Published in final edited form as:

Brain Res. 2013 October 2; 1533: . doi:10.1016/j.brainres.2013.08.011.

MicroRNA overexpression increases cortical neuronal vulnerability to injury

Jessie S. Truettner, Dario Motti, and W. Dalton Dietrich*

Miami Project to Cure Paralysis, Department of Neurological Surgery, University of Miami Miller School of Medicine, Miami, FL 33136-1060, USA

Abstract

Previously we reported that several microRNAs (miRNA) are upregulated following experimentally induced traumatic brain injury (TBI) using both *in vivo* and *in vitro* approaches. Specific miRNAs were found to be sensitive to therapeutic hypothermia and may therefore be important targets for neuroprotective strategies. In this study we developed plasmid constructs that overexpress temperature sensitive miRNAs: miR-34a, miR-451, and miR-874. These constructs were transfected into cultured cortical neurons that were subjected to stretch injury using a cell injury controller device. Levels of expression of genes associated with stress, inflammation, apoptosis and transcriptional regulation were measured by qRT-PCR. mRNA levels of cytokines interleukin 1- (*IL1-*) and tumor necrosis factor alpha (*TNF-*) as well as heat shock protein 70 (*HSP70*) and *Caspase 11* were found to be increased up to 24 fold higher than controls in cells overexpressing these miRNAs. After moderate stretch injury, the expression of *IL1-*, *TNF-*, *HSP70* and *Caspase 11* all increased over control levels found in uninjured cells suggesting that overexpression of these miRNAs increases cellular vulnerability. miR-34a directly inhibits *Bcl2* and *XIAP*, both anti-apoptotic proteins. The observed increase in *Caspase 11* with over-expression of miR-34a indicates that miR-34a may be inducing apoptosis by reducing the levels of antiapoptotic proteins. miR-34a is predicted to inhibit *Jun*, which was seen to decrease in cells overexpressing this miRNA along with *Fos*. Over expression of several miRNAs found to be induced by TBI *in vivo* (miR-34a, miR-451 and miR-874) leads to increased vulnerability in transfected neurons. Therapeutic hypothermia blunts the expression of these miRNAs *in vivo* and antisense silencing could be a potential therapeutic approach to targeting the consequences of TBI.

Keywords

microRNA; Traumatic brain injury; miR-34a; miR-451; miR-874; Transfection

1. Introduction

MicroRNAs are a class of non-coding regulatory RNAs that have wide-ranging effects on the translation of many proteins and cellular functions in general (Cao et al., 2006; Ivey and Srivastava, 2010; Lee et al., 2002; Madathil et al., 2010; Sempere et al., 2004). These non-coding regulatory RNAs therefore provide a mechanism for the regulation of protein expression levels of various targeted genes (Redell et al., 2011; Qureshi and Mehler, 2010). Because microRNAs can suppress the translation of target genes by binding to their

Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.brainres.2013.08.011>.

mRNAs, their role in gene regulation in health and disease is an area of active investigation (Conley and Alexander, 2011; Esau and Monia, 2007; Madathil et al., 2010; Nakanishi et al., 2010; Wang et al., 2010).

Traumatic brain injury (TBI) is a complex and devastating clinical condition that affects approximately 1.5 million U.S. citizens each year. Following TBI, a cascade of pathomechanisms is activated that leads to increased cell vulnerability and long term neurological deficits. These injury processes include excitotoxicity, inflammation, apoptosis and reactive oxygen species (Dietrich and Bramlett, 2010; Truettner et al., 2007). Various studies have investigated the molecular and cellular processes that are activated after TBI including alterations in microRNA expression after experimental TBI (Lei et al., 2009; Liu et al., 2009; Redell et al., 2009, 2013; Truettner et al., 2011). In a study by Lei et al. (2009), alterations in miRNA expression patterns were reported in the cerebral cortex after fluid percussion (FP) brain injury. In another study, Redell et al. (2009) investigated altered expression in the hippocampus after controlled cortical impact injury. In both studies, unique miRNA profiles in vulnerable brain regions after trauma were documented. In a recent study by Truettner et al. (2011), microarrays were also used to evaluate the effects of trauma on 388 rat microRNAs. In that study, 47 microRNAs were significantly different between TBI and sham at 7 h after TBI, including 15 higher in TBI and 31 lower. After 24 h, 15 microRNAs still differed significantly from control values. In a recent study by Redell and colleagues, miR-21 expression was significantly upregulated in the hippocampus peaking at 3 days post injury and returning to near sham levels by 15. In that study, 99 potential target genes were identified that possessed miR-21 binding sites within their 3 prime untranslated regions. Further analysis documented an overrepresentation of genes involved in enzyme-linked receptor signaling, transcriptional regulation and developmental processes. Taken together, these studies emphasize the relative importance of microRNAs in the pathophysiology of TBI.

Previous work has reported the beneficial effects of therapeutic hypothermia in models of TBI (Dietrich and Bramlett, 2010; Tomura et al., 2012). Indeed, therapeutic hypothermia is one of the most powerful cytoprotective strategies demonstrated in models of cerebral ischemia and trauma. In models of TBI, posttraumatic hypothermia has been reported to protect against irreversible neuronal injury, reduce overall contusion volumes and axonal injury and improve behavioral outcome. Additional studies have indicated that mechanisms underlying the hypothermic effects include a number of injury cascades including excitotoxicity, free radical generation, apoptosis and inflammatory processes. In terms of inflammation and apoptosis, posttraumatic hypothermia differentially regulates the expression of inflammatory cytokines, pro-apoptotic proteins and stress response genes after TBI (Kinoshita et al., 2002; Truettner et al., 2005a, 2005b; Vitarbo et al., 2004). Currently it is unclear exactly how hypothermia may have a regulatory control over gene expression.

Limited data are available regarding the effects of posttraumatic hypothermia on patterns of miRNA expression. In a previous study from our group (Truettner et al., 2011) the effect of posttraumatic hypothermia on expression of a variety of microRNAs was investigated following fluid percussion brain injury. Interestingly, some microRNAs that were differentially regulated by TBI showed a temperature sensitivity to hypothermia verified by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). These findings emphasize that early hypothermia treatment could be affecting traumatic outcome by targeting temperature-sensitive microRNAs involved in basic cell processing events.

Because of the beneficial effects of hypothermia in preserving neuronal viability after TBI, we sought in the present investigation to determine whether the overexpression of selective microRNAs that are affected by therapeutic hypothermia would alter the vulnerability of

cultured cortical neurons subjected to stretch injury. Several of the miRNAs sensitive to hypothermic treatment following TBI identified in our previous study (miR-34a, miR-874 and miR-451) were targeted for analysis in the present study. We report that overexpression of these micro-RNAs induced by TBI leads to increased stress and vulnerability of transfected cells following an *in vitro* stretch injury that mimics some of the biophysical characteristics of a TBI.

2. Results

2.1. Effect of overexpression of miRNAs

Primary neuronal cultures were transfected with plasmid constructs to over express miR-34a, miR-451 and miR-874, plus the vector alone as a control and plated. Three days after transfection, many cells were seen to be expressing GFP (Supplemental Fig. 1). In contrast, neurons cultured for 14 days and then transfected had very low transfection rates, <0.1%, and were therefore not used for further experiments (data not shown). One day after transfection, the media was changed and 3–5 days later conditioned media was removed and media and cells were harvested for RNA extraction. Quantification of miRNA expression in non-transfected as well as transfected cells, including an empty vector control was performed by Real Time RT-PCR and normalized to reference small molecule U6. Cultures that were transfected with the empty vector had undetectable levels of the 3 miRNAs miR-34a, miR-451, and miR-874 (Table 2). The cells transfected with the overexpressing constructs all showed elevated levels of these miRNA as compared to the empty vector. Levels of microRNAs were elevated in both the cells and the conditioned media at various levels but higher in the cells. miR-34a showed a very robust overexpression of over 1800 fold with miR-451 and miR-874 lower but still highly expressed (7.78 fold and 46.85 fold increase, respectively). The amount of miRNAs present in the media as a proportion of the amount in the cells is indicative of the levels of secretion of these miRNAs. miR-34a was the least secreted microRNA with 150 fold greater amounts in the cells than the media. miR-451 was present at a higher level in the media with the cells measuring about 7 fold more miR-451 than the media. miR-874 was present at the highest levels in the media with the cells having only a 4.35 fold excess in miRNA levels (Table 2).

To estimate the consequences of miRNA over expression in the absence of injury, we causing cellular stress or dysregulation, the expression of genes known to be upregulated in response to different types of stress or dysregulation were measured by qRT-PCR. *IL1-* and *TNF-* are inflammatory molecules that are frequently upregulated in response to many cellular pathologies including trauma and ischemia (Truettner et al., 2007, 2009). In comparison to the vector alone transfected cell, those cultures with overexpression of these miRNAs were seen to have increased levels of both of these cytokines. *IL1-* was slightly elevated by 3.7 fold in the miR-34a cells and 4.3 fold in the miR-874 cells. The cells overexpressing miR-451 had almost 11 fold higher levels of *IL1-* ($p<0.05$) (Fig. 1A). Similarly, *TNF-* mRNA levels were increased in the miR-34a and miR-874 transfected cells by 6.4 and 6.2 fold, respectively. In addition, *TNF-* was highly upregulated, almost 24 fold higher than control in the cells transfected with the miR-451 plasmid ($p<0.05$) (Fig. 1B). All mRNA measurements were normalized to an internal housekeeping gene (*GAPDH*).

HSP70, a molecular chaperone known to increase expression in response to miss-folded proteins and other cellular stresses was increased in all three cultures that had over-expressing miRNAs (Fig. 1C). miR-34a resulted in the highest levels of over expression of *HSP70* and miR-874 the least with an approximate 3 fold increase ($p<0.05$). *Caspase 11*, a pro-apoptotic gene, was higher in all three transfected cultures ranging from 3 to 7 fold higher in transfected cells compared to the empty vector control (Fig. 1D).

In contrast, the two transcription factors *Fos* and *Jun* did not show any significant changes in expression in the miRNA transfected cells compared to vector alone transfected cells except for in the miR-34a transfection in which *Fos* was slightly but significantly higher ($p<0.05$) (Fig. 1E and F).

2.2. Stretch injury

Neuronal cultures that had been transfected to overexpress miR-34a, miR-451, and miR-874 plus the vector control were subjected to mild (0.4–1.4 psi) stretch injury and harvested 3 h after injury. As an indirect measure of the amount of stress to the cells the stretch injury caused, levels of *HSP70*, a chaperone protein known to increase after many physiological insults, was measured by qRT-PCR. There was little if any changes in the levels of *HSP-70* mRNA in any of the transfected cells as shown in Fig. 2. Three hours after injury, all 3 groups, miR-34a, miR-451 and miR-874 cells had less than a 2 fold change (1.06, 1.49 and 0.88, respectively) as compared with uninjured cells. Based on these data, we injured the cells at a higher stretch level corresponding to a moderate injury (3.3–3.9 psi). Four days after transfection with vector, miR-34a, miR-451 or miR-874 cultures were injured and harvested 3 h later. Relative expression of a number of genes associated with stress, apoptosis, inflammation and transcriptional activation were measured by qRT-PCR. After normalization to *GAPDH*, the levels of expression of *IL1-*, *TNF-*, *HSP70*, and *Caspase 11* were compared between non-injured transfected cells and injured transfected cells (Fig. 3). In cells transfected with miR-34a, these genes were all higher in injured cells: *IL1-*: 51 fold, *TNF-*: 467 fold, *HSP70*: 89 fold and *Caspase 11*: 25 fold. Cells overexpressing miR-451 had increased levels of these genes as well, but at much lower levels: 13, 194, 44 and 1.4 fold, respectively. Cells transfected with miR-874 showed large differences between uninjured and injured cells. *IL1-* was increased by 110 fold, *TNF-* was upregulated at 175 fold higher, *HSP70* was upregulated 89 fold, and *Caspase 11* increased by 28 fold.

In uninjured transfected cells, the levels of *Jun* and *Fos* increased very modestly, neither reaching a two fold increase (Fig. 1E and F). Likewise in vector alone cells after moderate stretch injury, the levels of *Fos* and *Jun* were at 1.4–1.7 fold of uninjured. In contrast, in the miRNA transfected cells, *Jun* levels were down, though not approaching significance ($p<0.1$), to just 1/5 to 1/10 of uninjured levels (Fig. 3F). More significantly, the levels of *Fos* in the injured cells were only 1% of uninjured levels ($p<0.05$) (Fig. 3E).

3. Discussion

Traumatic brain injury is a major problem both to the victims and to society as a whole. Few treatments have been shown to have beneficial effects. For a review of clinical trials in the past 30 years (see Lu et al., 2012). Hypothermia is one of the few treatments to alleviate the effects of TBI (Pietrini et al., 2012; Sadaka and Veremakis, 2012; Varon et al., 2012). The mechanisms of action of hypothermic treatment remain largely unknown but most likely are multifactorial and involve cellular and molecular events (Dietrich and Bramlett, 2010; Truettner et al., 2005b). MicroRNAs are known to be involved with various biological processes and to influence the responses of cells to many pathological perturbations (Kosik, 2006; Krichevsky, 2007). We have previously shown that injury to neurons after TBI leads to changes in the levels of expression of specific miRNAs. Additionally, some of these changes can be ameliorated by hypothermic treatment that has been shown to be protective in TBI models (Truettner et al., 2011).

In an effort to try to understand how hypothermia may be acting through regulation of miRNA expression after injury, we have transfected neurons to overexpress some of these temperature sensitive miRNAs and investigated how the neurons respond genetically to injury. We investigated genes involved in the inflammatory response that are known to be

upregulated after TBI: *IL1-* and *TNF-* (Kinoshita et al., 2002; Vitarbo et al., 2004). *IL1-* and *TNF-* were expressed at higher levels in cells that over-expressed miRs 34a, 451 and 874. Levels of these miRNAs were previously reported to increase after injury in neurons. When overexpressed in cultured neurons without any injury, these miRNAs led to cellular stress responses involving the inflammatory cytokines *TNF-* and *IL-1*. Although these cytokines are not known to be direct targets of these miRNAs, this finding would indicate that the over-expression of these miRNAs may directly or indirectly lead to an enhanced inflammatory response in neurons.

Additionally when transfected cells were stretch injured, the levels of both cytokines increased dramatically compared to the uninjured cells. There may well exist a positive feedback loop involving miR-34a which is induced by both *TNF-* and *IL-1*. miR-451 has been found to regulate a subset of pro-inflammatory cytokines and to be induced by viral infection in dendritic cells (Rosenberger et al., 2012) and by injection of LPS into mice (Hsieh et al., 2012). When overexpressed in the esophageal carcinoma cell line EC9706, miR-451 inhibited expression of Bcl2 (B-cell lymphoma 2) and AKT (RAC-alpha serine/threonine-protein kinase) and promoted apoptosis (Wang et al., 2012).

HSP70 is a chaperone protein that is upregulated after injury to stabilize miss-folded proteins and protect the cell from further injury. Its expression is activated by numerous injuries to the brain including trauma, hypoxia and ischemia (Gifford et al., 2004, 2008; Kim et al., 2012; Truettner et al., 2007, 2009). *HSP70* is not a known direct target of the miRNAs investigated in the present study, but was assessed as a marker for cellular stress. The over-expression of miR-34a, miR-451 and miR-874 alone did not significantly change the expression level of *HSP70* indicating that these miRNAs are most likely not affecting protein aggregation or miss-folding. However, when the cells were subjected to moderate stretch injury, *HSP70* increased dramatically in all cases.

Caspase 11 was reported to go up after injury in cells that were transfected with miR-34a and miR-874 but not miR-451. *Caspase 11* is associated with the inflammasome and is a pro-inflammatory gene leading to *Casp1* activation of *IL1-B* (Kayagaki et al., 2011). *Caspase 11* (also known as *Casp 4*) is involved in the end stages of apoptosis. miR-34a downregulates both Bcl2 and XIAP (X-linked inhibitor of apoptosis protein), both anti-apoptotic proteins and miR-34a is activated by p53, which is pro-apoptotic. The observed increase in *Caspase 11* with over-expression of miR-34a may be inducing neuronal apoptosis while miR-34a is reducing the levels of anti-apoptotic proteins.

One target of miR-874 is *PPPICA* (Serine/threonine-protein phosphatase PP1-alpha catalytic subunit) which encodes the catalytic subunit of PP1a. PP1a forms complexes with other regulatory subunits and regulates cellular activities including apoptosis, cell cycle and signal transduction (Ceulemans and Bollen, 2004; Cohen, 2002). PP1a has also been reported to be involved in the cellular process of recovery from stress, but promotes apoptosis if cells are too damaged to repair (Ceulemans and Bollen, 2004). In a study of squamous cell carcinoma, miR-874 was highly downregulated (Nohata et al., 2011). In our study, miR-874 was highly upregulated after injury perhaps leading to increased apoptosis. We have previously reported that this miRNA as well as miR-34a are temperature sensitive, with moderate hypothermia reducing levels of both miRNAs after trauma (Truettner et al., 2011). Interestingly, previous studies have shown that therapeutic hypothermia reduces apoptosis after TBI (Lotocki et al., 2006) and cerebral ischemia (Zgavc et al., 2013).

Fos and *Jun* are both components, along with *ATF* (activating transcription factor) of the transcription factor activator protein 1 (AP-1). In addition to its function as an activator of gene transcription, AP-1 can also function to repress the expression of genes. Many

cytokines, chemokines, growth factors as well as hormones and environmental stresses have been shown to regulate AP-1 at both the transcription level and also at the mRNA stability level (for a review see Meng and Xia, 2011). miRNA clusters are co-transcribed and the clustered miRNAs have been reported to target many genes within a signaling pathway. In addition, miRNA clusters have been shown to target AP-1 directly as well as target genes in cell signaling cascades that activate AP-1 (Becker et al., 2012; Cui et al., 2006). Cui et al. (2006) reported that miRNAs target signaling networks more than other cellular processes. Of the targeted proteins, half were nuclear proteins (likely transcription factors) with the balance being intracellular proteins, receptors and ligands. Of the miRNAs they studied, most were predicted to target downstream effectors such as second messengers and transcription factors, more so than upstream ligands and receptors, housekeeping or structural genes.

miR-34a, which decreased in cells overexpressing this miRNA, is predicted to bind to and inhibit *Jun*. miR-34a also targets *p53* which in turn regulates *Fra-1* (fos related antigen 1) expression via miR-34a inhibition (Wu et al., 2012). In a breast cancer study, miR-34a levels were elevated leading to a decrease in *Fra-1* (Yang et al., 2012). A melanoma, a number of miRNAs have been shown to regulate *Jun* expression including miR-125b (Yang et al., 2012). Similarly, *Fos* expression is suppressed by miR-139 in hepatocellular carcinoma (Fan et al., 2013) and by miR-181a (Wu et al., 2012). It is clear that miRNAs directly regulate AP-1 members *Jun* and *Fos* in addition to the signaling pathways that control *Jun* and *Fos* expression. In the present study, the decrease in transcription of these genes after stretch injury reported in the transfected neurons may indicate that there are complex changes occurring within these cells and that many different genes and pathways are involved. miRNAs are known to maintain basal levels of immediate early genes (IEGs) such as *Jun* and *Fos* low and may be further suppressing them after physical damage (Avraham et al., 2010).

In addition to acting cytoplasmically to control translation and degradation of mRNAs, microRNAs are known play a role in influencing other cells by being secreted (Rayner and Hennessy, 2013). Extracellularly, they are generally either packaged in exosomes or more commonly can be found vesicle free in association with highly stable molecules such as Ago2 (Argonaught) or even as free miRNAs (Arroyo et al., 2011; Turchinovich et al., 2011). MicroRNAs have been found in several bodily fluids including plasma, saliva, CSF, tears, and breast milk. Weber et al. (2010) profiled the miRNA spectrum in 12 bodily fluids and were able to identify over 450 different miRNAs extracellularly. This diverse extracellular distribution would indicate that miRNAs may have functional roles in influencing surrounding cells and tissues. In our study, we found that these miRNAs were indeed secreted in both large (miR-451 and miR-874) and in relatively small (miR-34a) quantities.

In conclusion, over expression of several miRNAs (miR-34a, miR-451 and miR-874) previously reported to be induced by TBI (Truettner et al., 2011) leads to increased stress and vulnerability of transfected cultured neurons. Some of the known mechanisms affected by these miRNAs include inflammatory processes and apoptosis. Further studies to determine specific gene targets will help clarify the direct actions of these miRNAs after TBI. Therapeutic hypothermia blunts the expression of these miRNAs *in vivo*, and antisense silencing may mimic the positive influences of hypothermia. Future studies will investigate the possibility of antisense miRNA silencing as a potential therapeutic approach to targeting the detrimental consequences of TBI.

4. Experimental procedures

4.1. Neuronal cultures

Neuronal cultures were obtained by dissection of cerebral cortices from embryonic rats (E18). The tissue was disrupted into a cell suspension by gentle trituration, transfected immediately and plated onto poly-*L*-lysine coated plates in N5 medium containing 5% neurotrophic factor from horse serum as described (Kaufman and Barrett, 1983; Keane et al., 1992). Control, non-transfected cells were plated directly after trituration. Cultures were maintained at 37 °C in a 5% CO₂ incubator. This method of isolation and culture media have previously been shown to yield an almost pure neuronal culture (Kaufman and Barrett, 1983).

4.2. Neuronal transfections

Prior to plating dissociated neurons after harvest, cells were transfected with overexpressing plasmids or empty vector as control using the Amaxa Rat Neuron Nucleofector Kit (Lonza) according to manufacturer's protocol. Briefly, cells are pelleted and resuspended in 600 µl transfection media plus 18% additive. An amount of 4 µg of plasmid DNA was added per 100 µl media per well for a 6 well plate and transferred to a cuvette and electroporated using the Amaxa Nucleofector 2 device. After adding 500 µl warm media, cells are plated and incubated at 37 °C and assessed for green fluorescent protein (GFP) expression the following day. Control transfections were exactly as described without the electroporation step. One day after transfection, the media was changed to eliminate excess plasmid in the media.

4.3. Stretch injury

Primary cortical neuronal cultures were grown on poly-*L*-lysine coated Bioflex plates (FlexCell Int.) 3–5 days after plating, neurons were stretched with the Cell Injury Controller II (Custom Design & Fabrication Inc.) which uses a pulse of compressed gas to transiently deform a SILASTIC membrane and the adherent cells (Ellis et al., 1995). Cells were injured using a 50ms pulse with an injury pressure of 3.8 atm, corresponding to a moderate injury. Three or 24 h after injury, cells were harvested and RNA was extracted as described below. Non-stretched wells were used as a control.

4.4. MicroRNA synthesis and cloning

Pre-microRNA sequences for miR-34a, miR-451, and miR-874 were based on published sequences (miRBase). The minimum possible complete loop that included the mature form of the microRNA was synthesized as a single-stranded DNA oligomer (Integrated DNA Technologies, USA) with flanking Bbs1 restriction sites. Each pre-microRNA was cloned into the Bbs1 site of the UI4-GFP-SIBR vector (Chung et al., 2006) which was kindly provided by Dr. David Turner (University of Michigan, USA). Each clone was verified both by test digest and by sequencing. See Table 1 for sequences of oligos. To confirm the correct overexpression of the mature form of the microRNAs, the constructs were transfected into neuronal cultures and after 2 days, the RNA was subjected to qRT-PCR targeting only the mature form of the microRNA to measure amount of overexpression as compared with non transfected or empty vector controls (data not shown).

In this study, the empty vector plasmid used as a control contained the GFP gene in addition to the same intron and digestion sequence as the miRNA constructs. The use of a scrambled miRNA as control is not optimal because of the potential of the scrambled sequence to have unknown targets. However, scrambled sequences are most effective in siRNA since you know that scrambled construct does not target a precise sequence. Additionally, use of a

miRNA that is presumed not to have an effect on the experiment in question is not desirable since all potential targets of every miRNA in neuronal cultures are not known.

4.5. RT-PCR

RNA was extracted using the *mirVana* miRNA Isolation Kit (Ambion) according to manufacturer's protocol five days after transfection. Briefly, media was removed and cells were harvested by scraping into 600 µl of cell lysis buffer. After addition of 60 µl of miRNA Homogenate Additive, cells were incubated on ice for 10 min. After adding 600 µl of phenol, the homogenate was centrifuged and the aqueous layer removed and 1.25 vol EtOH was added and the mixture was spun through filter cartridges and washed several times. RNA was eluted in dH₂O and concentrations determined by optical density at 260 nm.

For assessing the amount of miRNAs, 50 ng of RNA was reverse transcribed to cDNA with the miRCURY LNA Universal RT microRNA PCR kit (Exiqon) and amplified by quantitative PCR with SYBR Green Master Mix and primers for individual miRNA mature forms or reference gene U6 (Exiqon).

For assessing expression of mRNAs for *HSP70*, *Caspase 11*, *IL1- β* , and *TNF- α* , *Fos*, *Jun* and reference gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), RNA was reverse transcribed using random primers and Reverse Transcription Reagents (Applied Biosystems) followed by qPCR with SYBR Green Master Mix or TaqMan master mix and primers. The primers used for *Caspase 11* (F: CTTACAGTGCGAAAGAACT, R: GGTCCACACTGAAGAATGTCT), *IL1- β* (F: CATCTTTGAAGAAGAGCCCG, R: GGGATTTTGTGCTTGCTTGT), *TNF- α* (F: TGCCTCAGCCTCTTCTCATT, R: TGTGGGTGAGGAGCACATAG), and *Fos* (F: CTCTTCAGCGTCCATGTTCA, R: CCACATGTCGAAAGACCTCA). PCR for *HSP70*, *GAPDH*, and *Jun* used TaqMan primers from Applied Biosystems. Quantitative PCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystem). Levels of expression were calculated with the 2^{-C_t} method. Individual miRNA and mRNA quantities in transfected cells were normalized to the reference gene GAPDH (for mRNAs) or U6 (for miRNAs) and then compared to quantities in untransfected cells. Injured cells were also compared to uninjured cells after 3 h. All samples were run in triplicate and each primer pair was optimized to insure the PCR reaction was in the linear, quantitative range. Fold change *versus* control values were plotted as means and SEM of triplicates. The controls were assigned the value of 1 and significance was determined by a Student's *t*-test as previously reported (Bijkerk et al., 2012; Duggal et al., 2012; Yablonska et al., 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Doris Nonner for her expertise in isolating and culturing rat primary cortical neurons for this study. The authors have no conflicts of interest to report. This study was supported by grants from NIH: RO1 NS042133 and RO1 NS30291.

references

Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanian EL, Stirewalt DL, Tait JF, Tewari M. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Nat. Acad. Sci. U. S. A.* 2011; 108(12):5003–5008.

- Avraham R, Sas-Chen A, Manor O, Steinfeld I, Shalgi R, Tarcic G, Bossel N, Zeisel A, Amit I, Zwang Y, Enerly E, Russnes HG, Biagioni F, Mottotese M, Strano S, Blandino G, Borresen-Dale AL, Pilpel Y, Yakhini Z, Segal E, Yarden Y. EGF decreases the abundance of microRNAs that restrain oncogenic transcription factors. *Sci. Signal.* 2010; 3:ra43. [PubMed: 20516477]
- Becker LE, Lu Z, Chen W, Xiong W, Kong M, Li Y. A systematic screen reveals MicroRNA clusters that significantly regulate four major signaling pathways. *PLoS One.* 2012; 7:e48474. [PubMed: 23144891]
- Bijkerk R, de Bruin RG, van Solingen C, Duijs JMGJ, Kobauashi K, van der Veer EP, ten Dijke P, Rabelink TJ, Goumans MJ, van Zonneveld AJ. MicroRNA-155 functions as a negative regulator of RhoA signaling in TGF- β -induced endothelial to mesenchymal transition. *MicroRNA.* 2012; 1:2–10.
- Cao X, Yeo G, Muotri AR, Kuwabara T, Gage FH. Noncoding RNAs in the mammalian central nervous system. *Annu. Rev. Neurosci.* 2006; 29:77–103. [PubMed: 16776580]
- Ceulemans H, Bollen M. Functional diversity of protein phosphatase-1, a cellular economizer and reset button. *Physiol. Rev.* 2004; 84:1–39. [PubMed: 14715909]
- Chung KH, Hart CC, Al-Bassam S, Avery A, Taylor J, Patel PD, Vojtek AB, Turner DL. Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. *Nucleic Acids Res.* 2006; 34:e53. [PubMed: 16614444]
- Cohen PT. Protein phosphatase 1—targeted in many directions. *J. Cell Sci.* 2002; 115:241–256. [PubMed: 11839776]
- Conley YP, Alexander S. Genomic, transcriptomic, and epigenomic approaches to recovery after acquired brain injury. *Platinum Met. Rev.* 2011; 3:S52–S58.
- Cui Q, Yu Z, Purisima EO, Wang E. Principles of microRNA regulation of a human cellular signaling network. *Mol. Syst. Biol.* 2006; 2:1–7.
- Dietrich WD, Bramlett HM. The evidence for hypothermia as a neuroprotectant in traumatic brain injury. *Neurotherapeutics.* 2010; 7:43–50. [PubMed: 20129496]
- Duggal J, Harrison JS, Studzinski GP, Wang X. Involvement of microRNA-181a in differentiation and cell cycle arrest induced by a plant-derived antioxidant carnosic acid and vitamin D analog doxercalciferol in human leukemia cells. *MicroRNA.* 2012; 1:26–33.
- Ellis EF, McKinney JS, Willoughby KA, Liang S, Povlishock JT. A new model for rapid stretch-induced injury of cells in culture: characterization of the model using astrocytes. *J. Neurotrauma.* 1995; 12:325–339. [PubMed: 7473807]
- Esau CC, Monia BP. Therapeutic potential for microRNAs. *Adv. Drug Deliv. Rev.* 2007; 59:101–114. [PubMed: 17462786]
- Fan Q, He M, Deng X, Wu WK, Zhao L, Tang J, Wen G, Sun X, Liu Y. Derepression of c-Fos caused by MicroRNA-139 downregulation contributes to the metastasis of human hepatocellular carcinoma. *Cell Biochem. Funct.* 2013; 31:319–324. [PubMed: 23001723]
- Gifford RG, Xu L, Zhao H, Carrico W, Ouyang Y, Qiao Y, Sapolsky R, Steinberg G, Hu B, Yenari MA. Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury. *J. Exp. Biol.* 2004; 207:3213–3220. [PubMed: 15299042]
- Gifford RG, Han RQ, Emery JF, Duan M, Pillet JF. Regulation of apoptotic and inflammatory cell signaling in cerebral ischemia: the complex roles of heat shock protein 70. *Anesthesiology.* 2008; 109:339–348. [PubMed: 18648242]
- Hsieh CH, Rau CS, Jeng JC, Chen YC, Lu TH, Wu CJ, Wu YC, Tzeng SL, Yang JC. Whole blood-derived microRNA signatures in mice exposed to lipopolysaccharides. *J. Biomed. Sci.* 2012; 19:1–11. [PubMed: 22212150]
- Ivey KN, Srivastava D. MicroRNAs as regulators of differentiation and cell fate decisions. *Cell Stem Cell.* 2010; 7:36–41. [PubMed: 20621048]
- Kaufman LM, Barrett JN. Serum factor supporting long-term survival of rat central neurons in culture. *Science.* 1983; 220:1394–1396. [PubMed: 6857258]
- Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S, Zhang J, Lee WP, Roose-Girma M, Dixit VM. Non-canonical inflammasome activation targets caspase-11. *Nature.* 2011; 479:117–121. [PubMed: 22002608]

- Keane RW, Tallent MW, Podack ER. Resistance and susceptibility of neural cells to lysis by cytotoxic lymphocytes and by cytolytic granules. *Transplantation*. 1992; 54:520–526. [PubMed: 1412733]
- Kim N, Kim JY, Yenari MA. Anti-inflammatory properties and pharmacological induction of Hsp70 after brain injury. *Inflammopharmacology*. 2012; 20:177–185. [PubMed: 22246599]
- Kinoshita K, Chatzipanteli iK, Vitarbo E, Truettner JS, Alonso OF, Dietrich WD. Interleukin-1beta messenger ribonucleic acid and protein levels after fluid-percussion brain injury in rats: importance of injury severity and brain temperature. *Neurosurgery*. 2002; 51:195–203. [PubMed: 12182417]
- Kosik KS. The neuronal microRNA system. *Nat. Rev. Neurosci*. 2006; 7:991–920.
- Krichevsky AM. MicroRNA profiling: from dark matter to white matter, or identifying new players in neurobiology. *Sci. World. J*. 2007; 7:155–166.
- Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J*. 2002; 21:4663–4670. [PubMed: 12198168]
- Lei P, Li Y, Chen X, Yang S, Zhang J. Microarray based analysis of microRNA expression in rat cerebral cortex after traumatic brain injury. *Brain Res*. 2009; 1284:191–201. [PubMed: 19501075]
- Liu N-K, Wang X-F, Lu Q-B, Xu X-M. Altered microRNA expression following traumatic brain injury. *Exp. Neurol*. 2009; 219:424–429. [PubMed: 19576215]
- Lu J, Gary KW, Neimeier JP, Ward J, Lapane KL. Randomized controlled trials in adult traumatic brain injury. *Brain Inj*. 2012; 26:1523–1548. [PubMed: 23163248]
- Lotocki G, de Rivero Vaccari JP, Perez ER, Alonso OF, Curbelo K, Keane RW, Dietrich WD. Therapeutic hypothermia modulates TNFR1 signaling in the traumatized brain via early transient activation of the JNK pathway and suppression of XIAP cleavage. *Eur. J. Neurosci*. 2006; 24:2283–2290. [PubMed: 17074049]
- Madathil SK, Nelson PT, Saatman KE, Wilfred BR. MicroRNAs in CNS injury: potential roles and therapeutic implications. *Bioessays*. 2010; 33:21–26. [PubMed: 21053309]
- Meng Q, Xia Y. c-Jun, at the crossroad of the signaling network. *Protein Cell*. 2011; 11:889–898. [PubMed: 22180088]
- Nakanishi K, Nakasa T, Tanaka N, Ishikawa M, Yamada K, Yamasaki K, Kamei N, Izumi B, Adachi N, Miyaki S, Asahara H, Ochi M. Responses of microRNAs 124a and 223 following spinal cord injury in mice. *Spinal Cord*. 2010; 48:192–196. [PubMed: 19621023]
- Nohata N, Hanazawa T, Kikkawa N, Sakurai D, Fujimura L, Chiyomaru T, Kawakami K, Yoshino H, Enokida H, Nakagawa M, Katayama A, Harabuchi Y, Okamoto Y, Seki N. Tumour suppressive microRNA-874 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. *Br. J. Cancer*. 2011; 105:833–841. [PubMed: 21847129]
- Pietrini D, Piastra M, Luca E, Mancino A, Conti G, Cavaliere F, De Luca D. Neuroprotection and hypothermia in infants and children. *Curr. Drug Targets*. 2012; 13:925–935. [PubMed: 22512392]
- Qureshi IA, Mehler MF. Emerging role of epigenetics in stroke: Part 1: DNA methylation and chromatin modifications. *Arch. Neurol*. 2010; 67:1316–1322. [PubMed: 21060009]
- Rayner KJ, Hennessy EJ. Extracellular communication via microRNA: lipid particles have a new message. *J. Lipid Res*. 2013; 54:1174–1181. [PubMed: 23505318]
- Redell JB, Liu Y, Dash PK. Traumatic brain injury alters expression of hippocampal microRNAs: potential regulators of multiple pathophysiological processes. *J. Neurosci. Res*. 2009; 87:1435–1448. [PubMed: 19021292]
- Redell JB, Zhao J, Dash PK. Altered expression of microRNA-21 and its targets in the hippocampus after traumatic brain injury. *J. Neurosci. Res*. 2011; 89:212–221. [PubMed: 21162128]
- Redell JB, Moore AN, Grill RJ Jr, Johnson D, Zhao J, Liu Y, Dash PK. Analysis of functional pathways altered following mild traumatic brain injury. *J. Neurotrauma*. 2013; 30:752–764. [PubMed: 22913729]
- Rosenberger CM, Podymnugin RL, Navarro G, Zhao GW, Askovich PS, Weiss MJ, Aderem A. miR-451 Regulates dendritic cell cytokine responses to influenza infection. *J. Immunol*. 2012; 189:5965–5975. [PubMed: 23169590]
- Sadaka F, Veremakis C. Therapeutic hypothermia for the management of intracranial hypertension in severe traumatic brain injury: a systematic review. *Brain Inj*. 2012; 26:899–908. [PubMed: 22448655]

- Sempere LF, Fremantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol.* 2004; 5(R13):1–11.
- Tomura S, de Rivero Vaccari JP, Keane RW, Bramlett HM, Dietrich WD. Effects of therapeutic hypothermia on inflammasome signaling after traumatic brain injury. *J. Cereb. Blood Flow Metab.* 2012; 32:1939–1947. [PubMed: 22781337]
- Truettner JS, Alonso OF, Dietrich WD. Influence of therapeutic hypothermia on matrix metalloproteinase activity after traumatic brain injury in rats. *J. Cereb. Blood Flow Metab.* 2005a; 25:1505–1516. [PubMed: 15959464]
- Truettner JS, Takamoto S, Dietrich WD. The effect of therapeutic hypothermia on the expression of inflammatory response genes following moderate traumatic brain injury in the rat. *Mol. Brain Res.* 2005b; 138:124–134. [PubMed: 15922484]
- Truettner JS, Hu B, Alonso OF, Bramlett HM, Kokame K, Dietrich WD. Subcellular stress response after traumatic brain injury. *J. Neurotrauma.* 2007; 24:599–612. [PubMed: 17439344]
- Truettner JS, Hu K, Liu CL, Dietrich WD, Hu B. Subcellular stress response and induction of molecular chaperones and folding proteins after transient global ischemia in rats. *Brain Res.* 2009; 1249:9–18. [PubMed: 18996359]
- Truettner JS, Alonso OF, Bramlett HM, Dietrich WD. Therapeutic hypothermia alters microRNA responses to traumatic brain injury in rats. *J. Cereb. Blood Flow Metab.* 2011; 31:1897–1907. [PubMed: 21505482]
- Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res.* 2011; 39:7223–7233. [PubMed: 21609964]
- Varon J, Marik PE, Einav S. Therapeutic hypothermia: a state-of-the-art emergency medicine perspective. *Am. J. Emerg. Med.* 2012; 30:800–810. [PubMed: 21570229]
- Vitarbo EA, Chatzipanteli K, Kinoshita K, Truettner JS, Alonso OF, Dietrich WD. Tumor necrosis factor alpha expression and protein levels after fluid percussion injury in rats: the effect of injury severity and brain temperature. *Neurosurgery.* 2004; 55:416–424. [PubMed: 15271250]
- Wang T, Zang WQ, Li M, Wang N, Zheng YL, Zhao GQ. Effect of miR-451 on the biological behavior of the esophageal carcinoma cell line EC9706. *Dig. Dis. Sci.* 2012; 58:706–714. [PubMed: 23053883]
- Wang WX, Wilfred BR, Madathil SK, Tang G, Hu Y, Dimayuga J, Stromberg AJ, Huang Q, Saatman KE, Nelson PT. miR-107 regulates granulin/progranulin with implications for traumatic brain injury and neurodegenerative disease. *Am. J. Pathol.* 2010; 177:334–345. [PubMed: 20489155]
- Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, Galas DJ, Wang K. The microRNA spectrum in 12 body fluids. *Clin. Chem.* 2010; 56:1733–1741. [PubMed: 20847327]
- Wu J, Wu G, Lv L, Ren YF, Zhang XJ, Xue YF, Li G, Lu X, Sun Z, Tang KF. MicroRNA-34a inhibits migration and invasion of colon cancer cells via targeting to Fra-1. *Carcinogenesis.* 2012; 33:519–528. [PubMed: 22198213]
- Yablonska S, Hoskins EE, Wells SI, Khan SA. Identification of miRNAs dysregulated in human foreskin keratinocytes (HFKs) expressing the human papillomavirus (HPV) type E6 and E7 oncoproteins. *MicroRNA.* 2013; 2:2–13.
- Yang S, Li Y, Gao J, Zhang T, Li S, Luo A, Chen H, Ding F, Wang X, Liu Z. MicroRNA-34a suppresses breast cancer invasion and metastasis by directly targeting Fra-1. *Oncogene.* 2012; 24:1–10.
- Zgavc T, De Geyter D, Ceulemans AG, Stoop W, Hachimi-Idrissi S, Michotte Y, Sarre S, Kooijman R. Mild hypothermia reduces activated Caspase-3 up to 1 week after a focal cerebral ischemia induced by endothelin-1 in rats. *Brain Res.* 2013; 1501:81–88. [PubMed: 23357472]

Fig. 1. qRT-PCR in uninjured transfected cells 4 days after transfection. Fold change in expression of *IL1-* (A), *TNF-* (B), *HSP70* (C), *Caspase 11* (D), *Fos* (E), and *Jun* (F) in cells overexpressing miR-34a, miR-451 and miR-874 as compared to empty vector control. Mean and SEM of 3 replicates for each sample. miR-451 increased the expression of all 6 genes the most. miR-34a and miR-874 also increased the expression of all these genes except *Jun* but at lower levels. The cytokines *IL1-* and *TNF-* and *HSP70* and *Caspase 11* had greater increases in expression than the IEGs *Fos* and *Jun* which increased only 1.4 to 2.2 fold over the vector only cells. * $p < 0.05$, ** $p < 0.01$, Student's t -test.

Fig. 2. qRT-PCR of *HSP70* 3 h after mild stretch injury. Mild stretch injury does not have a significant effect on expression of *HSP70* in transfected cells.

Fig. 3. qRT-PCR 3 h after stretch injury. Fold change in expression of *IL1-β* (A), *TNF-α* (B), *HSP70* (C), *Caspase 11* (D), *Fos* (E), and *Jun* (F) in cells overexpressing miR-34a, miR-451 and miR-874 stretch injured as compared to noninjured controls. Mean and SEM of 3 replicates for each sample * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ paired Student's *t*-test. miR-34a and miR-874 overexpressing cells subjected to stretch injury induced *IL-1* and *TNF-α* at very high levels. Expression of *IL-1* and *TNF-α* in miR-451 cells were at lower, but still very high levels as compared to vector controls (A) and (B). *HSP70* was induced at very high levels in all cells as compared to non-injured controls (C). *Caspase 11* was induced by stretch injury in miR-34a and miR-874 expressing cells, but not in miR-451 cells

(D). *Fos* and *Jun* expression were suppressed almost to undetectable in the cells expressing all three microRNAs as compared to vector expressing cells after injury (E) and (F). *Fos* was down to only 3% of vector controls and *Jun* was 12–18% of vector only controls.

Table 1

Oligos used for pre-miRNA cloning.

miRNA	Forward	Reverse
SYBR-rno-miR-34a	GCTGTTGGCAGTGCTTAGCTGGTTGTGTGAGTATTAGCTAAG GAAGCAATCAGCAAGTATACTGCCCTAC	TCCTGTAGGGCAGTATACTTGCTGATGCTTCCTTAG CTAATACTCACACAACCAGCTAAGACACTGCCAAA
SYBR-rno-miR-451	GCTGAATGGCGAGGAAACCGTTACCATTACTGAGTTTAGTAATG GTAATGGTTCTCTTGCTGCTC	TCCTGAGCAGCAAGAGAACCATTACCATTTACTAAA CTCAGTAATGGTAACGGTTTCCTCGCCATT
SYBR-rno-miR-874	GCTGCGGCCCCACGCACCAGGGTAAGAGAGACTCGCTTCCTGC CCTGGCCCGAGGGACCGAC	TCCTGTCGGTCCCTCGGGCCAGGGCAGGAAGCGAG TCTCTTTACCCTGGTGCGTGGGGCCGC

Black nucleotides are flanking sequences for BbsI restriction sites, red nucleotides are part of the mature form of the microRNAs, blue nucleotides form the loop of the hairpin, and green nucleotides are genomic portions to complete the complementarity of the hairpin.

Table 2

qRT-PCR of miRNA expression levels in transfected cells.

Sample	FC vs. vector	FC cell/media
Vector	1	
miR-34a media	12.13	
miR-34a cell	1807.78	149.09
miR-451 media	1.11	
miR-451 cell	7.78	7.01
miR-874 media	10.78	
miR-874 cell	46.85	4.35

Fold change (FC) of expression of miR34a, miR451 and miR-874 in both cells and conditioned media 4 days after transfections compared with cells and media from a control, vector transfected group (column 2). The control group is set equal to one and the fold change of miRNAs is compared to that. miR-34a was measured in cells transfected with the miR34a-GFP plasmid and miR451 and miR874 likewise are measured in cultures transfected with those plasmids, respectively. miR-34a had the highest level of expression at over 1800 times greater than the control. All 3 constructs had higher levels of expression in the cells than in the media. FC cell/media indicates the amount of miRNA secreted into the media as relates to the amount of miRNA in the cell 4 days after transfection. miR-34a had 149 times more of this miR in the cells than in the media, miR-451 did not appear to be significantly secreted and miR-874 had 4.35 times more in the cells than the media.