=12; p<0.01, ANOVA). Primary human neuronal-glial (HNG) cell co-cultures stressed with AD-derived ECF also displayed an up-regulation of these miRNAs, an effect that was quenched using the anti-NF-κB agents caffeic acid phenethyl ester (CAPE) or 1-fluoro-2-[2-(4-methoxy-phenyl)-ethenyl]-benzene (CAY10512). Increases in miRNAs were confirmed independently using a highly sensitive LED-Northern dot-blot assay. Several of these NF-κB-sensitive miRNAs are known to be up-regulated in AD brain, and associate with the progressive spreading of inflammatory neurodegeneration. The results indicate that miRNA-9, miRNA-125b, miRNA-146a and miRNA-155 are CSF- and ECF-abundant, NF-κB-sensitive pro-inflammatory miRNAs, and their enrichment in circulating CSF and ECF suggest that they may be involved in the modulation or proliferation of miRNA-triggered pathogenic signaling throughout the brain and central nervous system (CNS).

Keywords: Alzheimer's disease (AD), CAPE (caffeic acid phenethyl ester), CAY10512 (1-fluoro-2-[2-(4-methoxy-phenyl)-ethenyl]-benzene), inflammatory signaling, micro RNA, NF-κB

Introduction

Alzheimer's disease (AD) represents a common, progressive and irreversible immune and inflammatory disorder of the human central nervous system (CNS) whose socioeconomic impact and incidence are reaching epidemic proportions [1, 2]. The molecular-genetic basis for AD initiation and progression is still not well understood. AD typically initiates within the hippocampal region, and more generally the limbic system or Paleo-mammalian brain, progressively advancing into anatomically-linked neocortical association areas [2-7]. Intercellular spreading, the basis for AD 'staging' and propagation, have been attributed to various age-related extracellular physiological factors such as brain cell-secreted cytokines, protein aggregates such as tau, phosphorylated tau and Aβ42 peptides or their fragments, hypoxia and interrelated neurovascular factors, and other small molecule pathogenic factors, including those found in the cerebrospinal fluid (CSF), and blood-borne neurotoxic elements from the

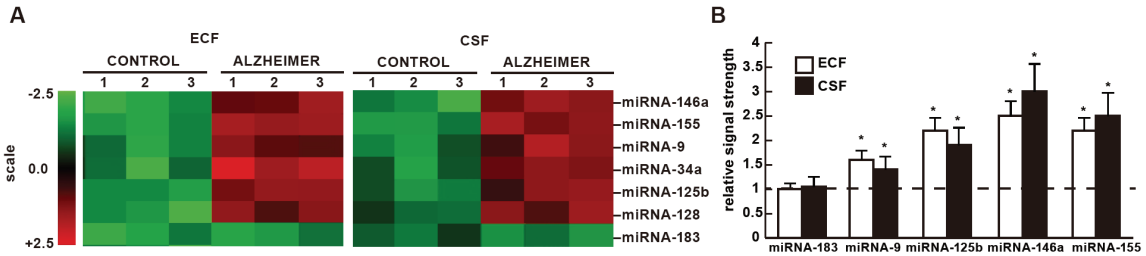

Figure 1. miRNA array analysis of control and AD ECF and CSF; A: human neocortical extracellular fluid (ECF; derived from high speed AD and age-matched tissue supernatants) or cerebrospinal fluid (CSF) were analyzed for miRNA speciation and mean abundance using fluorescent reporter miRNA arrays [13, 19-23]. miRNA array data for 6 of the most abundant miRNAs found in AD ECF or CSF (N=3 representative samples shown) compared to age-matched controls (N=3 samples shown) are shown in [A] and quantified in [B]; these extracellular fluids exhibit elevations in miRNA-9, miRNA-125b, miRNA-146a, miRNA-155 and other AD-enriched small non-coding RNAs (sncRNAs; miRNA-34a, miRNA-128) compared to age-matched controls or unchanging miRNAs such as miRNA-183 [13, 20]. Post-mortem intervals (PMIs) for age-matched control or AD human brain tissue ECF were all less than 3 hr; all tissues were from the medial temporal lobe neocortex; the study group (controls N=6; AD N=6) of tissues exhibited no significant differences in age (mean plus one standard deviation = 72.4±6.5 vs 72.5±7.5 yr, $p<0.85$), PMI (mean 2.1±2.1 vs 2.2±1.4 hr, $p<0.95$), RNA $A_{260/280}$ indices (2.1±0.5 vs 2.05±0.4, $p<0.95$) or RNA 28S/18S (1.5 vs 1.45, $p<0.92$) age-matched control and AD, respectively. RNA integrity numbers (RIN) were ≥ 8.8; no significant differences in total RNA yield between the control and AD groups were noted; in [B] a dashed horizontal line at 1.0 indicates a control miRNA-183 signal in the ECF for ease of comparison. miRNAs were analyzed by LC Sciences (Houston, TX) using miRNA array panels containing 1898 individual human miRNA targets; N=6, $^{*}p<0.01$ (*ANOVA*) [16-19, 70].

environment [6-11]. Key to AD processes are disruptions in the innate immune response and deviations or increases in neuroimmune markers or inflammatory signaling [11-16].

Alterations in tau, phosphorylated-tau and Aβ42 peptide abundance in AD CSF versus age-matched controls have already been extensively studied and reviewed [14-19]. In the current study, we examined the levels of Aβ42 peptides, using ELISA, and small non-coding RNAs (sncRNA), using fluorescent miRNA arrays and LED-Northern dot blot analysis, in human CSF and in short PMI AD and age-matched control tissue-derived extracellular fluid (ECF) (**Figure 1**). The ECF represents the physiological fluid in the extracellular space surrounding brain cells and is physiologically contiguous with the CSF [17-19]. In these studies we observed no significant differences in the total Aβ42 peptide load in AD CSF versus age-matched controls. In contrast we observed significant increases in the abundance of several miRNAs in both ECF and CSF, including specific pro-inflammatory miRNAs that included miRNA-9, miRNA-125b, miRNA-146a and miRNA-155. Previous studies have shown that all of these miRNAs are derived from NF-κB-regulated pre-miRNA transcripts and their up-regulation is implicated in modifying innate immune and inflammatory responses in AD brain [19-25]. These increases were independently confirmed using a novel, highly sensitive, LED-Northern dot blot analytical technique [26-29]. Interestingly, miRNA-9, miRNA-125b, miRNA-146a, miRNA-155 and other miRNAS were also found to also be secreted into the cell culture medium by AD ECF-stressed human primary neuronal-glial (HNG) co-cultures. Anti-NF-κB strategies using caffeic acid phenethyl ester (CAPE), or 1-fluoro-2-[2-(4-methoxyphenyl)-ethenyl]-benzene (CAY10512) were able to quench this pathological effect. These data cumulatively support the contention that selective miRNAs are increased in the conditioned medium surrounding stressed human brain cells and are abundant in AD ECF and CSF. Anti-NF-κB-based therapeutics may provide a novel and efficacious strategy to stem miRNA release and the potential spreading of aberrant and pathogenic inflammatory signals that characterize the AD process.

Materials and methods

Materials

1-ethyl-3-3-di-methyl-aminopropyl carbodiimide (EDAC) was used according to the manufacturer's specifications (Molecular Probes; E-2247, Invitrogen, Carlsbad CA) and as previously described [26-29]. Caffeic acid phenethyl

ester (CAPE; C8221; MW 284.31 Da; solubilized in DMSO; Sigma-Aldrich, St. Louis MO), an active component of propolis (an organic resinous mixture that honey bees collect from botanical sources), and the polyphenolic transstilbene resveratrol analog CAY10512 [1-fluoro-2-[2-(4-methoxyphenyl)-ethenyl]-benzene; MW 228.3 Da; Cayman Chemical, Ann Arbor MI] were used as high efficacy NF-κB inhibitors [20-23, 29]. Both CAPE and CAY10512 were solublized in DMSO at ~1 mg/ml and were used at a final concentration of 5 uM according to instructions and protocols supplied by the manufacturer, and by previously published reports from this laboratory [13, 20, 29]. All other reagents, biochemicals used in these experiments were obtained from independent commercial suppliers and were used without further purification [16-23].

Alzheimer's Disease (AD) and age-matched control neocortical samples

A collection of AD and age-matched control neocortical tissues and CSF samples were carefully selected from multiple domestic and international brain bank sources. Thanks are extended to the many families and physicians who contributed to the human brain bank resources used in these studies. AD and age-matched control human temporal lobe neocortical tissue and CSF were obtained from brain and tissue repositories including the LSU Health Sciences Center archives, the University of Oregon Health Sciences Center (OHSC), NIH collaborators and researchers, and the Institute for Memory Impairments and Neurological Disorders at the University of California at Irvine (MIND-UCI). CERAD/NIH criteria were used to categorize all AD tissues in accordance with established guidelines; all AD tissues used here had a clinical dementia rating (CDR) of 2 or 3, and genotyping indicated at least one ApoE4 allele in all samples [19-25]. All human neocortical tissues were used in strict accordance with the IRB/ethical guidelines of each donor institution [21, 22]. Human brain ECF, consisting of the extracellular fluid surrounding brain cells, highly similar in composition to CSF and derived by a medium speed centrifugation method that leaves whole brain cells intact [23, 24, 29]. Total RNA used in these experiments were obtained from brain tissues having a post-mortem interval (PMI: death to brain freezing interval) of 3 hrs or less, and tissue pH with ranges from 6.7 to 6.9 [20-22]. Both tissue-derived ECF and CSF were analyzed for total miRNA using miRNA arrays and LED-Northern dot blots as previously described [19-25, 29]. All AD and control cases were from the superior temporal lobe; the mean (+/- one standard deviation) age of the control brain group (N=6) was 72.4+/-6.5 yr and the post-mortem interval (PMI; death to brain freezing interval) was 3 hr or less (mean 2.1 hr); the mean age of the AD group (N=6) was 72.5+/-7.5 yr and each PMI was also 3 hr or less (mean 2.2 hr; see legend to **Figure 1**). There were no significant differences in the age, sex, PMI or total RNA quality or yield (see below) between the AD and control tissue groups.

Human primary neuronal-glial (HNG) co-cultures

HNG cell lines, derived from cryopreserved normal human neural progenitor cells (PT-2599; Lonza-Clonetics Cell Systems, Walkersville MD) were cultured in 6-well (3.5 cm diameter) plates (Costar 3506, Corning Life Sciences, Acton, MA) at 5% CO_2, 20% O_2 and 37°C in an HNG maintenance medium (HNGMM) supplemented with 2.5% serum containing hFGF (human fibroblast growth factor), NSF-1 (neuronal survival factor 1), hEGF (human epidermal growth factor) and GA-1000 (gentamicin-amphotericin B G/A 1000) as previously described [11-13, 19-22, 28]. The ApoE genotype of these cultures was derived from donors having an ApoE genotype of E3/E4. HNGMM was completely changed every 3 days of culture. At 2 weeks of growth there were approximately 40% neurons and 60% astroglia at 60% cell confluency (**Figure 2A**). Based on previous dose-response analysis [19-24], after additions of AD-ECF (1ug/uL; containing about 30 ug ECF-derived protein in HNGMM, cells were cultured for an additional 0, 24, 48, 72 and 96 hrs after which total RNA and miRNA fractions were isolated and analyzed [21, 22, 27].

Total RNA, protein extraction and quality control

Total protein and RNA were simultaneously isolated in a dedicated protein- and RNA-extraction laminar hood using TRIzol reagent (Invitrogen; Carlsbad CA) and/or an Ambion mirVana RNA isolation kit according to the manufacturer's instructions (Invitrogen, Carlsbad CA). Protein

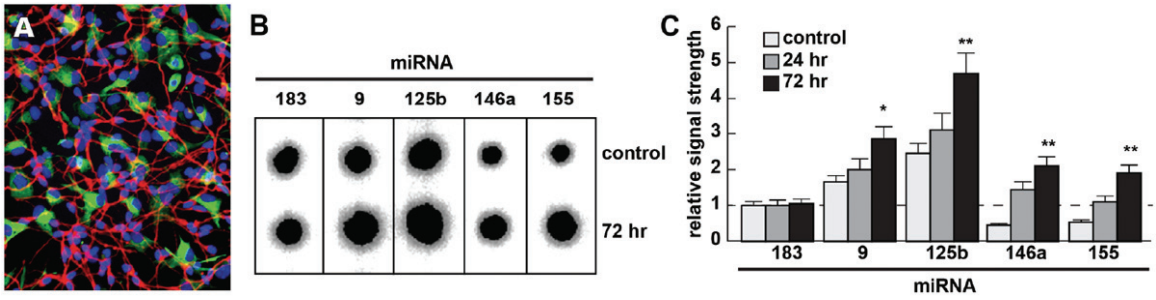

Figure 2. Analysis of miRNA in ECF-stressed HNG cells; A: human neuronal-glial (HNG) primary cells 2 weeks in co-culture; 20x; cells are triple stained using antibodies to glial fibrillary acidic protein (GFAP), a glial-specific marker (green fluorescence; λ_{max}=556 nm), with βTUBIII, a neuron-specific marker (red; λ_{max}=702 nm) and Hoescht 33258 to highlight cellular nuclei (blue; λ_{max}=461 nm) [3, 11-13, 19-23]; human primary neurons do not culture well by themselves so are co-cultured with primary glial cells [21, 22]; B: HNG cells were stressed with AD-derived ECF (containing ~30 ug total protein; see text) for 5 time-points (0, 24, 48, 72 and 96 hrs); no additional increases were observed after 72 hrs of ECF treatment; LED-Northern dot blots of miRNA-9, miRNA-125b, miRNA-146a and miRNA-155 in control (0 hr) and AD ECF-stressed (72 hr) HNG cells using an unchanging miRNA-183 as an internal control marker; C: LED-Northern-based graphed results of miRNA-9, miRNA-125b, miRNA-146a, miRNA-155 and a control miRNA-183 in ECF-stressed HNG cells indicating relative levels at 0 (control), 24 and 72 hrs; dashed horizontal line at 1.0 indicates miRNA-183 control levels for ease of comparison; N=6; $^{*}p<0.05$; $^{**}p<0.01$ (*ANOVA*).

concentrations were determined using dotMETRIC microassay as previously described (sensitivity 0.3 ng protein/ml; Chemicon-Millipore, Billerica, MA) [3, 13, 20-22, 29, 32]. As an index of tissue integrity, RNA quality was assessed using an Agilent Bioanalyzer 2100 (Lucent Technologies/Caliper Technologies; Mountainview CA). In a typical total RNA analysis 1 uL, containing ~1 ug of total RNA was loaded and analyzed on a 6000 Nano Labchip [19-24]. All total RNA samples analyzed in these experiments had RNA integrity numbers of ≥8.8, 28S/18S ratios >1.4 and $A_{280/260}$ >2.0 [19-23, 27].

Analysis using miRNA array, LED-Northern dot blot analysis, RT-PCR and anti-miRNAs

miRNA labeling, hybridization and miRNA array-based analysis were performed as previously described [12, 19-25]. LED-Northern dot blot analysis was performed using a modified Bio-Dot microfiltration blotting device (LED = locked nucleic acids - 1-ethyl-3-3-dimethylaminopropyl carbodiimide (EDAC) - digoxigenin; LNA, EDAC and DIG; detection limit = 0.05 fM of a single miRNA species; apparatus #170-6545, BioRad Life Science Research, Hercules, CA) as previously described [26, 28]. LED-Northern dot blots represent a significant advancement over classical Northern dot blotting techniques because they utilize an LNA-stabilized miRNAs covalently linked to a nylon-based membrane matrix (EDAC) and are probed using DIG-labeled small RNAs with fluorescent reporters [26-29].

Analysis of ECF and CSF for Aβ42 peptides and miRNA

The concentrations of Aβ40 and Aβ42 peptides were quantified using a human amyloid ELISA kit (Sigma, St. Louis MO) and a human amyloid β (1-x) assay kit(American Research Products, Waltham MA) as previously described [30, 31]. After reactions, the plates were immediately measured at 450 nm by using SpectraMax M3 Microplate reader (Molecular Devices, Sunnyvale CA). In consideration that the molecular weight of a typical 22 nucleotide miRNA is [(22 x 320.5) + 159] ~ 7.2 kDa, human ECF and CSF were desalted and concentrated 10- to 30-fold using spin columns (Centricon) containing an ultracel YM-3 membrane with a 3 kDa dalton MW cutoff (final protein concentration ~1ug/uL; Amicon-Centricon 4203; EMD Millipore Corporation, Billerica, MA). Both ECF and CSF were subjected to miRNA array analysis using a human MRA-1001 miRNA microfluidic chip analytical platform, and LED-Northern analysis using a modified dot blot apparatus and vacuum manifold BioRad as previously described [12, 26-29].

Data analysis and interpretation

All miRNA array and LED Northern dot blot data were analyzed using methods previously devel-

oped and published by this laboratory [19-25, 29-31]. Statistical procedures for Aβ42 (ELISA) and miRNA abundance (miRNA aray and LED-Northern assay), were analyzed using a two-way factorial analysis of variance (*p*, ANOVA) using programs and procedures in the SAS language (Statistical Analysis Institute, Cary, NC). Only *p*-values less than 0.05 (ANOVA) were considered to be statistically significant [21-24]. Figures were generated using Excel 2011 (Microsoft, Redmond, WA) and Photoshop CS2 ver 9.0.2 (Adobe, San Jose, CA).

Results

Aβ42 peptide and total miRNA in human CSF and ECF

Consistent with previous reports, Aβ40 peptides were present in abundance 21- to 38-fold greater than Aβ42 peptides in both control and AD CSF samples [14-18]. While there was a trend for both Aβ40 and Aβ42 peptide to be decreased in AD CSF versus age-matched control samples, neither reached statistical significance (mean age ~72 yr; N=12; p>0.05, ANOVA; **Table 1**). On the other hand, based on mass, total miRNA content was much more abundant; total miRNA content was several hundred-fold greater than Aβ40 or Aβ42 peptides in both control and AD CSF or ECF. Importantly, there were no significant differences in age, post-mortem interval (PMI; interval from death to brain freezing), or total RNA quality between the control and ECF or CSF samples.

Up-regulation of miRNAs in AD CSF and ECF

AD and age-matched control CSF and ECF were subsequently studied for specific miRNA content. In AD CSF or ECF, miRNA-9, miRNA-125b, miRNA-146a, and miRNA-155 exhibited the greatest significance of up-regulation compared with age-matched controls (N=6; $p<0.01$, ANOVA) (**Table 1**). These increases ranged from 1.4-fold (for miRNA-9) to almost 3-fold (for miRNA-146a) over age-matched control. A secondary group of miRNAs, consisting, in part, of miRNA-34a and miRNA-128, were also found to be up-regulated compared with age-matched controls but their abundance was much more variable from sample to sample (N=6; $p<0.05$, ANOVA) (**Figure 1A**). At this time we can neither confirm nor deny the presence of other significantly enriched miRNAs or other small non-coding RNAs (sncRNAs) in AD CSF or ECF which may be related to the genetics, or epigenetics, of individual AD cases or to other pathological factors (**Figure 1B**). Again, there were no significant differences in age, post-mortem interval (PMI; interval from death to brain freezing), or RNA quality between either the control or AD brain tissue-derived CSF or ECF groups.

Up-regulation of miRNAs in AD-derived ECF-stressed HNG primary co-cultures

AD-derived ECF and CSF contain a complex mixture of glucose, protein, creatinine, lactate and other constituents, including inflammatory cytokines such as IL-1β and neurotoxic Aβ42 peptides [4-6, 32-34]. Interestingly IL-1β and Aβ42-peptide together show synergistic effects on pro-inflammatory miRNA induction in brain cells in culture, and have been widely used as an AD-relevant physiological stressor in *in vitro* models of AD [12, 21, 22]. We therefore next studied the effects of AD-derived ECF on miRNA induction of miRNAs in a two week old primary culture of HNG cells (**Figure 2A**) using a novel LED-Northern analytical technique (**Figure 2B**) [26-29]. ECF treatment of HNG cells showed a graded increase in selective miRNA induction that peaked at 72 hrs. ECF treatment of HNG cells for 72 hrs represents a relatively chronic application of stress to HNG cells as occurs physiologically during progressive, age-related AD neurodegenerative processes [20-22]. Using miRNA-183 in the same sample as an unchanging internal miRNA control, the levels of miRNA-9, miRNA-125b, miRNA-146a and miRNA-155 were found to increase between 2.4- and 4.5-fold over zero time controls by 72 hrs after stress treatment, and all of these results were highly significant (**Figure 2C**). No significant additional increases for these same miRNAs were noted after 72 hours (data not shown).

Down-regulation of miRNA abundance after treatment with NF-κB inhibitors

Previous studies have shown that miRNA-9, miRNA-125b, miRNA-146a and miRNA-155 and other interrelated pathogenic signals are each activated by the pro-inflammatory transcription factor NF-κB [13, 21, 25, 40]. NF-κB is a known positive regulator of transcription of a select group of primary pre-miRNAs [13, 22, 32]. Addition of either the NF-κB p65 translocation inhibitor caffeic acid phenethyl ester (CAPE), or the potent resveratrol analog 1-fluoro-2-[2-(4-

Table 1. ELISA-based analysis of Aβ40 and Aβ42 peptides, and miRVana™-based analysis of total

sample analyzed	Aβ40 peptide yield in pg/ul	Aβ42 peptide yield in pg/ul	total miRNA yield pg/μl
control CSF	7.5+/-3.5	0.3+/-1.1	108.5+/-12.3
AD CSF	6.3+/-2.7*	0.2+/-1.1*	135.6+/-13.1**

ELISA-based analysis of Aβ40 and Aβ42 peptides, and miRVana™-based analysis of total miRNA in AD versus control CSF; N=6 control and 6 AD, significance over age-matched controls; *p>0.05; **p<0.01 (ANOVA) [16-19, 70].

methoxyphenyl)-ethenyl]-benzene (CAY10512) were found to inhibit miRNA-9, miRNA-125b, miRNA-146a and miRNA-155 activation to at least 0.22-fold of their respective control values (**Figure 3**). These data are the first to show CAPE- and CAY10512-mediated inhibition of ECF-triggeredmiRNA up-regulation in brain cells which may have future therapeutic relevance for AD treatment (**Figure 3**).

Discussion and conclusions

A considerable amount of research work has been done in quantifying biological markers in AD CSF which may be diagnostic for AD, even during the prodromal or 'incipient phase' of patients with mild cognitive impairment [14-20, 36]. These AD-relevant markers have included total tau, phosphorylated tau and amyloid beta (Aβ) peptide abundance. Generally, decreases in Aβ42 peptides and small increases in total tau and phosphorylated tau have been widely reported in AD-derived CSF [15-18, 36, 37]. Due to the limited amount of CSF available for these studies we were only able to quantify Aβ40 and Aβ42 peptide concentrations using ELISA, and miRNA abundance using miRNA array and LED-Northern techniques [12, 13, 26-29, 31]. While we observe a general trend of a decrease of Aβ40 or Aβ42 peptides in AD CSF and ECF in these studies, these decreases were not found to reach statistical significance (N=12; $p>0.05$ ANOVA; **Table 1**). On the other hand miRNAs, constituting a family of ~21-24 nucleotide, non-coding, single-stranded non-coding RNAs, were found to be considerably more abundant than Aβ40 or Aβ42 peptides in the CSF and ECF (**Table 1**) [12, 13, 19-24, 29-31]. The major mode of biological action of miRNA is to bind to complimentary RNA sequences in the 3' un-translated region (3'-UTR) of messenger RNA (mRNA), and thereby act as a repressor of that mRNA's expression. It is now generally accepted that up-regulated miRNAs predominantly act to decrease their target mRNA levels, and hence down-regulate genetic information encoded by that target mRNA [14, 21-25, 34]. Of the approximately ~2000 human miRNAs currently identified, less than 100 miRNAs are abundantly expressed in the brain, and several of these have been reported to be rapidly inducible [40-42; unpublished]. While up-regulated miRNAs have the potential to reduce the transcript levels of their target mRNAs on a transcriptome-wide scale, in disease processes only selective miRNAs appear to be preferentially up-regulated. **Figure 1** and **Figure 2** shows expression of a family of potentially pathogenic miRNAs significantly up-regulated in AD CSF and ECF, and in AD-derived ECF-stressed HNG cells. Significant pathogenic constituents of AD-derived ECF are pro-inflammatory cytokines such as IL-1β and Aβ42 peptides; interestingly IL-1β and Aβ42 peptides together are synergistic in their induction of a pathogenic pro-inflammatory gene expression program in human brain cells in primary culture [5-8, 21-25]. **Figure 3** indicates that each of these up-regulated miRNAs is NF-κB-sensitive, and their up-regulation is effectively quenched by the NF-κB inhibitors CAPE or CAY10512. Interestingly, both peripherally applied Aβ42 peptides and NF-κB-regulated pro-inflammatory miRNAs have been shown to induce AD-type changes within brain cells in culture, including the dys-regulation of innate immune and pro-inflammatory signaling [13, 29, 41-44].

In summary, the five main conclusions of the data presented this study are: (a) that aging brain neocortical-derived ECF and CSF exhibit overlap in the speciation of their resident miRNAs; (b) that pro-inflammatory miRNAs are abundant in AD ECF and CSF and significantly higher in abundance in mass than Aβ40 or Aβ40 peptides; (c) that miRNA-9, miRNA-125b, miRNA-146a and miRNA-155 are similarly up-regulated in AD ECF and CSF compared to age-matched controls; (d) that these same small non-coding miRNAs are secreted by human neuronal-glial (HNG) cells in primary co-culture when stressed with AD-derived ECF; and (e) that as NF-κB-sensitive pro-inflammatory miRNAs, their abundance in AD ECF-stressed HNG

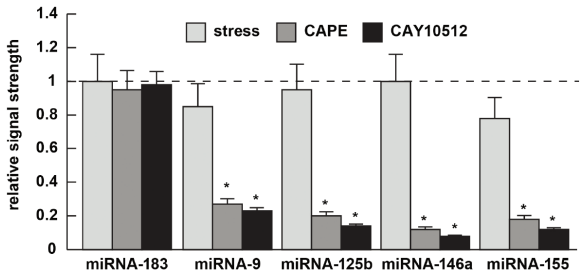

Figure 3. Down-regulation of miRNA-9, miRNA-125b, miRNA-146a and miRNA-155 by the NF-κB inhibitors CAPE and CAY10512; the presence of caffeic acid phenethyl ester (CAPE; Sigma-Aldrich, St. Louis MO), an active component of an organic resinous mixture that honey bees collect from botanical sources, or the polyphenolic transstilbene resveratrol analog CAY10512 down-regulated miRNA-9, miRNA-125b, miRNA-146a and miRNA-155 induction to ~0.1- to ~0.25-fold of controls strongly suggesting that these inducible miRNAs are derived from NF-κB-regulated genes; [13, 21, 29; see text; unpublished); N=5; $^{*}p<0.01$ (*ANOVA*).

cells can be attenuated using the NF-κB inhibitors CAPE and CAY10512.

These studies have some noteworthy limitations including small CSF and ECF sample size, although the post-mortem acquisition time for CSF and ECF (the later derived from post-mortem tissues) was relatively short (PMI mean~2.1 hrs) and the quality of the miRNAs analyzed was rigidly controlled. It will be interesting to further understand if different ApoE alleles, or if indeed other AD-relevant gene mutations or other epigenetic factors have any mechanistic effects on miRNA speciation and complexity in the CSF and ECF, or how brain mRNA 3'-UTR targets, and ensuing down-regulated gene expression, are affected by this soluble and circulating miRNA system. Interestingly, the presence of such a dynamic miRNA intercellular signaling system in circulating fluids such as the CSF and ECF suggests a potential paracrine role for miRNA throughout the entire human CNS. It will be extremely interesting to expand our understanding of the role of NF-κB with miRNAs and with other transcription factors and other molecular-genetic influences on specific miRNA-mRNA activation pathways to further understand their surprisingly dynamic and interactive roles, and their coordinate contribution to the neurogenetic regulation of brain cell aging in health, aging and degenerative disease. Even though the molecular-genetic basis for the proliferation of AD is still incompletely characterized, it seems reasonable that the quenching of intercellular disease-carrying signals, using anti-NF-κB compounds, may be an effective therapeutic strategy to contain essential pathological elements of the AD process, even if an effective treatment or cure for this complex neurological disease cannot be found [2, 45].

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