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Neurotrophin-3 mRNA a putative target of miR21 following status epilepticus

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Abstract

Status epilepticus induces a cascade of protein expression changes contributing to the subsequent development of epilepsy. By identifying the cascade of molecular changes that contribute to the development of epilepsy we hope to be able to design therapeutics for preventing epilepsy. MicroRNAs influence gene expression by altering mRNA stability and/or translation and have been implicated in the pathology of multiple diseases. MiR21 and its co-transcript miR21*, microRNAs produced from either the 5″ or 3′ ends of the same precursor RNA strand, are increased in the hippocampus following status epilepticus.

We have identified a miR21 binding site, in the 3′ UTR of neurotrophin-3 that inhibits translation. Neurotrophin-3 mRNA levels decrease in the hippocampus following SE concurrent with the increase in miR21. MiR21 levels in cultured hippocampal neurons inversely correlate with neurotrophin-3 mRNA levels. Treatment of hippocampal neuronal cultures with excess K^+Cl^-, a depolarizing agent mimicking the episode of status epilepticus, also results in an increase in miR21 and a decrease in neurotrophin-3 mRNA. MiR21 is a candidate for regulating neurotrophin-3 signaling in the hippocampus following status epilepticus.

Keywords

epilepsy; hippocampus; pilocarpine; microRNA; neurotrophin-3; miR21

1 Introduction

Epilepsy, a disorder of recurrent unprovoked seizures, has a lifetime prevalence of ~0.5%. Currently treatments are aimed at suppressing seizures but are not effective in 30% of patients and no medications to prevent or reverse the development of epilepsy have been developed. An improved understanding of which molecules contribute to the development

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of epilepsy will allow identification of therapeutic targets and anti-epileptogenic medications.

Pilocarpine treatment of rodents provokes a prolonged seizure, status epilepticus (SE), and leads to changes that eventually cause spontaneous seizures or epilepsy. This model has been used to identify suspected molecular and cellular changes contributing to the development of epilepsy. Molecular changes shown to influence the development of epilepsy include altered expression of neurotransmitters, neurotransmitter receptors, transcription factors, neurotrophins and neurotrophin receptors (Lin, *et al.*,2003, He, *et al.*, 2004, Raol, *et al.*,2006, Noe, *et al.*,2008). In several models of epileptogenesis, neurotrophin-3 mRNA (NT-3) levels decrease following an insult that leads to the development of epilepsy (Bengzon, *et al.*,1993, Schmidt-Kastner&Olson,1995, Mudo, *et al.*, 1996, Kim, *et al.*,1998). NT-3 deprivation decreases neurite outgrowth, neuronal cell survival and suppresses the development of epilepsy (Lowenstein&Arsenault,1996, Elmer, *et al.*,1997). To date, no one has identified the mechanism that suppresses NT-3 levels following SE.

MicroRNAs bind to the 3′ tails of mRNAs, decreasing protein levels either by blocking translation and/or by destabilizing mRNAs leading to increased degradation. MicroRNA genes are interspersed throughout the genome and following transcription of a primary transcript, pri-miRNA, the Drosha complex cleaves it to a pre-miRNA and finally a dicer containing complex cleaves it to mature ~21 base pare length miRNAs. Additional members of the microRNA-processing complex have been identified and add an additional layer of regulation of mature microRNA products (for review see (Nilsen,2007). MicroRNAs are controlled at a transcription, translation or stabilization level (Thomson, *et al.*,2006). Increased levels of miR21 can occur through specific activation of a post-translational complex of Drosha and SMAD, increasing miR21 production or stability (Davis, *et al.*,2008, Davis, *et al.2010*). MiR21 is an extensively studied microRNA, as it is widely increased in multiple cancers including those of the nervous system and following hypoxia (Kulshreshtha, *et al.*,2007, Gabriely, *et al.*,2008, van Rooij, et al. 2006). MiR21 has been shown to be increased in several brain injury models, stroke, trauma and as demonstrate here following SE (Buller, *et al.*,2010, Hu, *et al.*,2011, Redell, *et al.*,2011, Ziu, *et al.2011*). MiR21 targets to date have included proteins involved in neurotrophin signaling, sprouty family members SPY-1 and SPY-2 but not NT-3 (Sayed, *et al.*,2008, Thum, *et al.*,2008). Using a rodent epilepsy model, we show that miR21 is a candidate for regulating NT-3 mRNA following an episode of SE.

2 Results

A MicroRNA array of rat hippocampus was carried out using whole RNA solated from animals treated with high dose pilocarpine to induce SE or low dose pilocapine (control) and sacrificed at 4, 48 hours and 3 weeks following SE. MiR21 and 21* increased at several time points following SE (Table 1). We then carried out real-time PCR analysis of hippocampal RNA from a new set of animals to confirm the microRNA array results. MiR21 increased at 48 hours and 3 weeks following SE but not at 4 hours (Figure 1A, 4 hour SE mean 0.9 ± 0.2 SEM; 48 hours SE mean 2.4 ± 0.4; SEM; 3 weeks SE mean 2.4 ±

0.2 SEM; Kruskal-Wallis Dunn's Multiple comparison P *<0.5, **<0.01). In contrast miR21* was increased at 4 hours and 48 hours following SE but not at 3 weeks (Figure 1B, 4 hour SE mean 4.4 ± 0.7 SEM; 48 hour SE mean 7.5 ± 2.0 SEM; 3 week SE mean 1.85 ± 0.43 SEM; Kruskal-Wallis Dunn's Multiple comparison P **<0.01). The real-time PCR data closely mimic the time course of increase seen in the arrays. In both cases there appeared to be a difference in the timing of miR21 and 21* increases following SE.

We were interested in determining if the increase in miR21 could be responsible for a change in mRNA or protein expression following SE. Taking a bioinformatics approach using TargetScan 4.1 and Sanger miRBase we scanned for putative targets of miR21 and identified NT-3. Similar to prior studies, we found a decrease in NT-3 mRNA levels following SE. In our model the decrease did not occur at the 4-hour time point but was present at 48 hours and 3 weeks (Figure 2, 4 hour SE mean 1.1 ± 0.1 SEM; 48 hour SE mean 0.3 ± 0.1 SEM; 3 week SE mean 0.5 ± 0.1 SEM; Kruskal-Wallis Dunn's Multiple comparison P *<0.05), mirroring the increase in miR21 at 48 hours and 3 weeks.

To determine if miR21 can suppress translation of an mRNA containing the 3′ UTR of NT-3, we constructed a plasmid with a luciferase transcript and the 3′ UTR of NT-3. MiR21 but not a scrambled microRNA suppressed luciferase activity (Figure 3, CTL mean 1 ± 0.04 SEM; miR21 mean 0.4 ± 0.05 SEM; scrambled miR mean 1.4 ± 0.3 SEM; Kruskal-Wallis Dunn's Multiple comparison P ***<0.001). To confirm direct suppression by miR21 on translation of the NT-3 3′ UTR construct, we mutated the putative miR21-binding site in the NT-3 3′ UTR. Suppression of luciferase activity by miR21 was lost with co-transfection of the mutated NT-3 3′ UTR (Figure 3, Mutant mean 1 ± 0.1 SEM; miR21 1.1 ± 0.1 SEM; scrambled miR mean 0.9 ± 0.1 SEM).

To determine if the inverse relationship between miR21 levels and NT-3 mRNA levels in the hippocampus following SE occurs *in vitro* we measured miR21 levels and NT-3 mRNA levels in neonatal hippocampal neuronal cultures. Similar to the findings *in vivo*, cultures with higher levels of miR21 have lower levels of NT-3 mRNA (Figure 4, Slope=−0.73 ± 0.17, r squared 0.52 and slope non-zero P=0.0004). We also utilized an *in vitro* 'seizure' model using 20mM K^+Cl^- to depolarize neurons and discovered similar increases in miR21 and 21* in neuronal cultures. While there was no change at 24 hours after K^+Cl^-, both miR21 and 21* increased at 72 hours following K^+Cl^- treatment (Figure 5A, 24 hour post K^+Cl^- treatment miR21 1.3 ± 0.3 SEM; 72 hour post K^+Cl^- treatment miR21 2.1 ± 0.3 SEM; Figure 5C 24 hour post K^+Cl^- treatment miR21* 1.8 ± 0.4 SEM; 72 hour post K^+Cl^- treatment miR21* 2.2 ± 0.4 SEM Kruskal-Wallis Dunn's Multiple comparison P *<0.5). We find that the increase in miR21 is accompanied by a decrease in NT-3 mRNA at 72 hours (Figure 5B, 24 hour post K^+Cl^- treatment NT-3 1.4 ± 0.3 SEM; 72 hour post K^+Cl^- treatment NT-3 0.3 ± 0.05 SEM Kruskal-Wallis Dunn's Multiple comparison P *<0.5).

3 Discussion

Here we have shown that miR21 and miR21* increase in the hippocampus following pilocarpine induced SE. Prior studies have shown an increase in miR21 following hypoxia in heart tissue or with increased inflammation in the lungs (van Rooij, *et al.*,2006, Lu, *et al.*,

2009) and following hypoxia or trauma in the brain (Redell, *et al.*,2011, Ziu, *et al.*, 2011). Together with our findings of an increase in miR21 following SE, miR21 appears to be upregulated following biologic stresses in a variety of tissues (Kulshreshtha, *et al.*,2007). As brain hypoxia, trauma and SE all contribute to an increased incidence of epilepsy it will be interesting to determine if the increase in miR21 found in these models promotes or prevents the development of epilepsy.

The distinct time course for the increase in the two miR21 gene products, miR21 and 21* is intriguing, as miR21* increases at 4 hours, prior to the increase in miR21, at 48 hours and 3 weeks. Both products should be produced in equivalent concentrations from the same RNA molecule. The difference in miR21 and 21* levels at 4 hours suggests differential post-transcriptional processing of miR21 and 21* following SE. While microRNAs are small molecules, they have been shown to undergo post-translational regulation by SMADs outside of the nervous system. Our data would support future studies to determine if SMADs are regulating miR21 and 21* following SE.

The increase in hippocampal miR21 at 48 hours and 3 weeks coincides with a decrease in NT-3 mRNA, suggesting miR21 is a candidate for regulation of NT-3 mRNA levels. Using a bioinformatics approach we identified a highly conserved region in the 3′ tail of NT-3 that has a putative miR21-binding site. MiR21 blocked translation of an NT-3 3′UTR luciferase reporter construct and mutation of the miR21 seed pairing region eliminated miR21's ability to block translation. MiR21 is capable of blocking translation of mRNA's containing the NT-3 3′ UTR.

We have evidence *in vitro* that miR21 levels inversely correlate with NT-3 mRNA levels. In hippocampal neuronal cultures there is an inverse correlation between miR21 and NT-3 mRNA levels. Hippocampal neuronal cultures treated with the depolarizing agent K^+Cl^- increases miR21 and miR21* and coincides with a decrease in NT-3 mRNA. The data suggests that the increase in miR21 following SE could be responsible for down regulation in NT-3 mRNA at 48 hours and 3 weeks but further studies *in vivo* will be needed to prove this hypothesis.

NT-3 and BDNF are neurotrophic factors that have been implicated in the development of epilepsy in several rodent models. Mice with decreased levels of NT-3 develop epilepsy at a slower rate suggesting that suppressing NT-3 levels could be a therapeutic strategy for preventing epilepsy (Elmer, *et al.*,1997). In contrast, chronic infusion of NT-3 into the hippocampus can also delay epilepsy development as well as contribute to aberrant neuronal sprouting into the pyramidal cell layer (Xu, *et al.*,2002). A proposed reconciliation of these disparate results is based on findings that chronic administration of neurotrophins down-regulate their receptors and may result in overall decreased neurotrophin signaling (Binder, 2007). Decreased NT-3 levels following SE would suppress neurotrophin signaling and epilepsy development. The increase in miR21 after SE could be anti-epileptic if one of its primary targets is NT-3.

Seventy-two hours after K^+Cl^- treatment, mir21 and 21* increased and NT-3 decreased. In the K^+Cl^- treated neuronal cultures the housekeeping genes 4.5s and PPIA also decreased.

We think the decrease in house keeping genes was due to a subset of neurons dying following K^+Cl^- treatment, TUNEL staining was increased at 72 hours after K^+Cl^- treatment (data not shown). The neuronal cell loss complicates interpretation of the mRNA changes after the K^+Cl^- treatment, though the most straightforward explanation is that remaining neurons have an increase in miR21 and 21* and decrease in NT-3. In contrast to the *in vitro* decrease in the housekeeping genes following K^+Cl^- treatment there was no change in 4.5s or PPIA in the hippocampus following SE. There may be less overall cell loss or other compensation, following SE that preserves housekeeping RNA expression *in vivo* compared to K^+Cl^- treatment *in vitro*.

Whether the decrease in NT-3 mRNA is related to the increased cell death in the K^+Cl^- treated culture is not known. Treatment with excess NT-3, however, has been shown to be neuroprotective in cultures exposed to low glucose or elevated glutamate. Decreased NT-3 mRNA following traumatic brain injury has been implicated in neuronal cell loss (Cheng&Mattson,1994, Hicks, *et al.*,1997, Yang, *et al.*,2005). MiR21 via down regulating of NT-3 is a candidate for increased neuronal cell loss following SE. However, another study reported miR21 over-expression was neuroprotective following glucose and oxygen deprivation (Buller et. al.,2010). Future studies measuring neuronal loss after SE in animals lacking miR21 can clarify what roll it has in epileptogenesis and neuronal injury.

In addition to suppressing NT-3 mRNA, miR21 is also a candidate for inhibiting translation of hundreds of other mRNAs following SE that could also influence epileptogenesis (TargetScan 4.1 and Sanger miRBase). Furthermore, miR21 is one of almost a thousand microRNAs suggesting that microRNA control of translation in epilepsy provides a complex and fruitful area for further study.

4 Methods and Materials

Induction of SE

The Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia approved the experimental protocol. Adult male CD (a spraguedawley) rats from Charles River between 60 to 90 days of age underwent pilocarpine induced SE. All animals received methylscopalamine, 1 mg/kg intraperitoneal (IP) 30 minutes prior to pilocarpine to block peripheral cholinergic effects. SE was induced with pilocarpine (385 mg/kg, IP), with a half dose given 1 hour later if a stage V seizure has not occurred. Control rats received, $1/10^{th}$ dose or 38.5 mg/kg of pilocarpine IP to partially control for pilocarpine effects. The SE rats were monitored for appearance of stage V racine seizures,(Racine,1972) and receive a dose of valium 6mg/kg IP 1 hour after induction of a stage V (tonic clonic) seizure. If the animal was still having behavioral seizures 2 hours after the first dose of valium it recieved 3mg/kg dose of valium every 2 hours until the behavioral seizures resolved. Control animals receive valium doses similar to the SE group to try and control for the effects of valium treatment.

Rats were sacrificed 4 hours, 48 hours, and 3 weeks after the induction of status epilepticus. Rats were anesthetized with isoflurane and hippocampal tissue was dissected out and fast frozen, sonicated and RNA extracted using the mirVANA miRNA isolation kit (Ambion

Inc., Austin, Texas, USA). The quality and quantity of RNA are assessed on the Agilent Bioanalyzer for presence of 5, 5.8, 18S bands.

Array

An Exicon 10.2 array (Exicon,Denmark) was carried out following the companies protocol. Two micrograms of total hippocampal RNA from 4 hour, 48hour and 3week control and SE treated animals was labeled with Cy3 or Cy5 aminoallyl tailing to make the fluorescent probes using the Exicon labeling kit. We took a dye swap approach, a control-Cy3 and seizure-Cy5 animal is probed on an array then the same samples with the dyes switched, seizure-Cy3 and control-Cy5 are probed on an array (Tsai, *et al.*,2003, Altman,2005). Four samples for control and SE were done at each time point. A simple T test was performed for each sample.

Real time-PCR

Reverse transcription using random hexamers was performed using a Superscript II Reverse Transcription kit for NT-3 and PPIA, cyclophilin (Invitrogen, Carlsbad, California, USA). Specific stem loop miRNA primers from the Taqman MicroRNA Assays and reagents from the Reverse Transcription Kit (Applied biosystems) were used to transcribe miR21, 21* and 4.5s. Concentrations of RNA and cDNA were measured using a spectrophotometer (ND 1000, Thermo Fischer Scientific Inc, Wilmington, DE). Reactions were carried out in 394-well plates with 5 ml of the Taqman Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ), 0.5ml of the probe/primer mix for either the gene of interest (NTF3-Rn00579280_m1, or PPIA (Rn00690933_m1), 2.5ul of ddH2O, and 2ml of sample DNA per well. Concentrations of cDNA were diluted so that approximately 1000ηg-1100ηg of sample cDNA were added to each well. Each sample was run in two sets of triplicates, one set with the probe for the gene of interest and one for PPIA, cyclophilin. A standard curve for each probe was included on each plate using cDNA from the cortex of a control rat. For experiments measuring miR21 the MIMAT0000530 and miR21*, MIMAT0004711 probe sets from Applied Biosystems used the same protocol except the No AmpErase UNG (Applied Biosystems) enzyme was used and 4.5S ribonuclear small RNA as a loading control (Applied Biosystems). Real-time PCR assays were carried out on a SDS 7900HT model thermocycler (Applied Biosystems). The real-time PCR settings were 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 95°C for 1 minutes and 60°C for 1 minute. The housekeeping genes, 4.5s or PPIA (cyclophilin) for the hippocampus samples did not vary across treatment group for any of the time points.

Cell culture and Transfection

Hek 293 cells were maintained in Dulbecco's modified eagle's medium (Whittaker Bioproducts, Walkersville, MD) plus 10% FBS, penicillin and streptomycin (GIBCO BRL, Carlsbad, CA). The cells were split in 24 well plates a day before transfection at 50-60% confluency; transfections were carried out with 2μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per well. Controls were transfected with a pMIR REPORT Luciferase miRNA Expression Reporter Vector (Ambion) with the NT-3 3′ tail inserted between Spe1 and Sac1 sites (120ng). Treatment wells received 3ρmoles of miR21 oligo

5′UCAACAUCAGUCUGAUAAGCUA3′ or scrambled oligo 5′AUCAUGCAAUAGCAUCUAGCAU3′ (Integrated DNA Technologies, Corraville, IA) along with 120ng of luciferase NT-3 construct, all diluted in OptiMEM 1 reduced serum medium. All wells were co-transfected with B-gal pMIR-REPORT to normalize for transfection efficiency. A green fluorescent protein (GFP) expression vector served to visually asses transfection efficiency and typically >90% of the cells were transfected. Cell lysates were collected 24 hours post-transfection and luciferase assays were performed using the Luciferase/beta-Galactosidase Luciferase Assay Kit (Applied Biosytems). Luminescence was measured using microplate luminometer (Veritas, Turner Biosystems, Sunnyvale CA).

Neuronal Cultures

Hippocampi from E19 rat embryos are dissected from anesthetized pregnant Sprague–Dawley rats and trypsinized in Dulbecco's minimum essential medium (Whittaker Bioproducts) containing 0.027% trypsin at 4°C for 20min (Cummings, *et al.*,1996). They are triturated in media consisting of DMEM supplemented with 10%bovine calf serum (Hyclone Lab), 10%Ham's F12 with glutamine (Whittaker Bioproducts), and 50 U/mL penicillin–streptomycin (Sigma). Dissociated cells are plated at a density of 100,000 cells/mL in 35-mm petri dishes in Neurobasal medium (GIBCO) supplemented by B27 (GIBCO) and cultured at 37° C in a humidified 5%CO2 incubator. After three weeks they were treated with the addition of K^+Cl^- to a final concentration of 20mm for 3 hours or an equal volume of normal saline, then washed and fresh medium was added. Plasmid constructs: The pmiR-REPORT miRNA Expression Reporter Vector System (Ambion) was used to construct the NT-3 3′ luciferase constructs. The 3′UTR of Neurotrophin 3 was obtained from a PCR reaction of rat brain and cloned between the Spe1 and Sac1 sites and the sequence was confirmed. A mutant version of the 3′UTR was prepared by introducing 4 base pair changes to the putative miRNA (miR21) seed-pairing region using the Accuprime Supermix (Invitrogen). NT-3 Primer sequences: ACTAGTATTGGCATCTGTCCCCAC (Spe1 site underlined) Mutation primers: CGAATTTTATTGAGAAAAAAAGGCGGACATACTGTAAGGGTTGCCGAAG; CTTCGGCAACCCTTACAGTATGTCCGCCTTTTTTTCTCAATAAAATTCG(mutation sites are underlined) and GAGCTCTCCGATTTTAAGAAAGT (sac1 site underlined). The gel purified PCR product was cloned in Spe1 and Sac 1 site of the pmiR luciferase vector and confirmed by sequencing.

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Margie Maronsky and Mark Dichter for providing hippocampal neurons.

Abbreviations

SE	status epilepticus
NT-3	neurotrophin-3
IP	intraperitoneal

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Highlights

1. Following status epilepticus miR21 increases in the hippocampus.
2. Following status epilepticus neurotrophin-3 mRNA decreases in the hippocampus.
3. The 3′ UTR of neurotrophin-3 mRNA has a miR21 binding site.
4. In neuronal cultures miR21 levels are inversely related to neurotrophin-3 mRNA levels.

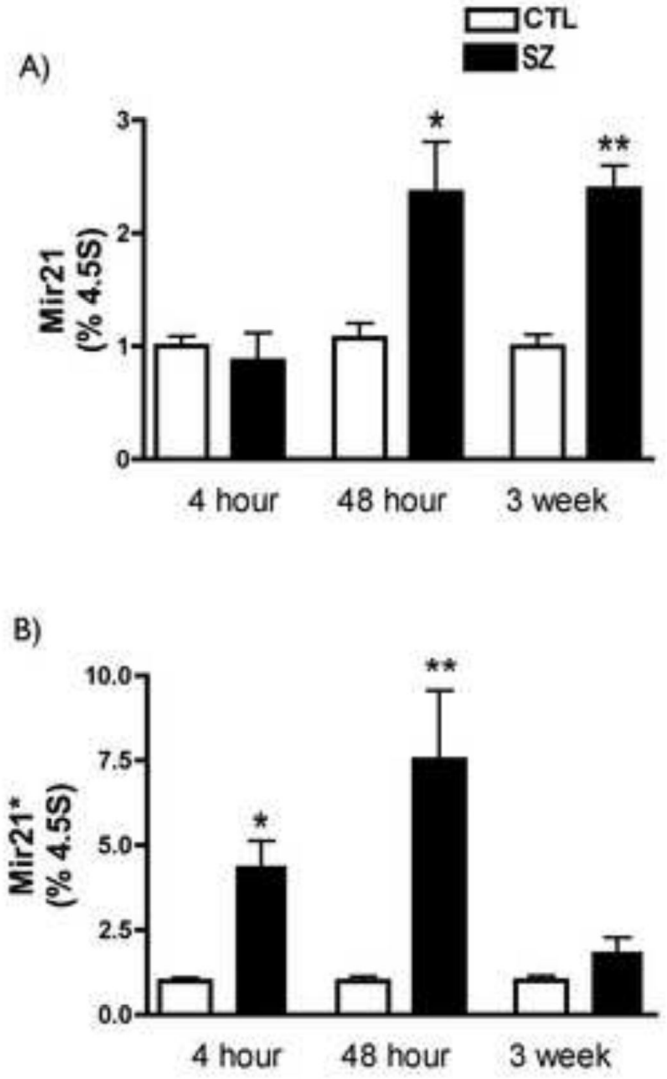

Figure 1.

MiR21 and 21* increase following SE but with distinct time courses. Real-time PCRs were performed on samples from whole rat hippocampus sacrificed 4, 48 hours, and 3 weeks after seizure. (A) MiR21 shows a significant increase at 48 hours and 3 weeks. (B) MiR21* shows a significant increase at 4 hours, peaking at 48 hours but is close to control levels by 3 weeks. MiR21 and 21* levels are expressed relative to house keeping small mRNA 4.5s used as a loading control. There was no difference in 4.5s levels between control and SE animals or between time points. A Kruskal-Wallis statistical test with a Dunn's multiple comparison post-test was carried out (P*<0.05 and P**<0.01 N=6-9 for each group). A Dunn's multiple comparisons post-test of miR21 and 21* in the SE animals identified a statistical difference at 4 hours (P<0.01).

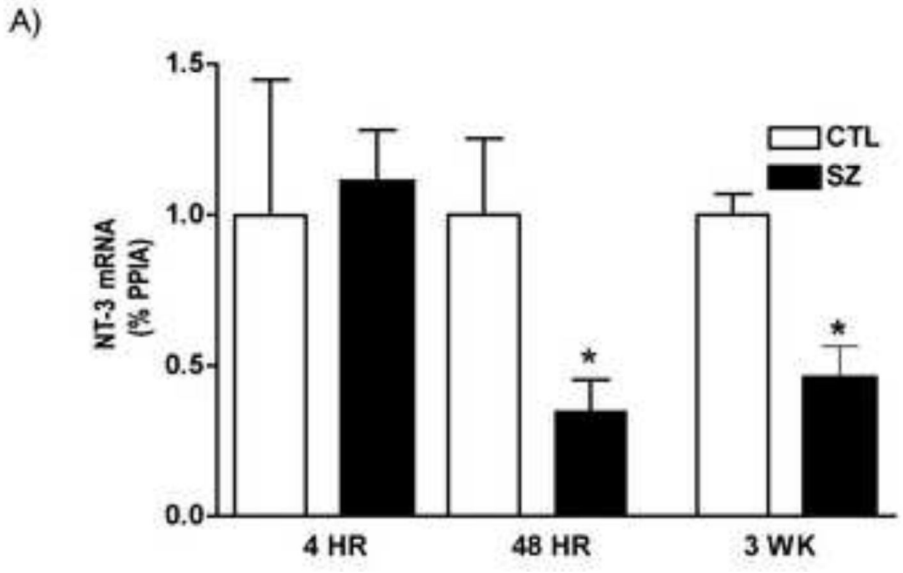

Figure 2.

NT-3 mRNA levels are decreased in the hippocampus at 48 hours and 3 weeks following SE. Real-time PCRs were performed on samples from whole rat hippocampus sacrificed 4, 48 hours, and 3 weeks after SE. NT-3 mRNA levels are expressed relative to house keeping mRNA PPIA (Cyclophilin) used as a loading control. There was no difference in PPIA between control and SE animals or between time points. A Kruskal-Wallis statistical test with a Dunn's multiple comparison post-test was carried out (P*<0.05N=6-9 for each group).

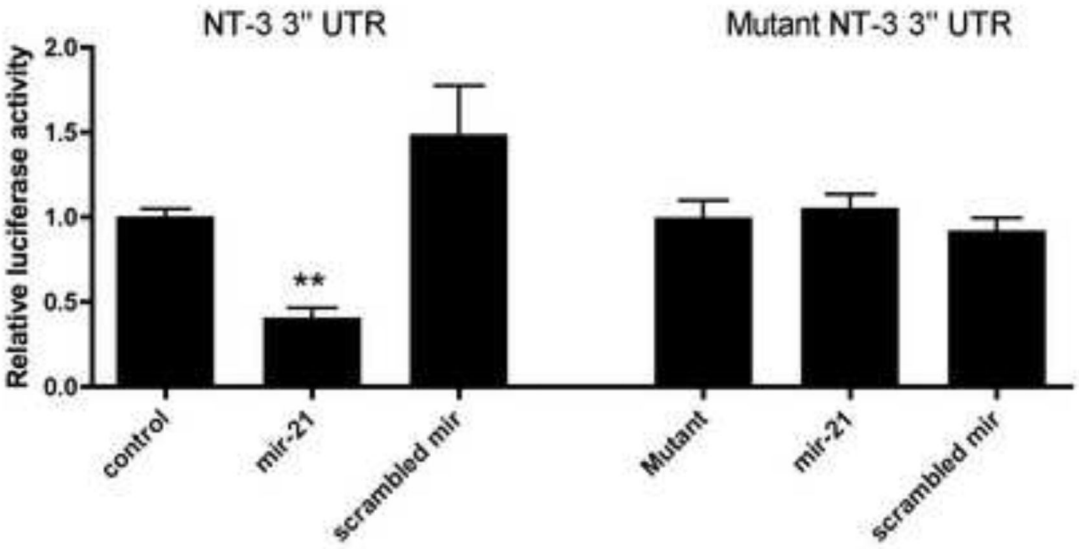

Figure 3.

The 3′ tail of NT-3 has a site that miR21 can use to repress translation. A) An artificial construct with the 3′tail of NT-3 attached to a luciferase reporter assay showed repressed luciferase activity when co-transfected with 3 ρmoles of miR21 but not a scrambled control microRNA. B) Mutation of the putative miR21 binding site destroys the ability of miR21 to suppress translation. A Kruskal-Wallis statistical test with a Dunn's multiple comparison post-test was carried out (P**<0.01 N=6 for each group).

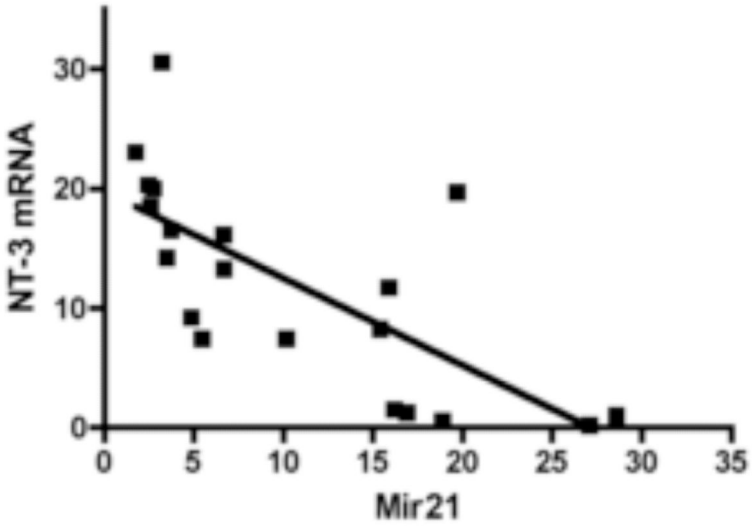

Figure 4.

In cultured hippocampal neurons there was variability in miR21 and NT-3 mRNA levels. Increased miR21 levels were associated with decreased NT-3 mRNA levels. Plotted are the real-time PCR levels of miR21 and NT-3 mRNA from single dishes of hippocampal neurons. Twenty samples from 3 different experiments are plotted. Slope=−0.73 ± 0.17, r squared 0.52 and slope non-zero P=0.0004.

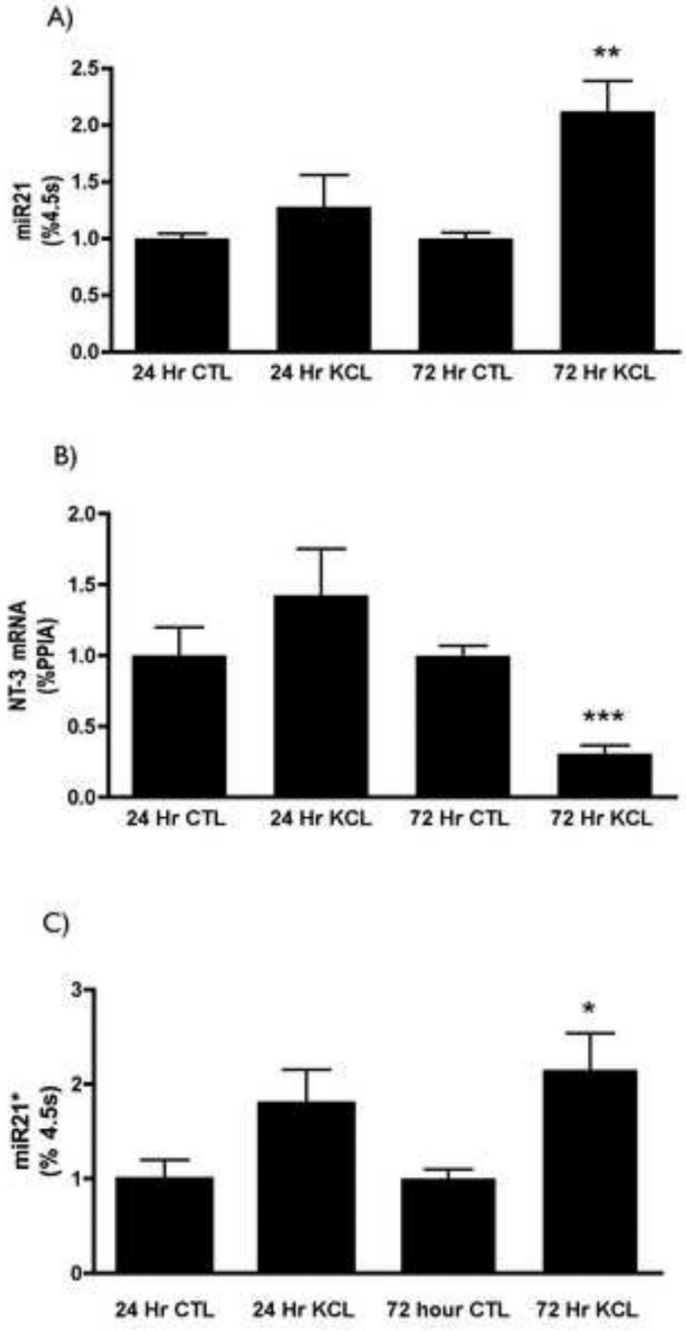

Figure 5.

MiR21 and 21* increased 72 hours after hippocampal neuronal cultures were treated with 20mM K^+Cl^- L for 3 hours. NT-3 mRNA decreased 72 hours after hippocampal neuronal cultures were treated with 20mM K^+Cl^- for 3 hours. Real time-PCR was performed on samples from rat hippocampal neuronal cultures 24 and 72 hours after a 3 hour incubation in 20mm K^+Cl^- or normal saline treatment. A) MiR21 is increased in hippocampal neuronal 72 hours post- K^+Cl^- treatment. B) NT-3 mRNA is decrease in at 72 hours in hippocampal neuronal 72 hours post- K^+Cl^- treatment. C) MiR21* is increased in hippocampal neuronal

72 hours post- K^+Cl^- treatment. MiR21 and 21* levels are expressed relative to house keeping small mRNA 4.5s used as a loading control. NT-3 mRNA levels are expressed relative to house keeping mRNA PPIA (Cyclophilin) used as a loading control. A Kruskal-Wallis statistical test with a Dunn's multiple comparison post-test was carried out (P*<0.05, P**<0.01, P***<0.001; N=6-14 for each group from 3 experiments).

Table

	4 HOUR DATA		48 HOUR DATA		3 WEEK DATA	
MicroRNA	SZ/CNTL	T-Test	SZ/CNTL	T-Test	SZ/CNTL	T-Test
hsa-miR-21	0.996	0.979	2.821	0.010	1.941	0.018
mmu-miR-21*	5.098	0.008	7.390	0.253	1.331	0.125