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miRNA-155 up-regulation and complement factor H (CFH) deficits in Down's Syndrome

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Abstract

Down's syndrome, a congenital disorder associated with cognitive impairment and early onset Alzheimer's disease, is a progressive genetic pathology resulting from full or partial triplication of chromosome-21. Down's brain is typified by activated microglia, increases in inflammatory signaling and an aberrant immune system. In these studies, a screening of micro-RNA (miRNA) from Down's brain and peripheral tissues indicated an up-regulation of a chromosome-21-encoded miRNA-155, and a decrease in the abundance of the miRNA-155 mRNA target complement factor H (CFH), an important repressor of the innate immune response. Stressed primary human neuronal-glial cells indicated both miRNA-155 increases and CFH down-regulation, an effect that was reversed using anti-miRNA-155. These findings suggest that immunopathological deficits associated with Down's syndrome can in part be explained by a generalized miRNA-155-mediated down-regulation of CFH that may contribute to both brain and systemic immune pathology.

Keywords

Alzheimer's disease; Down's Syndrome; gene dosage effects; inflammatory neurodegeneration; micro RNA (miRNA); miRNA-155; systemic inflammation

INTRODUCTION

Down syndrome, also known as trisomy 21, occurring at an incidence of approximately 1 in 1000 live births, is the most frequent survivable congenital chromosomal abnormality and the most frequent cause of congenital intellectual disability in humans [1–3]. While the clinical presentation of Down's is complex and variable, characteristic features such as progressive mental deterioration, increased risk for autoimmune disease, and early onset Alzheimer disease support the hypothesis that Down's syndrome is associated with premature aging and abnormalities in the innate immune response [2–7]. Early genetic and bioinformatics analysis suggested that much of the developmental pathology of Down's syndrome can be attributed to the extra chromosome 21 'gene dosage' effect, resulting from the 50% increase in expression of the ~300 chromosome genes, that leads to an imbalance of critical gene expression, which in turn initiates the Down's phenotype [2–3]. More current

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genetic evidence suggests that the Down's phenotype is most likely multi-genetic, and only a specific subset of the 300 or so trisomic genes, interacting with other disomic genes, mediates disease onset [4,5]. Recent analysis has shown that human chromosome-21 encodes a specific subset of micro RNAs (miRNAs) including let-7c, miRNA-99a, miRNA-125b-2, miRNA-155b and miRNA-802 [6; unpublished data]. The major mode of action of these small, non-coding RNAs is to interact, via base-pair complementarity, with the 3′-untranslated region (3′-UTR) of their target messenger RNAs (mRNAs), and in doing so decrease the expression of that particular target mRNA [7–12]. In fact, ribosome profiling has shown that up-regulated miRNAs act predominantly to decrease their target mRNA levels, and miRNA-mediated destabilization of mRNAs is the main reason for reductions in gene expression [10]. Of the approximately 1250 currently identified human miRNAs, individual human tissues appear to use only a small subset of this total number; for example the human neocortex probably utilizes less than 40 major miRNAs [8,11,12]. Interestingly, physiologically relevant stressors such as viruses, cytokines and oxidative stress are strong inducers of miRNA and ensuing effects on mRNA speciation, and again these effects are significantly cell and tissue specific, even within related cell types in the same tissue [8,9,11–15]. In these experiments we analyzed for trisomy-21 up-regulated miRNAs associated with Down's syndrome, and found that of the 5 miRNAs located on chromosome 21 only miRNA-155 was significantly up-regulated in the brain and in immunogenic organs. A bioinformatics-predicted target of miRNA-155 and immune system repressor, CFH was found to be down-regulated in Down's brain, liver and spleen. In primary co-cultures of human neuronal and glial (HNG) cells, stress-induced miRNA-155-mediated down-regulation of CFH was found to be reversible by using anti-miRNA-155. The results suggest that a miRNA-155-mediated down-regulation of CFH may be a consequence of trisomy-21 and may explain altered immune processes that contribute to the Down's phenotype.

MATERIALS AND METHODS

Reagents, Down's tissues and HNG cells

Reagents used in these experiments were obtained from commercial suppliers and were used without further purification [15–19]. Human recombinant TNFα (T6674; Sigma-Aldrich, S. Louis MO) was used as previously described [15]. Down's and age-matched control human brain, spleen and liver tissue, were obtained from brain and tissue repositories including the Institute for Memory Impairments and Neurological Disorders and the University of California at Irvine; tissues were analyzed for total miRNA and CFH abundance using Western analysis [8,9,12–14]. All Down's syndrome cases were adult; all brain tissues were from the temporal lobe, all were epilepsy-free and all had been previously trisomy-21-genotyped [22]; the mean (+/− one standard deviation) age of the control brain group (N=8; all female) was 48.1+/−8.2 yr and the mean post-mortem interval (PMI; death to brain freezing interval) was 2.8 hr; the mean age of the Down's syndrome group (N=5; 5 female) was 46.7+/−11.5 yr and mean PMI was 3.1 hr. There were no significant differences between the age, sex or PMI between the Down's and control tissue groups.

Total RNA and Protein extraction and quality control

Total RNA and proteins were simultaneously isolated using TRIzol (Invitrogen) [9,12–14]. RNA quality was assessed using an Agilent Bioanalyzer 2100 (Lucent Technologies/Caliper Technologies) [15,16]. Typically one uL of total RNA sample was loaded on the RNA chip (6000 Nano Labchip) and analyzed for quality control; RIN values were typically 9.0 [14–18]. Protein concentrations were determined using dotMETRIC microassay (sensitivity 0.3 ng protein/ml; Chemicon-Millipore, Billerica, Massachusetts, USA) [12,18].

miRNA array, Northern analysis RT-PCR and anti-miRNA-155 (AM-155)

miRNA labeling, hybridization, miRNA arrays, Northern dot blot analysis and RT-PCR analysis were performed as previously described [8,9,12–19]. Anti-miRNA, as locked nucleic acid (LNA) oligonucleotides, to let-7c, miRNA-99a, miRNA-125b, miRNA-802s, miRNA-155b (AM-155; 5′-aattacgattagcactatcccca-3′) and a scrambled control anti-miRNA-155 (AM-155sc; 5′-ttaacattagacgatatcccacc-3′; containing the same nucleotide sequence as AM-155, but in random nucleotide order) were purchased from Applied Biosystems/Ambion, Austin, TX or Exiqon Inc, Woburn, MA [11,16].

Western analysis of CFH and β-actin in Down's syndrome tissues

Western immunoblots were performed for quantification of CFH and β-actin protein in control and Down's tissues using human-specific primary antibodies directed against the control protein marker β-actin (3598-100; Sigma-Aldrich Chemical Company, St Louis, Missouri, USA) or human CFH (H-7; sc-166613 H-5; sc-166608; Santa Cruz Biotechnologies, Santa Cruz, California, USA) [15–17,19,20].

Statistical analysis and data interpretation

All miRNA array, Northern dot blot and RT-PCR data were analyzed as previously described [14,16]. Statistical procedures for protein (ELISA and Western) abundance 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) [14–16]. Only *p*-values less than 0.05 (ANOVA) were considered to be statistically significant. Figures were generated using Excel 2008 (Microsoft, Redmond, WA) and Photoshop CS2 ver 9.0.2 (Adobe, San Jose, CA).

RESULTS

Up-regulation of miRNA-155 in Down's syndrome

miRNA array, RT-PCR and Northern dot blot analysis (Fig. 1 and data not shown) did not detect miRNA-802. The abundance of the four detectable chromosome-21-encoded miRNAs was found to be in the ratio of let-7c:miRNA-99a:miRNA-125b:miRNA-155 of 20:8:3:1 when compared to an unchanging control miRNA-183 or 5S RNA in the same sample. However, only miRNA-155 was found to be significantly up-regulated in Down's tissues, and showed increases of 3.4-, 2.7- and 2.9-fold over age-matched controls in the brain, liver and spleen respectively (Fig. 1). Increases in miRNA-155 are therefore greater than could be explained by the 1.5-fold increases that the extra chromosome-21 gene dosage effects would be expected to contribute (Fig. 1).

Bioinformatics, miRNA-155 and CFH abundance, and anti-miRNA-155 effects

A bioinformatics search for miRNA-155 mRNA targets involved in immune signaling using the miRNA target prediction algorithms www.mirBASE.com and www.targetscan.org indicated that the miRNA-155-CFH mRNA-3′UTR interaction is highly stable, with a free energy of association (E_A) of −26.1 kcal/mol (Fig. 2); this prompted us to analyze CFH protein abundance in the Down's tissues. The results indicate significant decreases to 0.35-, 0.51- and 0.45-fold of controls in the brain, liver and spleen, respectively (Fig. 3). To examine the relationship between miRNA-155 up-regulation and CFH down-regulation we next analyzed the effects of cytokine TNFα-stressed HNG cells; these 2 week old co-cultures contain approximately 60% neurons and 40% glia whose growth and response to cytokine stress have been extensively described [9,12–18]. We find that TNFα-stressed HNG cells exhibited a significant miRNA-155 up-regulation coupled to CFH down-regulation, an effect that was quenched using anti-miRNA-155 (Fig. 4). Neither anti-let-7c,

anti-miRNA-99a, anti-miRNA-125b nor AM-155sc showed any such quenching effects (data not shown).

DISCUSSION

Genetically, 95% of Down's individuals have one complete extra copy of chromosome-21 and the remaining 5% have a partial duplication of this smallest, 47 million base pair chromosome [1–5]. Down's displays a complex and variable spectrum of pathological and clinical features, including systemic oxidative stress and inflammation, particularly in the brain and in tissues involved in the immune response [4–7,21–23]. Morphological and functional deficits in Down's brains include aberrations in inflammatory and immunological markers, altered neuronal cytoarchitecture and synaptogenesis [1–6]. In humans miRNA-155 is recognized as a central regulator of the innate immune system and is involved in various lymphoproliferative disorders [6,7,16,20–23]. In this study Down's brain, liver and spleen exhibited selective increases in miRNA-155; no other chromosome 21-encoded miRNAs attained this significance (Fig. 1). Note that normal human liver and spleen contain amongst the highest abundance of CFH of any solid tissues; these tissues further exhibit elevated miRNA-155 and reductions of CFH in Down's tissues (Fig. 1 & 3). Through genetic and bioinformatics analysis we found an exceptionally strong binding site for miRNA-155 in the CFH mRNA 3′-UTR; in this interaction 17/23 (74%) of nucleotides are perfectly complimentary and 4/23 (17%) are partially complementary, yielding an extremely stable miRNA-mRNA hybrid (Fig. 2). In stressed HNG cells increases in miRNA-155 correlated with CFH down-regulation, and an anti-miRNA-155 was shown to quench miRNA-155 abundance and restore CFH back to homeostatic levels (Fig. 4).

CFH (also known as adrenomedullin binding protein-1, β1H globulin, C3b inactivator-accelerator and H factor-1) is a crucial member of the regulator of complement activation (RCA) group of proteins encoded at the chromosome-1q21 RCA gene locus. This soluble 155 kDa glycoprotein is normally secreted by the liver, and reaches blood plasma concentrations of 500–800 ug/ml in the systemic circulation [16,20]. CFH is the second most abundant plasma protein, after serum albumen, and normally performs a systemic 'sentinel' function against spontaneous immune activation [20,21]. It is not clear if CFH is permeable to the blood-brain barrier; the brain may have an independent CFH supply secreted by neurons, astroglia and/or microglial cells [11,15]. CFH normally acts as a specific inhibitor of the C3 to C3b transition in the complement pathway; systemic CFH deficits are conducive to excessive and pathogenic complement activation associated with autoimmunity and sustained inflammatory responses [9,10,19–21]. An extra copy of chromosome-21 might be expected to contribute to a 1.5-fold increase in the expression of chromosome-21-encoded miRNAs, however, miRNA-155 levels were found to be increased 2.7-fold or greater (Fig. 1). A recent, related study also shows miRNA-155 to be the most significantly up-regulated chromosome-21 encoded miRNA in adult Down's prefrontal neocortex [6]. This elevation may be due to inter-genetic or epigenetic effects; alternately, miRNA-155 is under the regulatory control of the transcription factor NF-κB which is also up-regulated in Down's tissues [7,21–25]. Although in this study only adult female Down's tissues were examined, it will be interesting to analyze miRNA-155 and CFH levels in male and female, fetal and adolescent Down's samples to ascertain if there are any effects of gender, development or age. Although the miRNA-155-CFH mRNA-3′-UTR association is exceptionally stable, we cannot exclude that other miRNA-155-mRNA interactions may also occur *in vivo*. Lastly, CFH is significantly down-regulated in Alzheimer's disease, and predominantly by increased miRNA-146a; interestingly, the miRNA-146a and miRNA-155 recognition features in the CFH mRNA 3′-UTR show partial overlap and binding site homology (Fig. 2) [9,11–17; unpublished observations]. For the first time these data suggest that two different brain miRNAs, miRNA-146a and miRNA-155, contribute to the down-

regulation of CFH expression in two different neurological disorders. As anti-miRNA-155 significantly quenches induced miRNA-155 up-regulation with restoration of CFH to homeostatic levels, targeting immunological signaling involved in Down's syndrome using anti-miRNA-155 may reduce the susceptibility to inflammation and infections as typified by this tragic human disease.

CONCLUSION

Down's syndrome (trisomy-21), a congenital neurological disorder associated with immune deficits, progressive cognitive decline and increased risk for early onset Alzheimer's disease, is associated with the constitutive over-expression of an extra copy of genes encoded on chromosome-21. In these studies, a chromosome-21q21 encoded miRNA-155 was found to be significantly up-regulated, more-so than could be attributed to the chromosome-21 gene dosage effect alone, and a bioinformatics and experimentally confirmed miRNA-155 target, CFH mRNA, and CFH gene expression of this innate immune regulatory protein, was found to be significantly down-regulated in Down's tissues.

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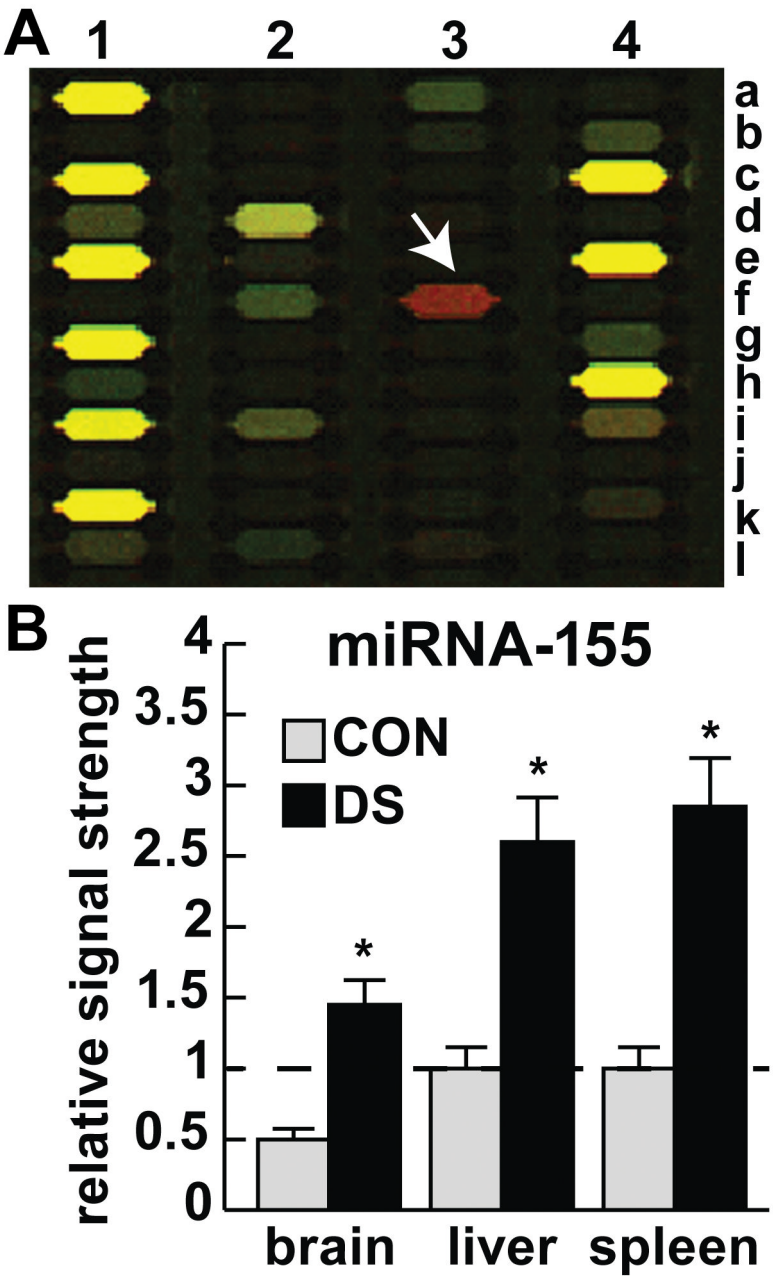

Fig 1. miRNA-155 is up-regulated in DS brain, liver and spleen using miRNA array and RT-PCR analysis

Typical miRNA array result in brain are shown in **(A)** and of an RT-PCR analysis in bar graph format in **(B)**; in **(A)** arrow points to increased miRNA-155 signal (position 3f) [8–12]; other chromosome-21-encoded miRNAs: let-7c (position 1a), miRNA-99a (position 2d), miRNA-125b (position 2i) were detected but showed no significant up-regulation over what would be expected from gene dosage effects alone (~1.15-1.5-fold increases; other positions in **(A)** are non chromosome-21 brain-abundant miRNAs) [8,11,12]; in these experiments miRNA-802 (position 4l) was not detected; an unchanging control miRNA-183 (loaded in triplicate) is at position (1g, 1i and 1k). Northern dot blot analysis showed similar miRNA expression trends (data not shown) [11,16]; **(B)** miRNA-155 abundance in Down's

brain, liver and spleen, respectively as determined by RT-PCR [8–12] compared to age-matched controls; a dashed horizontal line at 1.0 indicates relative miRNA-155 levels in control liver and spleen for ease of comparison; (N=5); *$p<0.01$ (ANOVA).

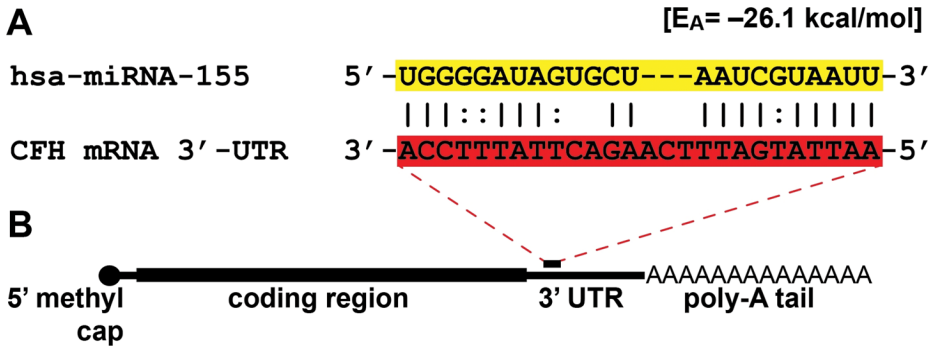

Fig 2. miRNA-155 CFH complementarity map and schematic structure of the human CFH mRNA

Homo sapiens micro RNA-155 (hsa-miRNA-155) **(A)** is a 23 nucleotide small RNA (highlighted in yellow) abundant in human brain, retinal and immune cells [8,11,16], with a high predicted affinity for the CFH mRNA 3′-UTR (highlighted in red); ribonucleotides involved in fully (|) or partially (:) complementary hydrogen bonding are indicated; an E_A = predicted energy of association for miRNA-mRNA interaction of −26.1 kcal/mol indicates strong intermolecular interaction [11,12]; **(B)** Structural features of the CFH mRNA drawn to approximate scale indicating the relative sizes of the 5′ leader sequence, coding region, 232 nucleotide 3′-UTR and poly-A tail; dashed lines indicate the approximate location of miRNA-155 interaction within the 3′-UTR. Interestingly a miRNA-146a binding site in the CFH mRNA 3′-UTR, known to be important in CFH down-regulation in Alzheimer's brain, partially overlaps with this miRNA-155 binding site [9,11,16; unpublished].

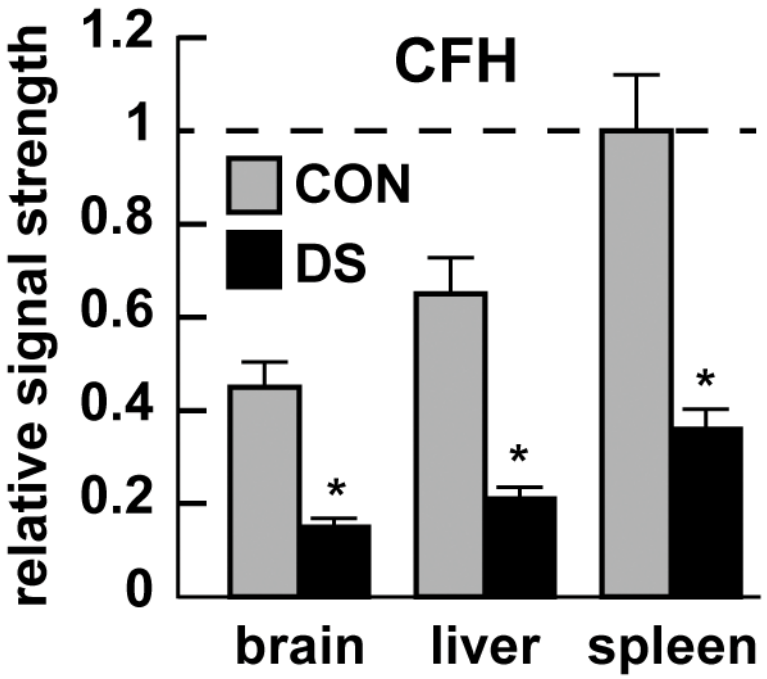

Fig 3. CFH protein down-regulation in Down syndrome

Control and Down's syndrome Western analysis shows significant reduction of CFH protein in Down's brain, liver and spleen when compared to β-actin signals in the same sample; CFH protein levels also showed a trend for down-regulation in retina and thymus (data not shown); a dashed horizontal line at 1.0 indicates relative CFH levels in control spleen for ease of comparison; control N=8; Down's syndrome N=5; **p*<0.01 (ANOVA).

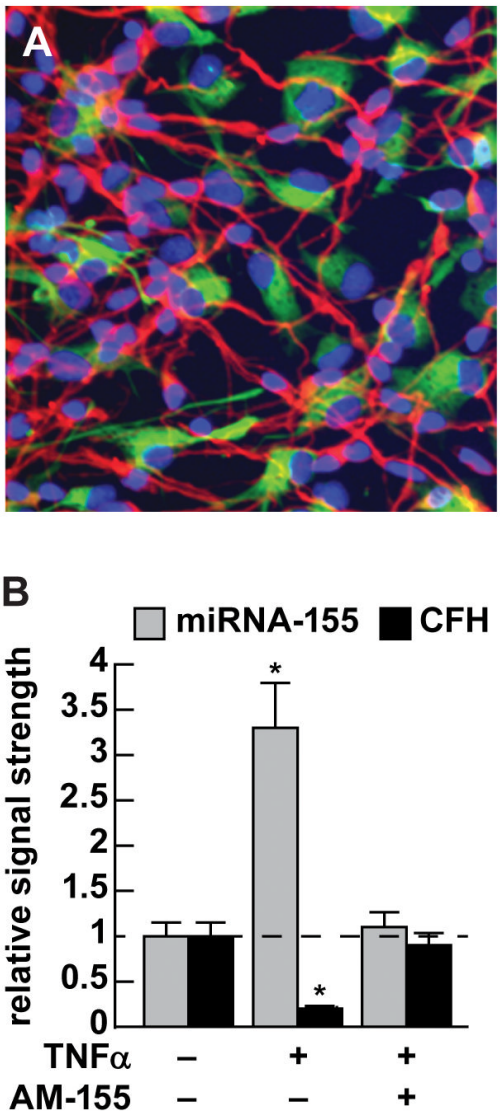

Fig 4. miRNA-155 up-regulation and CFH down-regulation in TNFα-stressed HNG cells; specific quenching of these effects by anti-miRNA-155 (AM-155)

(A) human neuronal-glial (HNG) cells in primary culture; neuronal cells are stained with neuron-specific β-tubulin (red; λ=690 nm), glial cells are stained with glial-specific glial fibrillary acidic protein (GFAP; green; λ=525 nm), and nuclei are stained with Hoechst 33258 (blue; λ=470 nm); HNG cells display basal expression of both miRNA-155 and CFH; magnification 20x; **(B)** abundance of CFH protein (compared to a control β-actin in the same sample) indicates a significant up-regulation of miRNA-155 and down-regulation of CFH after treatment with TNFα, and a reversal of this effect in the presence of AM-155; cytokine-treated HNG cells display significant increases in markers for inflammatory neurodegeneration [9,11,15–17]; a dashed horizontal line at 1.0 indicates relative miRNA-155 and CFH levels in control HNG cells for ease of comparison; N=5; relative signal strength compared to controls; *p<0.01 (ANOVA).