

Original Article

Mood stabilizer-regulated miRNAs in neuropsychiatric and neurodegenerative diseases: identifying associations and functions

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Received April 4, 2013; Accepted April 27, 2013; Epub May 24, 2013; Published June 1, 2013

Abstract: Identifying mechanisms to enhance neuroprotection holds tremendous promise in developing new treatments for neuropsychiatric and neurodegenerative diseases. We sought to determine the potential role for microRNAs (miRNAs) in neuroprotection following neuronal death. A neuronal culture system of rat cerebellar granule cells was used to examine miRNA expression changes following glutamate-induced excitotoxicity and neuroprotective treatments. Combination treatment with the mood stabilizers lithium and valproic acid provided near-complete protection from glutamate excitotoxicity. Numerous miRNAs were detected by microarrays to be regulated by the combined lithium and valproic acid treatment, and the following candidates were confirmed using real-time PCR: miR-34a, miR-147b, miR-182, miR-222, miR-495, and miR-690. We then verified the apoptotic actions of miR-34a mimic in a human neuroblastoma cell line (SH-SY5Y) under basal conditions and following endoplasmic reticulum stress. To gain insight into the function of these mood stabilizer-regulated miRNAs, we performed two separate analyses: a candidate approach using Ingenuity Pathway Analysis that was restricted to only our PCR-verified miRNAs, and a global approach using DIANA-mirPath that included all significantly regulated miRNAs. It was observed that the pathways associated with mood stabilizer-regulated miRNAs in our study (global approach) are strongly associated with pathways implicated in neuropsychiatric diseases such as schizophrenia. We also observed an overlap in the mood stabilizer-regulated miRNAs identified from our study along with dysregulated miRNAs in both neuropsychiatric and neurodegenerative disorders. We anticipate that these associations and overlaps implicate critical pathways and miRNAs in disease mechanisms for novel therapeutic treatments that may hold potential for many neurological diseases.

Keywords: microRNA, neuroprotection, glutamate excitotoxicity, lithium, valproic acid, mood stabilizers

Excitotoxicity is a pathological process that has been associated with numerous neurological diseases including stroke, traumatic brain injury, and other neurodegenerative diseases. It occurs when excessive stimulation caused by neurotransmitters acting on excitatory receptors, such as N-methyl-D-aspartate (NMDA) receptors, releases high levels of calcium ions into the cell. This perpetuates second messenger signaling mechanisms activating enzymes that damage cellular cytoskeleton, membrane, and DNA, leading to its demise. We sought to exploit this pathological process and investi-

gate the underlying neuroprotective mechanisms mediated by microRNA (miRNA).

miRNAs are small (~22 nts) non-protein coding RNAs that target mRNAs and usually lead to translational repression where they are anticipated to act as master regulators of the entire genome [1-3]. Some miRNAs are shown to be tissue-specific [4] and function in dendritic spine development [5], while others have less tissue-specific expression and function in numerous processes ranging from cell death and proliferation to developmental timing and

neuronal cell fate [6]. Dysregulation of miRNAs has also been found to be associated with CNS diseases including Alzheimer's disease [7], Parkinson's disease [8], schizophrenia [9], and others [10]. The regulation of miRNAs in the rat hippocampus and their potential for underlying the long-term actions of mood stabilizers lithium (Li) and valproic acid (VPA) has been reported [11]. Interestingly, some of these mood stabilizer-regulated miRNAs (e.g., miR-34a) were also found to target bipolar susceptibility genes (e.g., GRM7) under *in vitro* and *in vivo* conditions, supporting the notion that mood stabilizers partly modulate their targets via miRNA regulation. Mood stabilizers have also been shown to have neuroprotective effects in various models where their exact mechanisms remain elusive. In a glutamate-induced, NMDA receptor-mediated excitotoxicity model in primary neurons, both Li and VPA are neuroprotective [12]. The neuroprotective effects of Li are believed to be in part due to inhibition of NMDA receptor-mediated calcium influx [12, 13]. A proposed target for VPA is inhibition of histone deacetylases (HDACs). Chronic VPA treatment protected neuronal cultures from excitotoxicity induced by SYM 2081, a high-affinity ligand for kainate receptors, via HDAC inhibition as measured by increased acetylated histone levels [14]. A common anti-apoptotic target for both Li and VPA is B-cell lymphoma 2 (Bcl-2), which has been shown to be regulated *in vivo* following chronic Li and VPA treatment of the frontal cortex, and *in vitro* in primary neuronal cultures treated with lithium [15, 16]. Moreover, combined treatment with both Li and VPA produces synergistic neuroprotective effects in an aged primary neuronal culture model of glutamate excitotoxicity [17], and multiple enhanced benefits in mouse models of amyotrophic lateral sclerosis (ALS) [18] and Huntington's disease [19]. Therefore, we sought to investigate the miRNA mechanisms that may contribute to the neuroprotective effects of combined treatment with Li and VPA in a rat primary neuronal cell culture model.

Materials and methods

Neuronal culture studies

Cerebellar granule cell cultures (CGCs) were prepared from 8 day-old Sprague-Dawley rats, as described previously [17, 20]. Cells were cultured in serum-free B27/neurobasal medium

and plated at 1.6×10^6 cells/ml on 0.01% poly-L-lysine pre-coated plates. Cytosine arabinofuranoside (10 μ M) was added to the cultures 24 hours after plating to arrest the growth of non-neuronal cells. Cells were pre-treated with various agents (vehicle, 3 mM LiCl, 0.8 mM VPA, or 3 mM LiCl + 0.8 mM VPA for 6 days starting at 6 days *in vitro* (DIV) or 10 μ M MK-801, an NMDA receptor antagonist, for 30 minutes starting at DIV 12), and then exposed to 50 μ M glutamate for 2 hours to induce neurotoxicity. Previously, we have shown that 3 mM LiCl and 0.8 mM VPA combination treatment was optimal for neuroprotection against glutamate excitotoxicity in CGC [17]. Plates were prepared and treated in replicates of 6. After 2 hours of glutamate exposure, cells were harvested for total RNA isolation using the *mirVana* miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA concentration was determined using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Tech, Rockland, DE), then RNA samples were assessed for quality using Bioanalyzer (Agilent Technologies, Foster City, CA), and stored for further analysis at -80°C. For cell viability studies, cultures were exposed to 50 μ M or 100 μ M glutamate for 24 hours before viability quantification.

Measurement of cell viability

Cell viability was quantified by mitochondrial dehydrogenase activity to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), as described previously [21]. CGCs in 96-well culture plates were incubated with 125 μ g/ml MTT for 2 hours at 37°C. After medium aspiration, dimethylsulfoxide was added to cells to dissolve the formazan product, which was quantified spectrophotometrically at 540 nm. Results are expressed as a percentage of viability of the control cultures.

miRNA microarray hybridization and analysis

Total RNA (1 μ g) isolated with *mirVana* miRNA isolation kit (Ambion) was labeled using Flashtag RNA labeling Kit (Cat # FT10AFYB, Genisphere Inc., Hatfield, PA), as per manufacturer's instructions. Biotin-labeled total RNA was then used for Affymetrix GeneChip (Cat # 901325) miRNA hybridization. Four individual sample replicates were hybridized for each of the five treatment groups (20 arrays total) all on

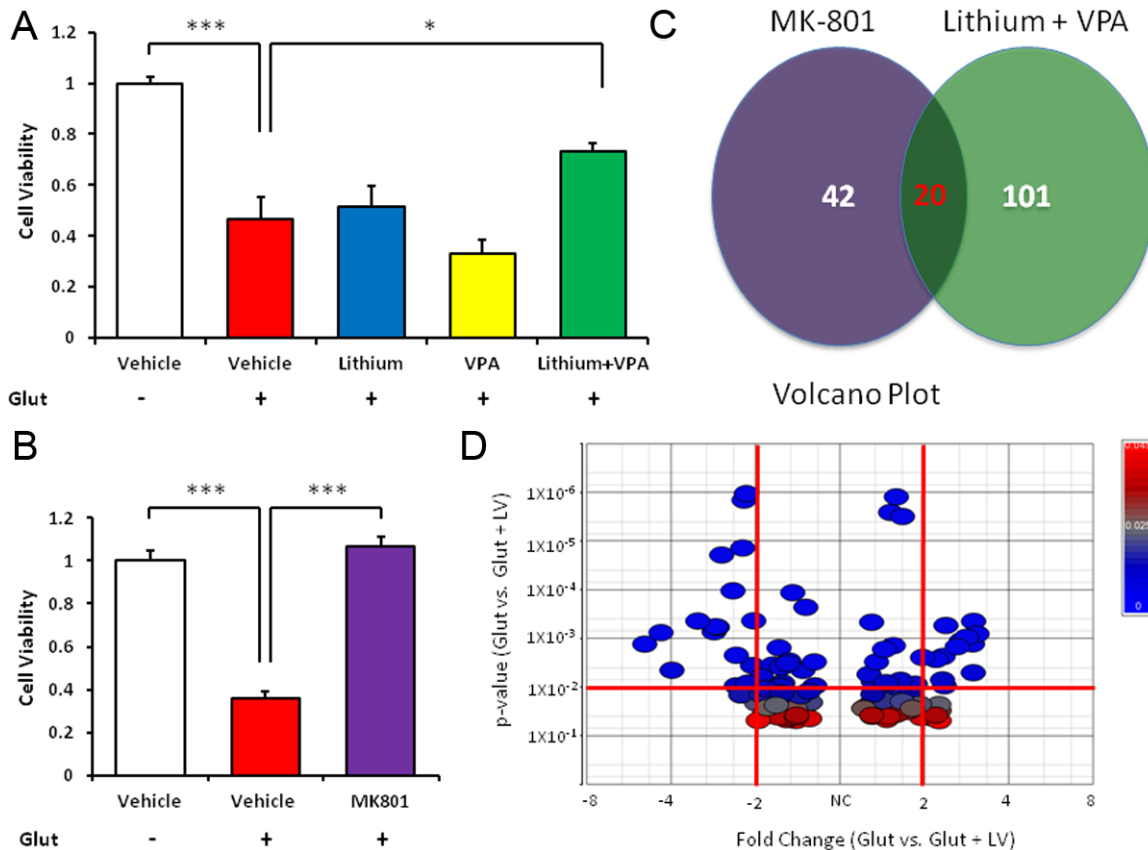


Figure 1. Profiling miRNA expression changes in cerebellar granule cells (CGCs) following two neuroprotective treatment conditions. A. Pretreatment for 6 days with 3 mM LiCl + 0.8 mM VPA protected CGC cultures against challenge with 50 μ M glutamate at 12 DIV, as measured by MTT assay ($n = 6$); and (B) Pretreatment for 30 min with 10 μ M MK-801 protected CGCs against challenge with 100 μ M glutamate at 10 DIV, as measured by MTT assay ($n = 12$); Student's t -test, $*p < 0.05$, $***p < 0.001$. C. Venn Diagram identifying miRNAs differentially regulated by either MK-801 or combination treatment with LiCl or VPA followed by glutamate challenge compared with glutamate challenge alone. Unadjusted p value < 0.05 ; fold regulation ± 1.2 . D. Volcano plot depicting differentially regulated miRNAs in combination treatment condition followed by 50 μ M glutamate challenge compared with glutamate challenge alone (± 1.2 fold change, unadjusted p value < 0.05). The red to blue color gradient indicates higher p -values. Red bars indicate a fold change of ± 2 and p value of 0.01.

the same day under the same conditions. Hybridized arrays were then washed, stained, and scanned as per manufacturer's instructions. For analysis, we utilized the miRNA quality control tool software to access labeling efficiencies using the RNA Spike Control Oligos. We also performed principle component analysis on our samples. miRNA data was analyzed by both GeneSifter and Partek.

Quantitative real-time PCR (qRT-PCR)

cDNA was synthesized from total RNA samples using the TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For all samples, separate primers for individual miRNAs were each used to reverse transcribe 5 ng

of total RNA, as specified by the manufacturer. The TaqMan® MiRNA Assay Kit (Applied Biosystems) was used for qRT-PCR amplification according to the manufacturer's instructions. Relative miRNA levels were normalized to U6 snRNA, a verified endogenous control. Samples were run in triplicates and PCR experiments were repeated for each of the 6-well culture plates.

SH-SY5Y transfection and flow cytometry analysis

Human neuroblastoma SH-SY5Y cells were maintained in a 1:1 mixture of F-12 and Minimal Essential Medium (Gibco, Carlsbad, CA) supplemented with 15% fetal bovine serum and 1%

Pen-Strep (Gibco). At 50% confluency, cells were transfected with 15 nM miR-34a mimic (Dharmacon, Thermo Fisher Scientific, Lafayette, CO) or the non-targeting control cel-miR-67 (Dharmacon) using PepMute siRNA Transfection Reagent (SignaGen Laboratories, Rockville, MD) as per manufacturer's instructions. Medium was changed at 24 hours, and at 48 hours cells were challenged with 100 μ M thapsigargin, an endoplasmic reticulum stressor. 24 hours later, cells were harvested after a 10 minute treatment with papain solution (Hank's balanced salt solution with bicarbonate (Gibco), 1 mM L-cysteine (Sigma, St. Louis, MO), 0.5 mM EDTA, and 20 U/ml papain (Worthington, Lakewood, NJ)), centrifuged at 1000xg for 5 minutes, and resuspended in cell sorting medium (145 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgCl_2 , 10 mM Hepes, 10 mM glucose, and 0.1% bovine serum albumin in sterile H_2O). Cells were then stained with Hoechst 33342 and propidium iodide (Vybrant Apoptosis Assay Kit #5, Molecular Probes, Eugene, OR) and subjected to flow cytometry to detect viable, apoptotic, and necrotic cell populations, as previously described [22].

Bioinformatic analysis

Ingenuity Pathway analysis (IPA) was performed on the six candidate miRNAs that were confirmed via qPCR, as described previously [23]. Briefly, we identified predicted mRNA targets for each candidate miRNA using miRWalk database (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html>) [24] and then these targets were analyzed using IPA. DIANA-mirPath, a web-based computational tool that factors the combinatorial effects of multiple miRNAs modulating a pathway, was performed to identify the potential pathways targeted by the whole Li/VPA miRNAome (all significantly regulated miRNAs by Li/VPA) in order to provide a global picture of miRNA function [25]. All Li/VPA regulated miRNAs that were available in DIANA-mirPath were used for the analysis.

Results

Profiling miRNA expression changes following neuroprotective treatments

We investigated the miRNA mechanisms that may underlie the neuroprotective effects of combination treatment with Li and VPA against

glutamate-induced, NMDA receptor-mediated excitotoxicity. Pretreatment for 6 days with 3 mM LiCl + 0.8 mM VPA significantly protected cerebellar granule cells (CGCs) against challenge with 50 μ M glutamate in an aged (12 DIV), highly vulnerable neuronal culture model (**Figure 1A**). Individual pretreatment with either LiCl or VPA was not protective (**Figure 1A**). In CGCs exposed to excitotoxic levels of glutamate, blocking NMDA receptors using 10 μ M MK-801 provided complete protection, as shown in **Figure 1B**. The glutamate excitotoxicity challenge plus neuroprotective treatments were then profiled using Affymetrix miRNA microarrays ([Supplemental Tables 1, 2 and 3](#)) where 121 and 62 miRNAs were regulated \pm 1.2 fold by Li and VPA combination or MK-801 treatment, respectively (**Figure 1C**). These non-overlapping and overlapping miRNAs that are regulated following neuroprotective treatments are listed in [Supplemental Table 4](#). Also shown are candidate neuroprotective miRNAs depicted in a volcano plot where they are plotted against fold change and *p*-value (**Figure 1D**).

Quantitative qRT-PCR confirmation

Top candidate miRNAs selected based on fold regulation, *p*-value, and literature support were then confirmed using quantitative real-time PCR (qRT-PCR). Samples used for this confirmation included 4 replicates used in our arrays and 2 independent replicates. We confirmed 6 top candidate miRNAs including miR-182, miR-222, miR-495, miR-34a, miR-690, and miR-147 (**Figure 2**). miR-182, miR-147, and miR-222 were shown to be exclusively upregulated following the combination treatment with Li and VPA when compared against all other groups. miR-690 was shown to be upregulated following glutamate excitotoxicity challenge and downregulated to control levels by both combination and MK-801 treatment. miR-34a and miR-495 were both shown to be downregulated following combination treatment.

miR-34a mimic induces apoptosis in SH-SY5Y cells

We next determined the functional effects of inducing miR-34a expression in a human neuroblastoma cell line, SH-SY5Y cells. The neuroblastoma cells were used in this study because they could be effectively transfected with molecules such as miRNA, as opposed to CGCs

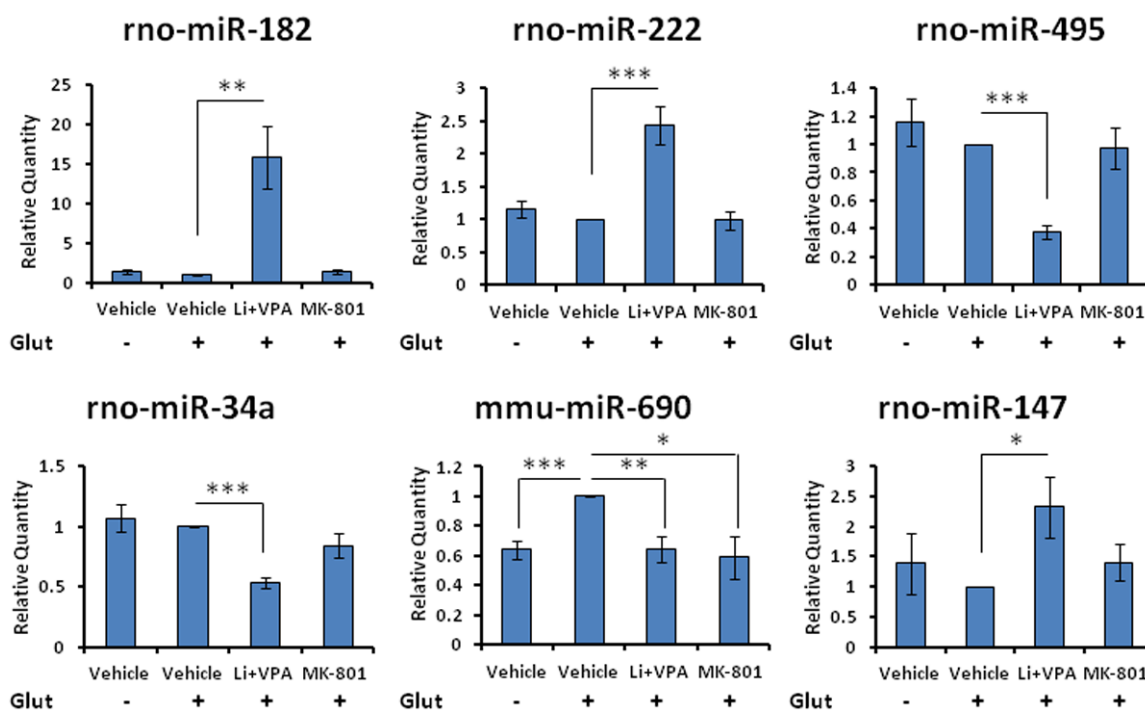


Figure 2. Quantitative real-time PCR (qRT-PCR) confirmation. Top miRNA candidates from microarray (N = 6/group; the experiments included 4 replicates used for array and 2 independent replicates) were confirmed via qRT-PCR. Each miRNA was measured under the following conditions: vehicle alone, vehicle with glutamate, LiCl and VPA combination with glutamate and MK-801 with glutamate. Student's t-test; *p < 0.05, **p < 0.01, ***p < 0.001, between indicated groups. Glut indicates glutamate challenge.

which were transfected with extremely low efficiency. Using a miR-34a mimic and non-targeting control (cel-miR-67), flow cytometry was used to detect cell viability and both apoptotic and necrotic cell death following successful transfection (Supplemental Figure 1). Transfection was performed using PepMute, a peptide-based delivery tool for miRNA mimics, siRNA, and DNA oligos. SH-SY5Y cells were first transfected with miR-34a mimic or control and then 48 hours later were challenged with vehicle or an endoplasmic reticulum (ER) stress agent (100 μ M thapsigargin). In SH-SY5Y cells, glutamate-induced cytotoxicity requires a large dose of glutamate (60 mM) and is not mediated through NMDA receptors but rather via oxidative stress mechanisms [26]. Then 24 hours later, cells were dissociated with papain solution and prepared for flow cytometry by staining with propidium iodide and Hoechst 33342. Our Vehicle + Control miRNA condition showed high cell viability and low rates of apoptotic and necrotic cell death, while Vehicle + miR-34a mimic potentiated apoptosis (Figure 3A). The induction of ER stress by thapsigargin also induced apoptosis where this apoptosis was

further augmented by the miR-34a mimic (Figure 3A). The miR-34a mimic under both baseline and thapsigargin conditions doubled the amount of apoptotic cell death (Figure 3B).

Pathway analysis

To gain insight into the miRNA mechanisms that may contribute to the neuroprotective treatments in our study, we performed dual analyses. The first analysis was a candidate approach using IPA where we restricted our samples to only our PCR-verified miRNAs (Figure 2). This analysis serves to identify top canonical pathways associated with the predicted mRNA targets of each neuroprotective miRNA candidate (Table 1). For instance, TGF- β signaling is a shared canonical pathway targeted by miR-34a, miR-182, and miR-690. This analysis also identified top molecular and cellular functions (Supplemental Table 5) for each candidate miRNA using predicted mRNA targets. Interestingly, 5 out of 6 miRNAs share cell death and survival as leading molecular and cellular functions. The second analysis used a global approach that included all significantly

Table 1. Top canonical Pathways associated with the predicted mRNA targets of neuroprotective miRNA candidates

microRNA	Canonical Pathways	p-value	Ratio
rno-miR-34a	PPAR Signaling	8.18×10^{-6}	10/82 (0.122)
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	1.17×10^{-5}	12/124 (0.097)
	Insulin Receptor Signaling	1.92×10^{-4}	10/118 (0.085)
	TGF- β Signaling	2.29×10^{-4}	8/78 (0.103)
	CDK5 Signaling	3.52×10^{-4}	8/83 (0.096)
rno-miR-147	Creatine-phosphate Biosynthesis	9.67×10^{-3}	1/5 (0.2)
	Thioredoxin Pathway	9.67×10^{-3}	1/5 (0.2)
	Phosphatidylethanolamine Biosynthesis II	1.54×10^{-2}	1/8 (0.125)
	Cardiac β -adrenergic signaling	2.24×10^{-2}	2/120 (0.017)
	Maturity Onset Diabetes of Young (MODY) Signaling	3.06×10^{-2}	1/16 (0.062)
rno-miR-182	TGF- β Signaling	6.98×10^{-4}	7/78 (0.09)
	RAR Activation	2.75×10^{-3}	9/154 (0.058)
	Induction of Apoptosis by HIV1	4.86×10^{-3}	5/58 (0.086)
	Pyridoxal 5'-phosphate Salvage Pathway	5.22×10^{-3}	5/59 (0.085)
	BMP Signaling Pathway	8.38×10^{-3}	5/66 (0.076)
rno-miR-222	CCR5 Signaling in Macrophages	7.76×10^{-7}	7/60 (0.117)
	Endothelin-1 Signaling	2.55×10^{-4}	7/154 (0.048)
	IL-1 Signaling	8.3×10^{-4}	5/85 (0.059)
	CXCR4 Signaling	1.23×10^{-3}	6/138 (0.043)
	Role of NFAT in Regulation of the Immune Response	1.28×10^{-3}	6/139 (0.043)
rno-miR-495	Glucocorticoid Receptor Signaling	3.75×10^{-4}	17/237 (0.072)
	eNOS Signaling	1.45×10^{-3}	10/115 (0.087)
	Inhibition of Angiogenesis by TSP1	1.82×10^{-3}	5/32 (0.156)
	Protein Kinase A Signaling	2.71×10^{-3}	19/333 (0.057)
	HGF Signaling	4.25×10^{-3}	8/92 (0.087)
mmu-miR-690	HMGB1 Signaling	4.67×10^{-4}	13/87 (0.149)
	Germ Cell-Sertoli Cell Junction Signaling	8.23×10^{-4}	17/140 (0.121)
	Colorectal Cancer Metastasis Signaling	8.27×10^{-4}	23/48 (0.106)
	CXCR4 Signaling	1.89×10^{-3}	16/138 (0.116)
	TGF- β Signaling	2.03×10^{-3}	11/78 (0.141)

Canonical pathways are shown below for PCR validated miRNAs. The right-tailed Fischer's exact test *p*-value calculated by IPA to determine the probability of each pathway assigned to each miRNA set is due to chance alone. The ratio indicates the number of miRNA-predicted targets out of the total molecules associated with a particular canonical pathway.

regulated miRNAs that were uploaded into DIANA-mirPath. This *in silico* computational tool performs an enrichment analysis of numerous miRNA target genes to investigate the combinatorial effect of co-expressed miRNAs in the modulation of all known KEGG pathways. From this global analysis, TGF- β signaling also appeared as a top pathway associated with mood stabilizer-regulated miRNAs in addition to numerous other KEGG pathways such as axonal guidance, focal adhesion, actin cytoskeletal regulation, and long-term potentiation (Supplemental Table 6).

Discussion

In this study, we identified specific neuroprotective miRNA candidates of which we confirmed the function of one of these candidates, miR-34a, in survival mechanisms. We then performed dual analyses that took either a candidate approach (6 miRNAs) or a global approach (entire Li/VPA miRNAome) to provide further information on single miRNA and global miRNA function, respectively. We focus our discussion on the current literature support for these 6 miRNA candidates with a specific emphasis on

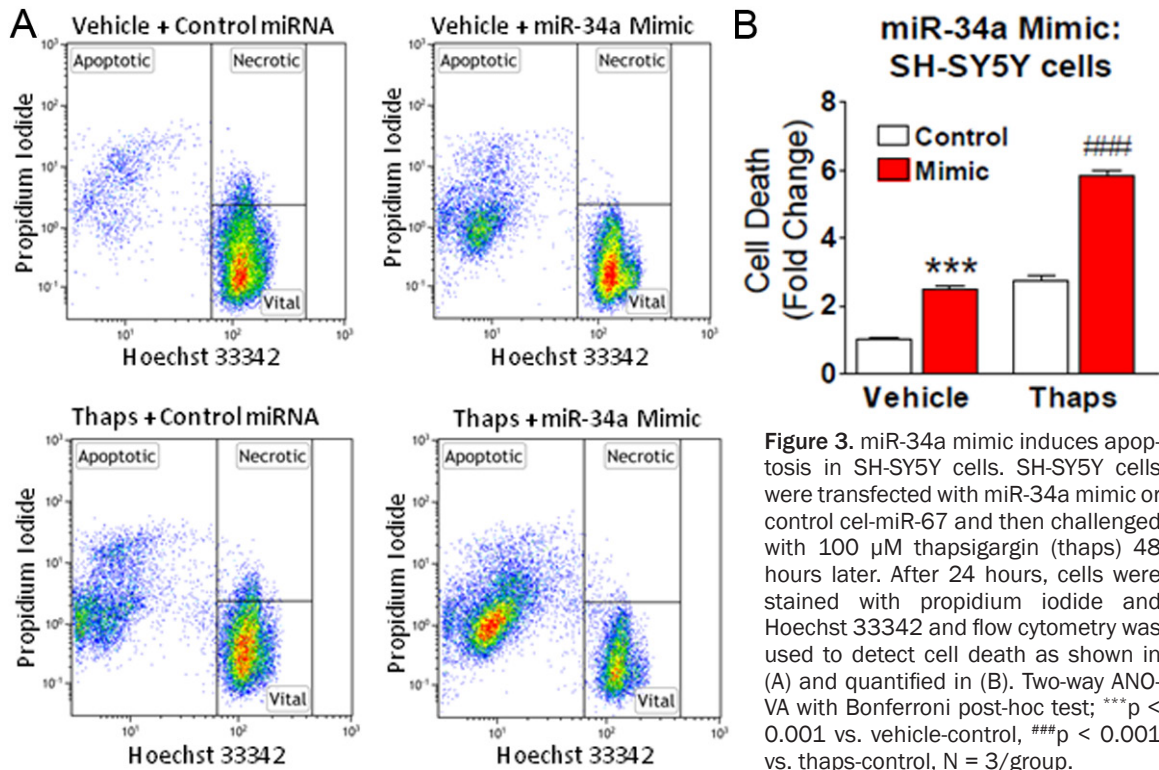


Figure 3. miR-34a mimic induces apoptosis in SH-SY5Y cells. SH-SY5Y cells were transfected with miR-34a mimic or control cel-miR-67 and then challenged with 100 μ M thapsigargin (thaps) 48 hours later. After 24 hours, cells were stained with propidium iodide and Hoechst 33342 and flow cytometry was used to detect cell death as shown in (A) and quantified in (B). Two-way ANOVA with Bonferroni post-hoc test; *** $p < 0.001$ vs. vehicle-control, ### $p < 0.001$ vs. thaps-control, $N = 3/\text{group}$.

neuronal function and targeted pathways, and then we discuss common mechanisms and pathways identified by our *in silico* analyses and their associations with neuropsychiatric and neurodegenerative diseases.

miR-34a has previously been implicated in survival mechanisms in an Alzheimer's disease (AD) mouse model where it is found to be upregulated and inhibit the neuroprotective protein Bcl-2 [27]. In a rat model of temporal lobe epilepsy, miR-34a is upregulated in the hippocampal temporal lobe, and *in vivo* administration of miR-34a antagomir inhibits activated caspase-3 protein expression and reduces neuronal death [28]. miR-34a has also been shown to function in survival signaling in calorie-restricted mice [29]. Collectively, these three studies illustrate the broad neuroprotective potential of miR-34a. Furthermore, these studies support our findings that downregulated miR-34a following neuroprotective Li/VPA combination treatment is neuroprotective in rat primary neuronal cultures (**Figure 1A**), while over-expression of miR-34a induces neuronal death in human SH-SY5Y cells (**Figure 3**).

Recently, some single nucleotide polymorphisms (SNPs) located in the 3' UTR of the amy-

loid precursor protein (APP) have been documented in Alzheimer's patients [30]. miR-147 down-regulates endogenous APP expression, and miR-147 binding was supported by luciferase-based assays [30]. SNP variant T171C, an AD-specific polymorphism located near the miR-147/APP binding site, significantly affects the activity of miR-147 and may potentially lead to augmented APP expression and cellular compromise [30]. Furthermore, miR-23a, miR-143, miR-324-5p, and miR-422a (all identified in our study- [Supplemental Table 4](#)), are also identified as potential players through miRNA-targeted SNP interactions contributing to AD [31]. In non-neuronal cells, miR-147 is a negative regulator of toll-like receptor (TLR)-associated signaling in macrophages [32], where knock-down of miR-147 increases inflammatory cytokine expression [32]. In addition to these actions, we predict the thioredoxin pathway, creatine-phosphate biosynthesis, and phosphatidylethanolamine biosynthesis as critical canonical pathways targeted by miR-147.

Ischemic preconditioning to oxygen glucose deprivation (OGD) is a technique that facilitates resistance to loss of blood supply in cells and can be an effective neuroprotective therapy. In a torpor model to investigate natural tolerance

Mood stabilizer-regulated miRNAs and neurodegenerative disease

Table 2. Lithium- and VPA- regulated miRNAs associated with neuropsychiatric (NSD) and neurodegenerative diseases (ND)

Regulated miRNAs by treatment	Neuropsychiatric disease (NSD)	Ref. (NSD)	Neurodegenerative Disease (ND)	Ref. (ND)
MK-801 and Li + VPA				
hsa-miR-1308				
hsa-miR-147b				
hsa-miR-199b-3p	AU	[46]		
hsa-miR-27b-star				
hsa-miR-29b	SZ, AU	[46, 47]	HD, PD, AD	[48-53]
hsa-miR-518e-star				
hsa-miR-589				
hsa-miR-591				
hsa-miR-628-5p				
mmu-miR-210	BD	[54]	AD	[55]
mmu-miR-448			AD	[55]
mmu-miR-449c				
mmu-miR-551b			AD	[56]
mmu-miR-690				
mmu-miR-697				
rno-miR-199a-3p	SZ	[47]	HD, PD, AD	[49, 55, 57]
rno-miR-363-star				
rno-miR-449a	SZ	[58]		
rno-miR-488				
rno-miR-708				
Li + VPA				
hsa-let-7e	SZ	[59]		
hsa-miR-100			HD, AD	[48, 55, 57]
hsa-miR-10a	AU, MD	[60, 61]	AD	[55]
hsa-miR-129-5p	AU	[62]		
hsa-miR-135b-star				
hsa-miR-138	BD, SZ	[54]	HD, AD	[55, 57]
hsa-miR-143			AD	[55]
hsa-miR-146a	MD	[61]	HD	[63]
hsa-miR-17	SZ	[47]		
hsa-miR-181a	BD	[54, 59]	AD	[55]
hsa-miR-181b	SZ	[59, 64]	AD	[65]
hsa-miR-182	AU, MD	[46, 66]		
hsa-miR-187				
hsa-miR-194	AU	[46]	AD	[55, 56]
hsa-miR-20a	SZ, MD	[59, 61]		
hsa-miR-22	BD, SZ	[54]		
hsa-miR-220a				
hsa-miR-222			HD	[48, 57]
hsa-miR-23a	AU	[46, 62]		
hsa-miR-302a				
hsa-miR-324-5p	MD	[61]		
hsa-miR-329				
hsa-miR-330-3p				

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hsa-miR-34a	SZ	[9, 58]	AD	[55, 65]
hsa-miR-371-5p			AD	[55]
hsa-miR-374b			PD	[49]
hsa-miR-374b-star				
hsa-miR-409-3p				
hsa-miR-422a			AD	[55]
hsa-miR-423-3p				
hsa-miR-431	AU	[62]		
hsa-miR-487b				
hsa-miR-497	MD	[61]	AD	[55]
hsa-miR-518c-star				
hsa-miR-520a-5p				
hsa-miR-526a				
hsa-miR-543				
hsa-miR-599				
hsa-miR-652	SZ, AU	[43, 58, 62]		
hsa-miR-664-star				
hsa-miR-668				
hsa-miR-7	SZ, AU	[9, 62]		
hsa-miR-708				
miR-409-5p				
mmu-let-7e	SZ	[59]		
mmu-miR-100			HD, AD	[48, 55, 57]
mmu-miR-1194				
mmu-miR-122				
mmu-miR-129-5p	AU	[62]		
mmu-miR-146a	MD	[61]	HD	[63]
mmu-miR-17	SZ	[47]		
mmu-miR-181a	BD	[54, 59]	AD	[55]
mmu-miR-181b	SZ	[59, 64]	AD	[65]
mmu-miR-182	AU, MD	[46, 66]		
mmu-miR-187				
mmu-miR-199b	AU	[46]		
mmu-miR-22	BD, SZ	[54]		
mmu-miR-222			HD	[48, 57]
mmu-miR-23a	AU	[46, 62]		
mmu-miR-28			PD	[49]
mmu-miR-323-3p				
mmu-miR-330-star				
mmu-miR-337-3p				
mmu-miR-345-3p			AD	[55]
mmu-miR-34a	SZ	[9, 58]	AD	[55, 65]
mmu-miR-34b-5p			HD, PD	[67, 68]
mmu-miR-379				
mmu-miR-450b-5p				
mmu-miR-485			HD	[48]
mmu-miR-487b				
mmu-miR-495	AU	[46]		
mmu-miR-504	BD	[9]		

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mmu-miR-543				
mmu-miR-652	SZ, AU	[43, 58]		
mmu-miR-770-5p				
rno-let-7e	SZ	[59]		
rno-miR-126			PD, AD	[49, 55]
rno-miR-129	AU	[62]		
rno-miR-146a	MD	[61]	HD	[63]
rno-miR-181b	SZ	[59, 64]	AD	[65]
rno-miR-182	AU	[46, 66]		
rno-miR-187				
rno-miR-205	AU	[46]	HD, AD	[55, 57]
rno-miR-22	BD, SZ	[54]		
rno-miR-222			HD	[48, 57]
rno-miR-23a	AU	[46, 62]		
rno-miR-24-2-star				
rno-miR-28			PD	[49]
rno-miR-298			AD	[56]
rno-miR-330-star				
rno-miR-345-3p			AD	[55]
rno-miR-34a	SZ	[9, 58]	AD	[55, 65]
rno-miR-379				
rno-miR-409-5p				
rno-miR-429			AD	[55]
rno-miR-431	AU	[62]		
rno-miR-487b				
rno-miR-495	AU	[46]		
rno-miR-543-star				
rno-miR-652	SZ, AU	[43, 58]		
rno-miR-770				
rno-miR-7b				

The literature was surveyed for studies where miRNAs are listed below that met the following criteria: (A) They were found to be significantly associated with either NSD or ND; (B) They were examined in human samples (cell lines, blood, CSF, and brain tissue); (C) They were regulated in our study by combination treatment ($p < 0.05$, fold ± 1.2). Abbreviations: BD (bipolar disorder); SZ (schizophrenia); AU (autism); MD (major depression); HD (Huntington's disease); PD (Parkinson's disease); AD (Alzheimer's disease).

to brain ischemia, the miR-182 family was down regulated during the torpor phase compared to active animals [33]. Furthermore, in SH-SY5Y cells, repression of the miR-182 family increases tolerance to OGD-induced cell death [33]. miR-182 has been shown to be regulated under ischemic preconditioning and is implicated in neuroprotection following this procedure [34]. miR-182 has also been linked with modulating DNA repair mechanisms in breast cancer models [35]. Therefore, miR-182 has the potential to facilitate mechanisms of neuroprotection through ischemic preconditioning as well as regulation of DNA repair. We predict miR-182 to target TGF- β signaling and retinoic acid receptor (RAR) activation, among others.

Neurite outgrowth and immune function are two roles associated with miR-222. In dorsal root ganglia neurons, miR-222 facilitates neurite outgrowth via targeting phosphatase and tensin homologs deleted on chromosome 10 (PTEN) [36]. In profiling experiments of human epilepsy, miR-222 was regulated by medial temporal lobe epilepsy and implicated in targeting genes involved in the immune response [37]. Our predictions implicate miR-222 signaling in macrophages, endothelin-1 signaling, and NFAT regulation of the immune response among others.

Growth factor expression is integral for cell viability. Importantly, miR-495 may modulate

brain-derived neurotrophic factor (BDNF). The binding site of miR-495 is in close proximity to two BDNF 3'-UTR polyadenylation sites and miR-495 exhibits developmental and lamina-specific expression patterns in human prefrontal cortex that is reciprocal to some extent with BDNF's expression pattern [38]. We predict that miR-495 will also target glucocorticoid receptor signaling, hepatocyte growth factor (HGF) signaling, eNOS signaling and PKA signaling.

miR-690 has been relatively unexplored in terms of its neuronal function. Recently, it was found that miR-690 was elevated in the rat spinal cord following chronic constriction injury (CCI) in a neuropathic pain model [39]. We predict that miR-690 facilitates the following canonical pathways: HMGB1 signaling, TGF- β signaling and CXCR4 chemokine receptor 4 (CXCR4) signaling. Interestingly, induction of CXCR4 by VPA treatment was found to enhance migration of mesenchymal stem cells (MSCs) *in vitro*, and improve the functional recovery following transplantation of VPA-treated MSCs into rats after cerebral ischemia [40, 41]. Future studies on miR-690 should address its role on MSC migration.

Our study using a candidate approach (IPA analysis) identifies multiple canonical pathways (Table 1) associated with the six miRNAs that may account for their effects on neuronal survival. Interestingly, TGF- β signaling is linked with three of the six: miR-34a, miR-182, and miR-690. Using a global approach (DIANA-mirPath), TGF- β signaling also is identified as a top pathway associated with mood stabilizer-regulated miRNAs. TGF- β expression has been implicated following several brain injuries including cerebral ischemia, traumatic brain injury, and AD [42]. TGF- β signaling is also predicted to be upregulated in schizophrenia by miRNAs found in the dorsolateral prefrontal cortex [43]. In addition to TGF- β signaling, regulation of actin cytoskeleton, axonal guidance, focal adhesion, and long-term potentiation were other KEGG pathways predicted to be upregulated in this study. Interestingly, these pathways were predicted to be targeted by the Li/VPA miRNAome as analyzed by DIANA-mirPath (Supplemental Table 6). This suggests an association between miRNA predicted pathways from our *in vitro* study and the clinical study by Santarelli et al. TGF- β has also been

shown to provide neuronal protection against excitotoxic lesion [44], and in cortical neurons TGF- β signaling is regulated by lithium through GSK-3 inhibition [45]. This suggests that the neuroprotective effects of the Li/VPA combination treatment may modulate TGF- β signaling via miRNA regulation among other mechanisms in order to protect against glutamate insult.

In Table 2, we highlight mood stabilizer-regulated miRNAs associated with neuropsychiatric and neurodegenerative diseases. These candidates may be useful for further investigation into disease and treatment mechanisms. For instance, candidate miRNAs that emerge from this list that are dysregulated in both neuropsychiatric and neurodegenerative diseases and regulated by mood stabilizers (miR-10a, miR-20, miR-29b, miR-138, miR-199a-3p) may act via common mechanisms that, if targeted, can facilitate treatment in their related diseases. Further analysis of this list may also identify critical miRNA-regulated networks that contribute to neuropsychiatric or neurodegenerative diseases.

In conclusion, our study identifies miRNAs that are potential candidates for facilitating neuroprotection against glutamate-induced excitotoxicity in a neuronal model through global regulation of multiple targets and signaling pathways. We validated microarray results of top candidate neuroprotective miRNAs via q-PCR and then used a human neuronal cell line to further demonstrate functional effects of one of these candidates, miR-34a. We performed pathway analysis on the predicted targets of our neuroprotective miRNA candidates using both a candidate and global approach, and identified miRNA-mediated pathways that have associations with neurological diseases such as schizophrenia. Finally, we provide a comparison of the Li/VPA miRNAome identified by our study along with miRNAs found to be dysregulated in neuropsychiatric and neurodegenerative diseases.

Acknowledgments

We would like to acknowledge the support of the Intramural Research Program of the NIMH, NIH. We would also like to acknowledge the technical support kindly provided by Weiwei Wu for microRNA array processing, and the editorial suggestions by Peter Leeds.

Abbreviations

AD, Alzheimer's disease; ALS, Amyotrophic lateral sclerosis; APP, Amyloid precursor protein; Bcl-2, B-cell lymphoma 2; BDNF, Brain-derived neurotrophic factor; CCI, Chronic constriction injury; CGCs, Cerebellar granule cells; CXCR4, CXC chemokine receptor 4; ER, Endoplasmic Reticulum; HDAC, Histone deacetylase inhibitor; IPA, Ingenuity Pathway Analysis; Li, Lithium; miRNA, microRNA; MSC, Mesenchymal stem cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMDA, N-methyl-D-aspartate; OGD, Oxygen glucose deprivation; qRT-PCR, Quantitative real time PCR; SNPs, Single nucleotide polymorphisms; VPA, Valproic acid.

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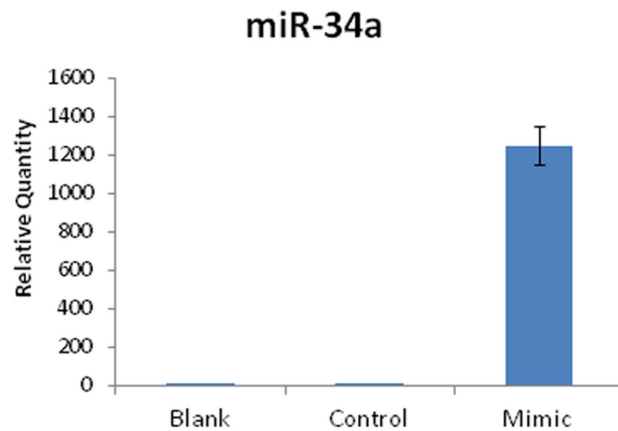
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Supplemental Figure 1. Transfection efficacy of miR-34a mimic. Transfection of miR-34a mimic into SH-SY5Y cells robustly upregulates miR-34a at 24 hours versus no transfection (blank) and transfection with control construct cel-miR-67 (N = 3/group) confirmed via qRT-PCR.