MicroRNAs as Biomarkers for Mild Traumatic Brain Injury and Post-traumatic Stress Disorder

THESIS

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By

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CERTIFICATE

This is to certify that the thesis entitled **"MicroRNAs as Biomarkers for Mild Traumatic Brain Injury and Post-traumatic Stress Disorder"** and submitted by **RAGHAVENDAR CHANDRAN** ID No. **2008PH29001P** for award of Ph.D. of the Institute embodies original work done by her under my supervision**.**

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DEDICATION

I dedicate this thesis to my father Chandran Seshiah, my mother Ushadevi Chandran, my sister Dhivya Rekha Sridhar and her family. They have inspired me and supported me through every step of my life. Thank you all for your support, encouragement and love.

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LIST OF ABBREVIATIONS

TBI: Traumatic Brain Injury

PTSD: Post Traumatic Stress Disorder

CHI: Closed Head Injury

CCI: Controlled Cortical Impact

BOP: Blast Over Pressure

MiRNA: MicroRNA

UTR: Untranslated Region

Ago2: Argonaute 2

TRBP: TAR RNA-binding protein

ABSTRACT

Traumatic brain injury (TBI) and post-traumatic stress disorder (PTSD) are two different brain disorders which entirely differ in their causative factor; one being physical injury to the brain and while the latter arising from a psychological insult (traumatic stress) but still there is a an overlap of symptoms between the two disorders which may lead to difficulty in differential diagnosis of both of them. Morever, TBI itself is a heterogenous disorder with the injury differing based on the cause of injury or injury mechanism, severity of injury and other such factors. Mild TBI (mTBI) is the less severe form of TBI which is known as 'invisible injury' due to lack of effective diagnostic methods. MicroRNAs (miRNA) are small noncoding RNA molecules that have in recent years emerged as a major biomarker candidate as well as therapeutic targets for various diseases including that of the brain. Recent studies have emerged showing serum miRNAs to be effective biomarker candidates for detecting abnormal pathological conditions. The main objective was to study the changes in miRNA expression primarily in serum but also in brain tissue focusing on the injury site in the three different animal models of TBI and the traumatic stress animal model related to PTSD. Three different rodent models of TBI –closed head injury (CHI), blast TBI and controlled cortical impact (CCI) model were selected and the serum miRNA expression signatures were studied in detail. The serum miRNA expression patterns among the three models were found to be different. The weight drop model indicated transient behavioral deficits with absence of histological damage indicating a mild injury in all four severity grades of injury used and a thirteen serum miRNA signature was identified in 3 hr post injury. A temporal expression study in brains of mice post weight drop injury showed distinct miRNA expression between 24 hr and day 7 timepoint. In the CCI model, a temporal

expression pattern of miRNAs in both serum and brain was observed with a few miRNAs in 3 hr timepoint being common between CHI and the CCI model. Also, brain miRNA expression was found to be different between the weight drop and CCI TBI model probably indicating the heterogeneity in injury mechanism to be reflected in the miRNA expression. The blast model showed one of the miRNAs – let-7i upregulated in serum and cerebrospinal fluid. Finally, miRNA expression in serum and amygdala of a rat model of PTSD showed a panel of nine common miRNA signature in which atleast five miRNAs were shown by network analysis to be involved in fear conditioning. The major observation was the differential miRNA expression observed in the miRNA expression signatures in serum between the TBI models as well as in comparison with the PTSD model. The major findings of this study are:

i) Mild TBI in mice induces a unique 13 serum miRNA signature irrespective of injury severity at 3 hr post mild CHI and temporal changes in brain miRNA expression.

ii) Serum and brain miRNA expression are distinct from each other and also has a temporal pattern post mild CCI injury and also atleast nine common miRNAs were found to be common between the 3 hr time point serum miRNA signature for CHI and CCI model.

iii) Serum miRNA expression is modulated in rats following exposure to three serial blast overpressure of mild to moderate intensity and one of the miRNA let-7i is upregulated in both serum and cerebrospinal fluid.

iv) Serum and amygdala miRNA expression is modulated in rats post exposure to traumatic stress and the modulated miRNAs were predicted to be involved in PTSD pathophysiology but there is no common miRNAs between this PTSD model and the TBI animal models.

Chapter 1: Background of the Proposed Research

1.1: Traumatic Brain Injury

1.1.1: Definition

Traumatic brain injury (TBI) is defined as the damage to the brain tissue that is a result of direct impact to the head, rapid acceleration or deceleration, penetrating injury or exposure to an explosive blast (Sharma and Laskowitz, 2012).

1.1.2: Classification

TBI is a heterogeneous disorder that can be differentiated based upon the type of external force responsible for the brain damage or the severity of the injury which can be assessed based upon clinical symptoms (Sharma and Laskowitz, 2012). The major classification of TBI involved based on the mechanism of injury like focal and diffuse injury or closed and penetrating injury (Teuntje et al., 2010, Sayer, 2012) Another widely acknowledged method of classification involves the use of Glasgow coma scale (GCS) in which the TBI patient population is divided into the mild, moderate and severe injury groups (Maas et al., 2008). Also, few other parameters like the duration of loss of consciousness (LOC), alteration of consciousness (AOC), posttraumatic amnesia (PTA) and brain imaging outcome measured immediately at the time of injury are also involved in injury severity-based classification of TBI patients (Table 1) (Sayer, 2012).

Table 1: Criteria for rating severity of traumatic brain injury (Source: Sayer, *Annu. Rev. Med.*, 2012).

Criteria	Mild	Moderate	Severe
Structural Imaging	Normal	Normal or abnormal	Normal or abnormal
Loss of consciousness	0-30 min	>30 min and <24 h	>24 h
Alteration of consciousness	A moment up to 24 h	>24 h	>24 h
Posttraumatic amnesia	Up to 24 h	>1 day and <7 days	>7 days
Glasgow Coma Scale	13-15	9-12	<9

1.1.3: Prevalence

TBI has emerged as a major cause of mortality and morbidity worldwide. TBI has been estimated to affect around 1.7 million people with around 235,000 hospitalizations arising out of it annually in the U.S. At least, 50,000 deaths have been attributed annually to TBI and a recent estimate indicates over 3 million people surviving with permanent disabilities arising due to TBI (Summers et al., 2009). TBI has also been found to exact a high economic cost of US $60 billion annually in total lifetime direct medical costs and indirect productivity losses (Rockhill et al., 2012).

TBI has a higher prevalence rate in the military population and is known as the "signature injury" of the American war campaigns in Iraq and Afghanistan. The scenario in these wars characterized by factors like high exposure to bomb explosions, better survival rates due to improved body armor and better emergency care on battlefield has made survivors with TBI a much prominent factor compared to earlier military operations of the US armed forces (Sayer 2012). The estimated prevalence rates of TBI in veterans returning from the warzone range from 5 to 23 % and vary due to personal bias in self-reporting, difference in the standards of diagnostic criteria and sampling error (Vasterling et al., 2009).

1.1.4: Pathophysiology

TBI has a unique pathophysiology that it is split up into two phases. The initial phase widely known as the primary phase involves direct damage to the brain tissue due to the force of the impact of the external force causing the TBI and this leads to hemorrhage, tissue loss and axonal shearing. The secondary phase follows within hours to days of the primary injury and involves the activation of a range of cellular, metabolic and molecular level changes such as oxidative stress, inflammation and apoptosis. These changes lead to the secondary brain injury which consists of the spreading of the tissue damage to the surrounding healthy brain tissue which was previously unaffected by the initial physical primary brain injury (Thal et al., 2014). Various post-injury processes like vascular damage, metabolic imbalance and excitotoxicity have been known to come together in a cascading effect thereby enhancing the secondary processes like blood-brain barrier breakdown, inflammation, neuronal hyperexcitabilty and cerebral odema ultimately leading to the emergence of TBI associated deficits (Shlosberg et al., 2010) (Figure 1).

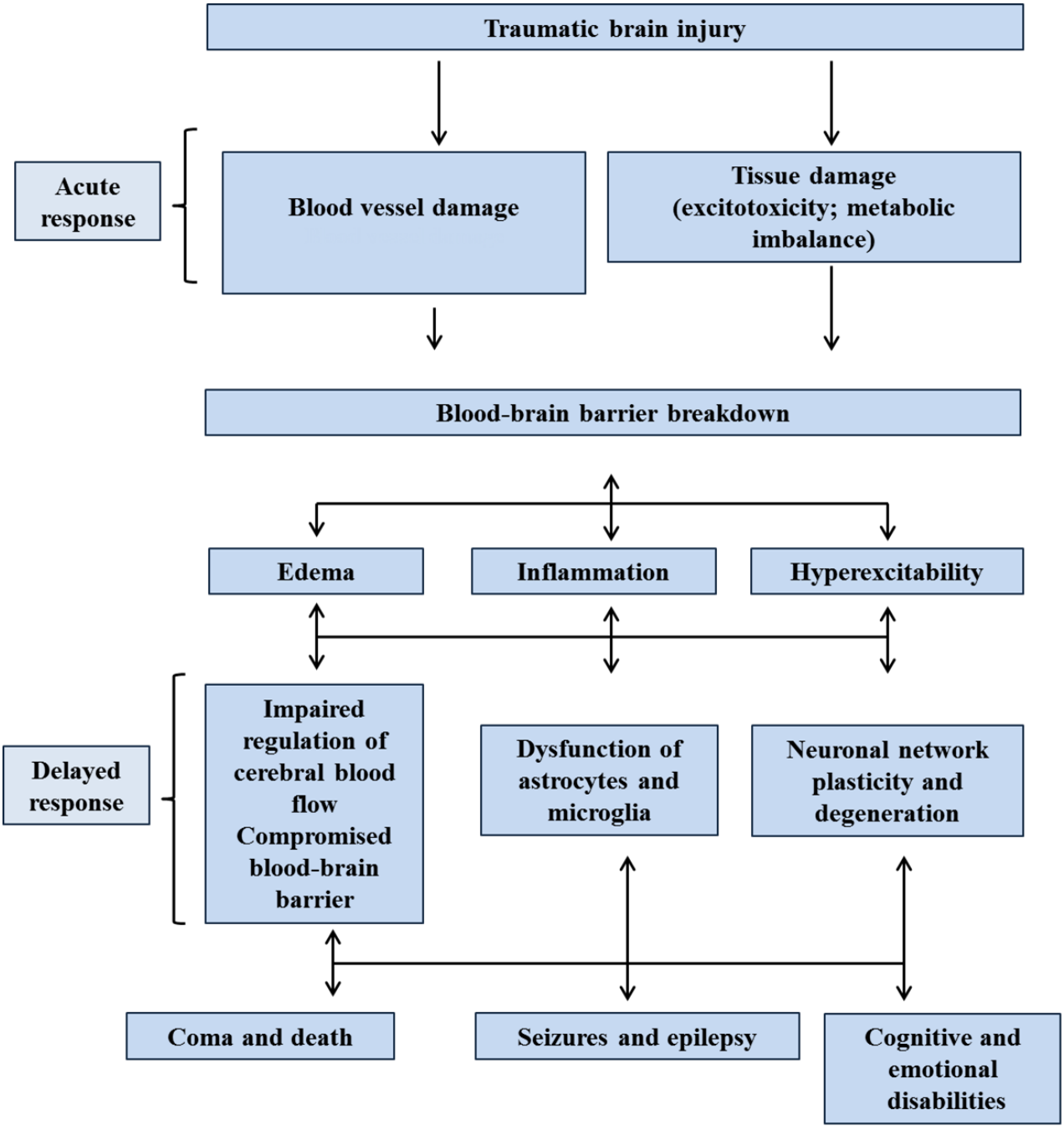

Figure 1: Major events in pathophysiology of TBI. The initial mechanical damage to brain tissue triggers a range of secondary processes that ultimately leads to the development of neurological deficits. (Source: Shlosberg et al., *Nature Reviews Neurology*, 2010.)

1.1.5: Clinical manifestations

The range of clinical symptoms arising due to exposure to TBI especially in soldiers have been grouped by Halbauer and team into five symptom clusters namely (1) cognitive dysfunctions (eg., memory and attention deficits), (2) neurobehavioral disorders (eg., depression and anxiety disorders), (3) somatosensory disruptions (eg., impaired smell, vision, hearing), (4) somatic symptoms (eg., headache and chronic pain) and (5) substance dependence (refers to uncontrolled overuse of alcohol or abuse of illegal narcotics) (Figure 2).

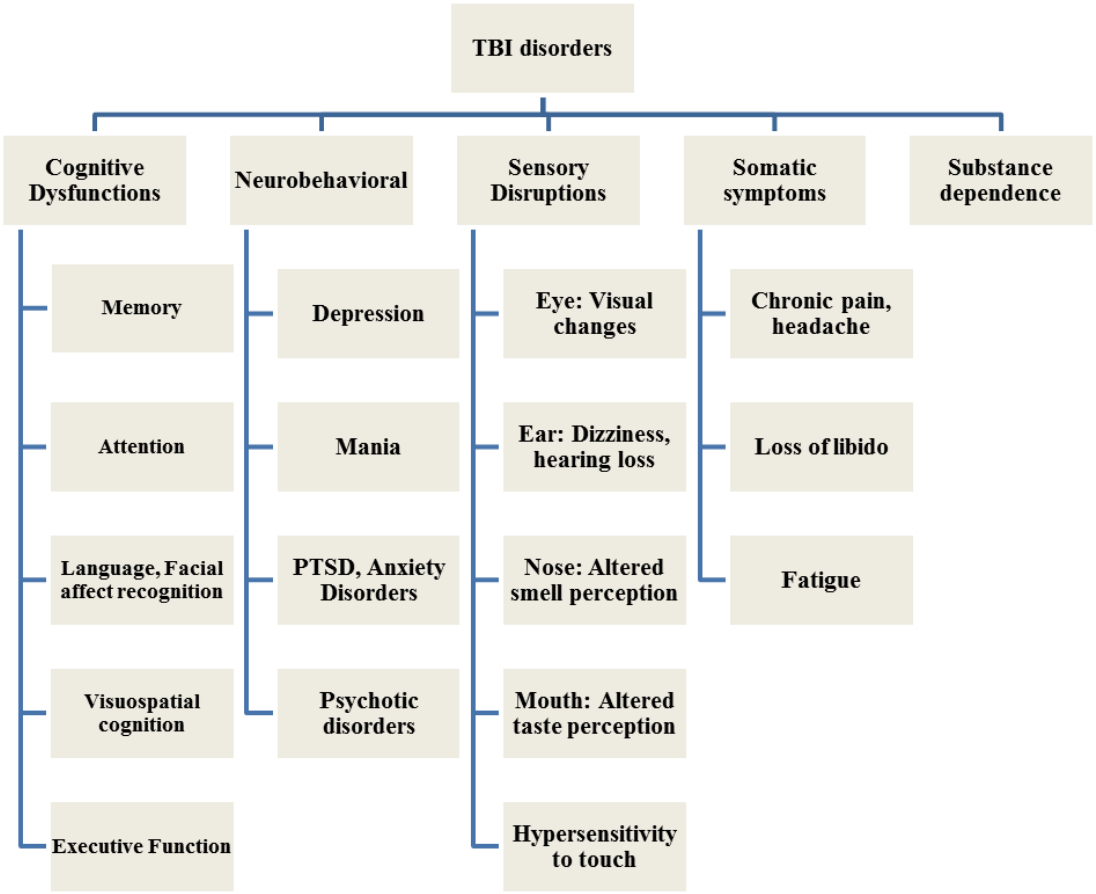

Figure 2: Clusters of clinical symptoms of TBI. The neuropsychiatric symptoms that are an outcome of TBI have been grouped separately into five clusters. Source: Halbauer et al., *J. Rehabil. Res. Dev.*, 2009.

1.1.6: Mild traumatic brain injury – Invisible injury

Mild traumatic brain injury (mTBI) is the type of traumatic brain injury in which there is a change or absence of consciousness of up to 30 minutes and may be present up to 24 hours after the injury (Vasterling, 2009). This type of injury is also referred to as concussion and is widely prevalent in both civilian and military populations (Redell et al., 2013). According to a 2012 Department of Defense report on TBI, mTBI constitutes nearly 80% of total cases of TBI in the US military (Figure 3).

mTBI can cause cognitive shortcomings not only in speed of information processing, attention, and memory in the immediate post injury period but also in motor skills and new problem-solving and general intellectual skills (Kennedy et al 2007). Due to similar neurological and cognitive deficits it is difficult to differentiate between mTBI and PTSD (Vasterling, 2007). In the absence of external wounds and specific biomarkers, mTBI is very difficult to diagnose (Belanger et al., 2005). The discovery of a specific biomarker for mild TBI can help in diagnosing it in different emergency situations.

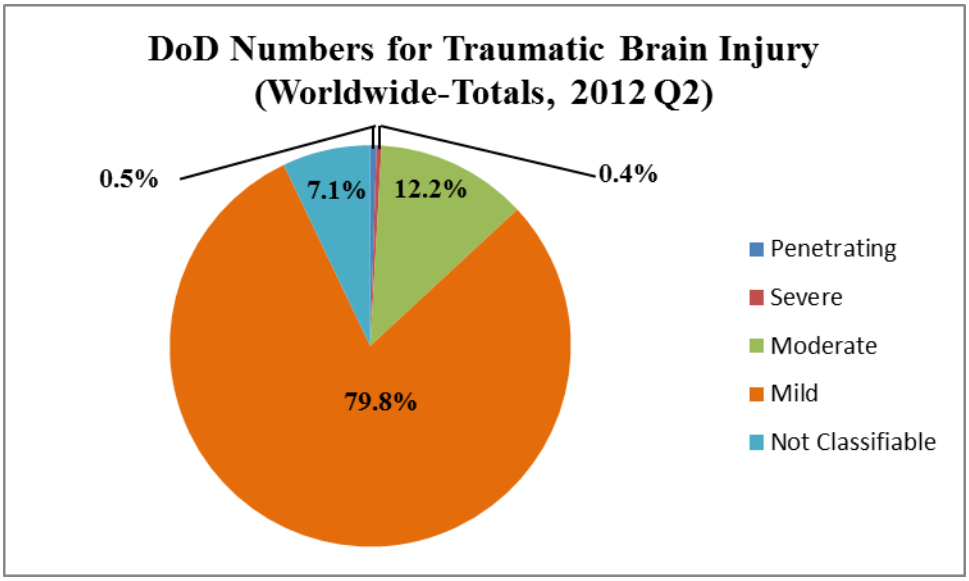

Figure 3: Number of mTBI cases in the US military. mTBI cases constitute nearly 80% of the total TBI population in US military (Source: www.health.mil).

1.1.7: Animal models of TBI

Animal models of TBI are needed to reproduce in the laboratory clinically relevant and standardised patterns of brain injury characterised by motor, neurobehavioral and cognitive dysfunction as seen in humans (Morganti – Kossmann et al, 2010). The amount of tissue damage needs to be proportional to injury severity obtained by variations of fixed parameters. For eg: Varying the falling height changes the severity of the injury produced in the weight drop model (Shapira et al, 1988). Experiments can be done to correlate behavioural, histological, and molecular changes in the injured brain with the varying parameter (Morganti – Kossmann et al, 2010). Rodents have been the most widely used animal model for studying TBI (Statler et al 2001, Morganti – Kossmann et al, 2010, Albert-Weissenberger and Siren, 2010). This is due to various reasons like:

- the simplicity of carrying out the brain injury/surgery
- ease of utilising larger groups
- limited costs and easy availability to researchers
- Use of transgenic rodents like knockout models for studying molecular processes
- baseline responses for a wide range of behavioural tests have been well studied

Animal models are the best means to study secondary injury processes in TBI, where the cellular and molecular processes which are the basis for the secondary injury can be studied in detail. The neurobehavioral responses of TBI and also the responses shown in the wake of therapeutic intervention by various agents can be studied only in animal models. A single model cannot reproduce the entire range of events that happen in TBI; therefore the choice of the model type depends on the objectives of the researcher. One of the most widely used criteria of selecting a specific model is whether the injury to be studied is a closed head or a penetrating injury both of which are produced by different model types. The three commonly used animal models for TBI which we have used in this study are

- weight-drop injury model
- controlled cortical impact (CCI) model
- blast TBI model

Weight Drop Injury model

Weight drop injury model, also known as closed head injury model was initially established by Shapira and co-workers (Shapira et al, 1988). In this model, the injury is produced by the fall of a rod with a specific mass on the closed skull (Figure 4A). Altering the height of the fall would generate varying degrees of injury. Although this particular model has less control over the injury procedure when compared to the other two models, it has a few advantages such as

- easy accessibility
- reproducibility
- cost effective
- low maintenance

The major disadvantages of the weight-drop model is the occurrence of 'rebound' injury which can cause unwanted secondary injury and the major advantage of this model is that it can be quickly performed under gas-anaesthesia which allows neurological scoring to be done immediately after injury (Chen et al 1996). Both focal and diffuse injuries can be done depending on the severity of the injury, in the case of this model by varying the falling height. As compared to the weight drop model which can produce only the focal injury, the diffuse injury is done using Marmarou's model or impact acceleration model in which the head is allowed to accelerate to model "whole head" motion resulting in a diffuse brain injury (Marmarou et al 1994) (Figure 4B).

Controlled Cortical Impact Injury model

Cortical impact injury is produced by a rigid impactor which under air pressure delivers a mechanical energy to the intact dura (Smith et al 1995) (Figure 4C). The severity of the injury can be controlled by altering the pressure acting on the impactor. This model creates a focal injury as compared to the entirely diffuse injury produced by the fluid percussion injury model. The major advantages of this injury model are the ease in controlling the injury severity by simple control/manipulation of pneumatic device. When compared to the weight drop model which is a gravity-based device prone to rebound effect, the CCI model lacks the risk from a rebound injury. The predominantly focal brain injury caused by CCI makes this model to a useful tool for studying the pathophysiology of the secondary processes induced by focal brain injury (Morales et al, 2005).Another widely known model that employs the use of pressurised

fluid to induce brain in jury is the fluid percussion in jury model (Morales et al., 2005) (Figure 4D).

Blast TBI model

Blast overpressure model is the widely used animal model to recreate blast induced neurotrauma**.** Pressurised gas is used to recreate a shock wave which is exposed on to the animal restrained inside the blast tube (Figure 5). By adjusting the pressure of the gas the intensity of the blast can be controlled. This model mainly focusses on the primary or secondary blast injury to the brain (Long et al., 2009, Cernak et al., 2011).

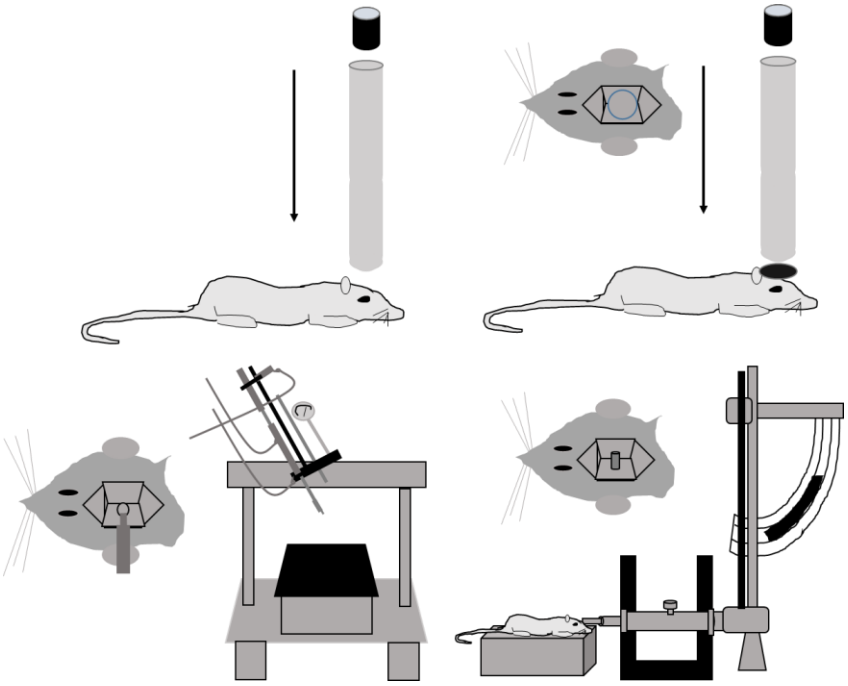

Figure 4: The four most commonly used experimental models of TBI: (A) the weight drop model, (B) the impact acceleration model, (C) the controlled cortical impact model, (D) the fluid percussion model (Source: Morales et al., 2005).

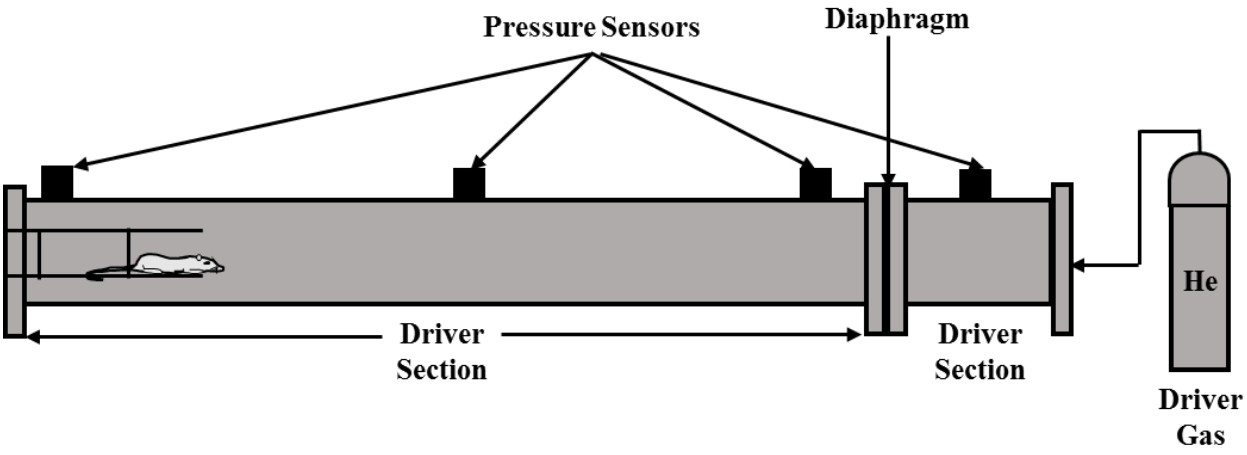

Figure 5: Blast overpressure tube used to recreate blast TBI. Pressurised gas is used to generate a blast wave that induces brain injury in the animal restrained in the blast tube (Source: Cernak et al., Neurobiol. Dis., 2012.)

1.2: Post-traumatic Stress Disorder

1.2.1: Definition

Post-traumatic stress disorder (PTSD) is the most frequent and widely prevalent anxiety disorder with large range of morbidity in the patient populations. Exposure to a single or a chain of traumatic events leads to development of a class of psychiatric diseases known as anxiety disorders. These anxiety disorders arise due to a range of molecular level changes like in the neurocircuitry involved in fear response (Pervanidou and Chrousos., 2010; Shin and Liberzon, 2010)

1.2.2: Prevalence

Different sample sets based on population affected with a particular type of disaster or a particular class of trauma has shown a range of estimates and is classified primarily based on the recent DSM guidelines which are a set of conditions for PTSD diagnosis. According to recent studies, it is estimated that approximately 7.3% of the global civilian population suffer from PTSD (Malan-Muller et al., 2013). PTSD is also a major psychological health issue in armed forces veterans who were previously involved in combat activities. Study shows that 10-30% of US veterans suffer from PTSD or other stress related disorders (Zhang et al., 2011). The clinical symptoms of PTSD may include feeling of helplessness, hypervigilance, irritability, exaggerated fear response or traumaspecific reenactment (Berna et al., 2012). Diagnosis of PTSD is currently based on symptoms determined from the patient's clinical history, examination of mental status, duration of symptoms and clinical symptom checklists or the patient self-report

1.2.3: Animal models of PTSD

Generally animal models for psychiatric disorders are difficult to model keeping in mind the complexity in their etiology as well as manifestation of their symptoms. But still the presence of common emotional processes between humans and mammals shows that still there is a possibility of employing animal models for psychiatric research. Another major point is that usually these animal models do not mimic the entire psychiatric component of humans but rather focus on just replicating core features of the specific psychiatric disorder in the form of endophenotypes (Siegmund and Wotjak., 2006). The main need for an animal model for PTSD arises due to the very limited knowledge on its pathophysiology in spite of abundant evidence on its physiological aspects. Various factors like inaccessibility to brain, ethical restrictions for

research on human subjects and an easier way to study the cellular and molecular mechanisms of PTSD pathophysiology aid in going for an animal model for studying PTSD.

The animal models differ mainly on the type of stressor used and the main factor is replicating the symptoms as faithfully as possible in them. The validity of a particular animal model for a disease especially that of a psychiatric disorder is normally based upon four different criteria.

1. Face validity – describes how closely the particular model reproduces the symptoms that are associated with the human disorder studied.
2. Construct validity – describes the level of similarity between the parameter measured in the animal model with that of the parameter focused in the human disorder and this usually insists that the cellular and molecular processes of the particular model is the same as that in the humans.
3. Predictive validity – describes how accurately the animal model can predict the outcome of a therapeutic strategy which has already shown success in human cases.
4. Discriminant validity – describes how strongly it can discriminate between those with and without PTSD (Daskalakis and Yehuda, 2014).

The above validity criteria seem to be very important in determining the suitability of the animal model for studying a specific disease.

1.2.4: Role of Amygdala in PTSD

Amygdala is an almond-shaped important brain structure that is basically a collection of nuclei (neuron clusters) located in the medial temporal lobe of the brain and is known to have anatomical connections with other brain regions (Pape and Pare, 2010) and is involved in tasks like controlling fear-related responses and the consolidation of emotional memory (Koenigs and Grafman et al., 2009). Amygdala is one of the most important brain structure highly involved in the pathophysiology of PTSD (Rauch et al., 2006). The other two important brain structures involved in mediating PTSD development are the hippocampus and prefrontal cortex (Rauch et al., 2006). Still there is a lack of information on the molecular level changes occurring in the amygdala that triggers the onset and mediates the persistence of PTSD and its symptoms. Mainly, there is a dearth of knowledge in relation to the molecular changes especially at the microRNA/epigenetic level which occur in the amygdala that lead to the development of PTSD and sustaining of a few of its chronic symptoms in certain patients.

1.2.5: PTSD and mTBI

The recent wars in Afghanistan and Iraq have led to an increase in the number of current service members as well as veterans with exposure to mild TBI and a current diagnosis of PTSD. This has led to an increased focus on studying the co-morbidity between both the disorders. In civilian cases, the co-morbidity between both the disorders can be observed in incidents involving a violent physical assaults or car crash (Tanev et al., 2014).

The comorbidity between TBI and PTSD is characterized by presence of a spectrum of common clinical features such as sleep disturbance, depression, anxiety, and irritability, difficulty in concentrating, fatigue, suicidality, chronic pain, and alterations in arousal. The overlap of symptoms between the both the disorders of PTSD and mTBI is thought to occur due to overlap in neural mechanisms leading to common brain regions like amygdala getting affected in both the disorders (Ruff et al., 2010) (Figure 6).

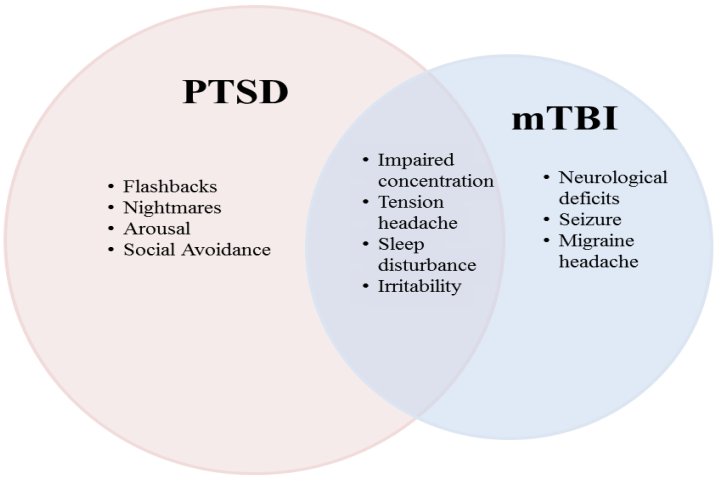

Figure 6: Overlap of symptoms between PTSD and mild TBI (Source: Ruff et al., 2010, F1000 Med. Reports).

1.3: MicroRNAs

1.3.1: Definition

MicroRNAs (miRNAs) are small non-coding RNAs of 20-24 nucleotides in length which regulate gene expression by binding to complementary sequences of mRNAs (Bartel et al., 2004; Ambros et al., 2001). A single miRNA can target multiple mRNAs and also vice versa can occur with a single mRNA being a target of various miRNAs which makes these miRNAs control different networks within the cell and thus influence various pathways (Bhalala et al., 2013). MicroRNAs have been known to play a major role in brain development and function and subsequently unique global expression patterns of miRNAs have been observed in response to specific brain diseases or injury (Sun et al., 2014; Bhalala et al., 2013; Liu and Xu., 2011; Xu et al., 2010; Cao et al., 2006).

1.3.2: Biogenesis of microRNAs

The generation of microRNAs involves a well-studied pathway known widely as the canonical pathway for microRNA biogenesis (Carroll and Schaefer, 2013) (Figure 7). The first step involves the formation of primary miRNA transcripts which are transcribed from exonic, intronic or intergenic regions of genomic DNA using RNA Polymerase II. These pri-miRNAs fold in an imperfect manner forming hairpin loop structures which are recognised and cleaved by a protein complex consisting of two proteins - Drosha and DGCR8 give rise to pre-miRNAs. The pre-miRNAs are then exported out of the nucleus by Exportin-5 protein to the cytoplasm (Carroll and Schaefer, 2013). Dicer protein further processes the hairpin loop pre-miRNAs into a double stranded RNA intermediate. These double stranded RNA intermediate consists of a biologically active mature miRNA strand and the complementary strand (miRNA*) which subsequently get degraded in most of the conditions. The mature microRNA strand (guide strand) then gets associated with the RISC protein complex (RNA induced silencing complex) which consists of the Dicer, Argonaute 2 (Ago2) and the TAR RNA-binding protein (TRBP) (Bhalala et al 2013).

The guide strand in the RISC complex then directs it to the complementary target sites of the target mRNA which is normally located in its 3' UTR region. The interaction between the miRNA and the target mRNA is primarily determined by the presence of the 'seed sequence', a region covering nucleotides 2-7 at the 5' end of the miRNA that has complementarity with the binding sites in the mRNA. The target mRNA is completely degraded in the presence of a

perfect match between the miRNA seed sequence and the mRNA target site. In most of the cases, there is a partial complementarity leading to either mRNA destabilization or translational repression (Carroll and Schaefer, 2013, Bhalala et al 2013).

Non-canonical pathways of microRNA biogenesis which stray away from the sequence of Drosha/DGCR8 followed by Dicer processing of the precursor microRNAs have also been observed in a few cases in nature. One of the pathways, the mirtron pathway involves synthesis of pre-miRNAs without the involvement of the Drosha/DGCR8 protein complex by direct splicing of precursor RNA molecules. Another pathway involves the direct entry of tRNA and sno-RNA derived RNA molecules into the RISC complex skipping the Drosha/DGCR8 processing step. Finally, a non-canonical pathway independent of the Dicer processing step and involving Ago proteins for pre-miRNA cleavage has also been found and characterised (Carroll and Schaefer, 2013).

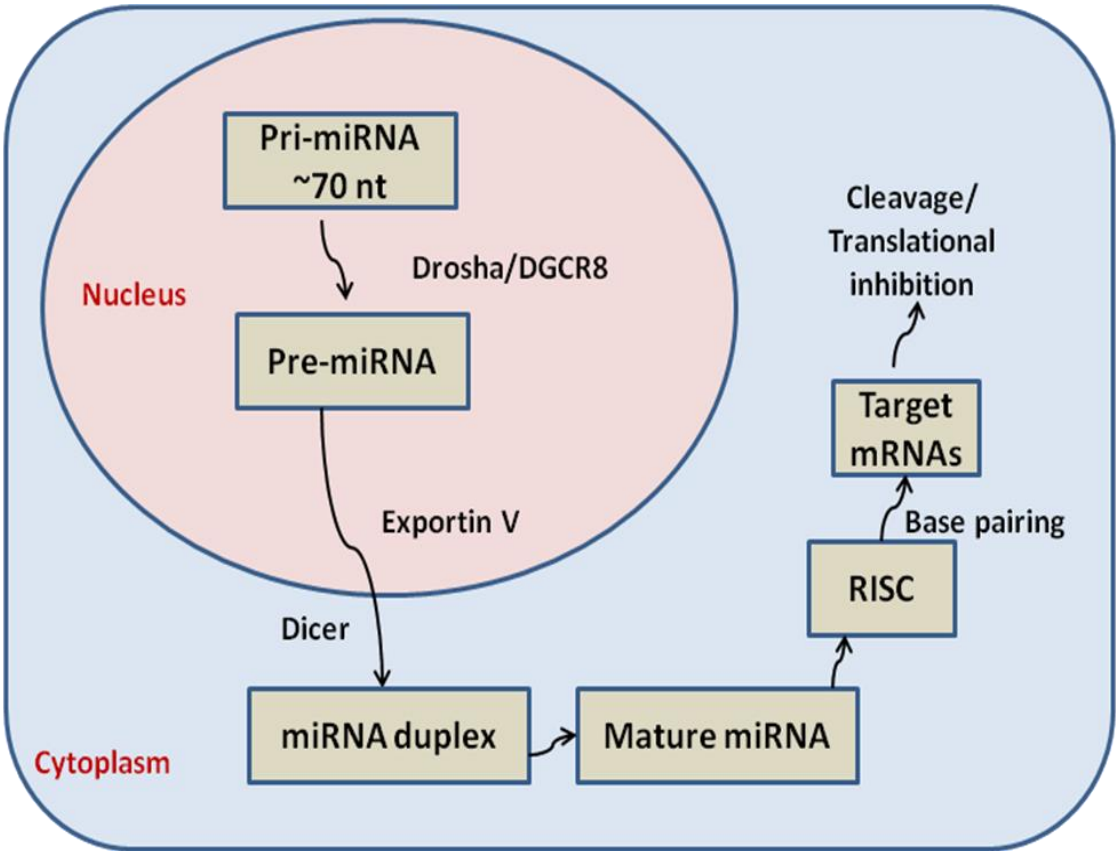

Figure 7: Canonical pathway of microRNA biogenesis. The pri-miRNA transcribed in the nucleus is processed by the Drosha protein to pre-miRNA which is transported out of the nucleus by Exportin V. In the cytoplasm, the miRNA duplex is generated by the action of Dicer which unfolds to give the mature miRNA that is incorporated into the RISC complex. The RISC acts upon the target mRNAs leading to their degradation (Adapted from Bhalala et al., 2013).

1.3.3: Role of microRNA in brain function

MicroRNAs are known to be found in large quantities in the brain and have been found to play an important role in brain development and function. Various studies show that they are differentially distributed in the different brain regions and different neuron types. Also, within the neuron they have been found to be localised in different regions depending on their specific role and function in brain physiology (Soreq et al., 2014, Follert et al., 2014).

Studies on dicer-deficient zebrafish were the first to prove the essential role of microRNAs in brain physiology. Further studies on deficiency of Dicer and Ago2 in various animal models showed the primary role of microRNAs in CNS development in general (Wang et al., 2014). This was also supported by the fact that there is a change in the miRNA expression levels and patterns associated with various neurological disorders in both animal models and human cases. For instance, different studies using human and animal studies on neurodegenerative disorders show different patterns of microRNA expression and associated gene targets (Junn and Mouradian, 2012) (Table 2). The role of miRNAs in brain physiology in general can be used for developing RNA-based therapies for brain-related diseases/disorders (Soreq et al., 2014, Follert et al., 2014).

Table 2: Differential pattern of brain miRNA expression in human and animal studies of neurodegenerative disorders (Source: Junn and Mouradian, *Pharmacology and Therapeutics*, 2012).

Disease	Decreased miRNAs	Increased miRNAs	mRNA target	Reference
Alzheimer's disease	miR-29a/29b-1,miR-9		BACE1	Hebert et al., 2008, Shioya et al., 2010, Nunez-Iglesias et al., 2010
Alzheimer's disease	miR-107, miR-103,miR-23b		BACE1	Wang, et al., 2008, Nelson & Wang, 2010, Wang et al., 2011
Alzheimer's disease		miR-9, miR-138,miR-125b	N/A	Lukiw, 2007
Alzheimer's disease	miR-9	miR-29a/29b-1	N/A	Cogswell et al., 2008
Parkinson's disease	miR-133b		Pitx3	Kim et al., 2007
Parkinson's disease	miR-34b/34c		N/A	Minones-Moyano et al., 2011
Huntington's disease	miR-9/9*		REST/CoREST	Packer et al., 2008
Huntington's disease	miR-132	miR-29a, miR-330	p250GAP	Johnson et al., 2008
Amyotrophic lateral sclerosis	miR-206		HDAC4	Williams et al., 2009
Prion disease		miR-342-3p, miR-494	N/A	Montag et al., 2009
Prion disease	miR-338-3p, miR-337-3p	miR-342-3p, miR-320, let-7b/7dmiR-328, miR-191, miR-370	N/A	Saba et al., 2008

1.3.4: MicroRNA as biomarkers

Circulating microRNAs are a subset of microRNA of cellular origin found in a range of body fluids including blood and transported via different carrier molecules like exosomes, lipoproteins and protein complexes etc., (Figure 8). Their unique properties such as non-invasive detection, stability in vivo and in vitro, their appearance correlating with an abnormal physiological/pathological state, tissue origin specificity make them much sought after candidates for diagnosing various diseases including cancer (Figure 9) (Cortez et al., 2011, Zampetaki., 2012 ; Alevizos et al., 2010; Kosaka et al.,2010).

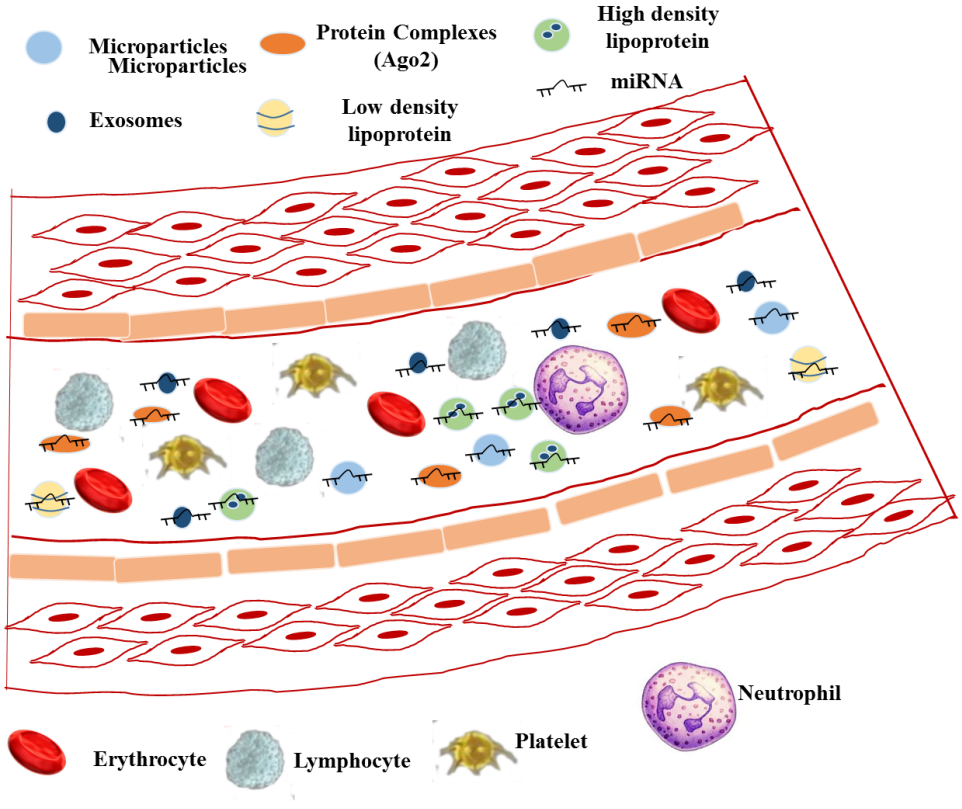

Figure 8: Modes of transport of circulating microRNAs. MicroRNAs are transported across the body especially in the blood stream via different routes like complexing with carrier proteins,lipids or enclosed in exosomes and microparticles (Source: Zampetaki., 2012)

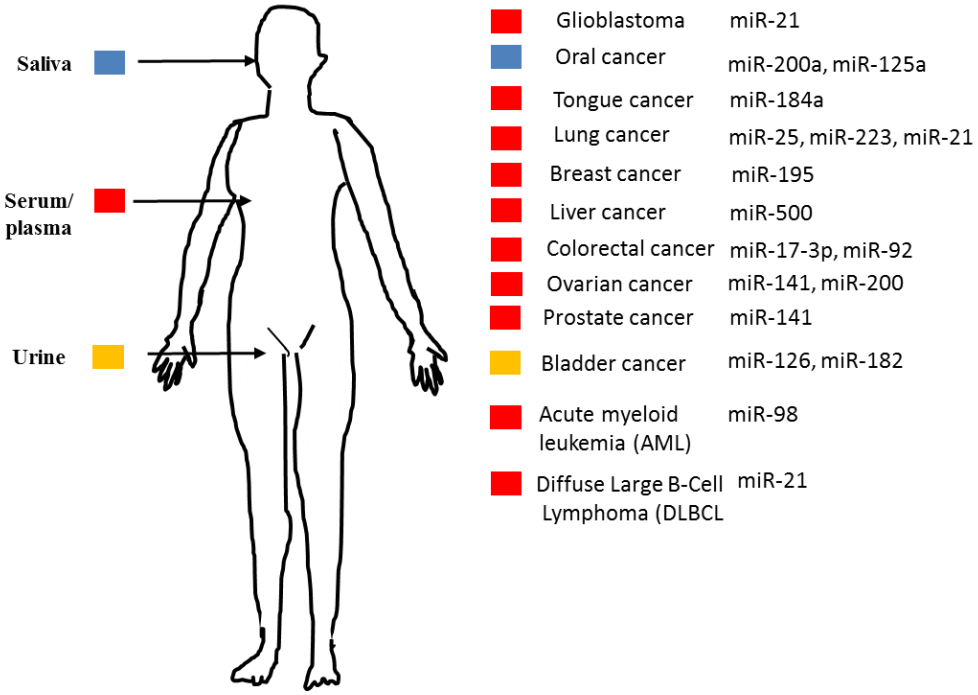

Figure 9: Circulating MicroRNA as biomarkers of different types of cancers. MicroRNAs detected in body fluids have been correlated with the presence of different types of cancers in various studies (Source: Kosaka et al., 2010)

Chapter 2: Material and Methods

2.1: Closed head injury procedure

Animals: Male C57BL/6J mice, 10-11 weeks old and weighing > 28 g before surgery (The Jackson Laboratory, Bar Harbor, ME, USA) were used for CHI procedure. The animals were first acclimatized for about a week to the new environment during which they were handled daily by the laboratory staff. The handling procedure involved gentle stroking and placing on the handler's arm and this was normally done to habituate animals to staff and lessen anxiety or stress before the behavioral tests. The animals were housed in standard rodent containers in a reverse light cycle room with controlled optimum temperature and humidity conditions as well as food and water being provided *ad libitum*. The reverse light cycle were adopted so as to perform the behavioral tests at their most active period based on the nocturnal nature of the test animals.

CHI procedure: CHI in mice was induced using a custom-built weight drop device (Figure 10A) (FJB Engineering, Rockville, Maryland, USA) based on the description given in Flierl et al., 2009 (Flierl et al., 2009). Two different rods of weight - 246 g and 333 g were used in the weight drop device. Each of the rods was dropped at two different fall heights- 2 cm and 3 cm above the parietal lobe of exposed mouse skull to recreate four different grades of injury (246 g/2 cm; 246 g/3cm; 333 g/2cm and 333g/3 cm) of closed head injury within the mild spectrum. The four different injury groups were renamed as injury severity 1 (IS1= 246 g/2 cm), injury severity 2 (IS2= 246 g/3 cm), injury severity 3 (IS3= 333 g/2 cm), and injury severity 4 (IS4= 333 g/3 cm). Rebound injury was minimized by immediately holding the rod manually after the primary hit.

Initially, the mice were subjected to anesthesia using a mixture of isoflurane and oxygen in a separate anesthesia box and were removed only after it did not show any response to toe pinch using tweezers. It was then transferred to the platform under the rod and anesthesia was maintained through a nose cone at a dose lower than that of in the earlier anesthesia box. The hair on the scalp between the ears was shaved off using a trimmer followed by sterilizing the scalp with 10% povidone-iodine (Betadine) solution (Purdue Products L.P., Stamford, CT). The skull was then exposed by making a single cut which was made along the mid line of the head using a small scissor. The exposed skull was then cleaned using 70 % Ethanol followed by

wiping dry using a fresh cotton applicator and the injury site was marked using a black felt tip marker for easy identification during the injury. The injury site was marked on the left parietal lobe located 2.5 mm from sagittal suture and 2.5 mm from lambda (Figure 10B). Injury was induced by a free falling metal rod with a rubber tip of 1 mm diameter (Figure 10A). Animals were resuscitated by gently massaging the thorax and providing oxygen through the nose cone. Once continuous breathing resumed, the animals were again put on mild anesthesia using the nose cone. Animals without skull fractures were included in the experiment and the scalp skin was sutured back using Vetbond Tissue Adhesive 1469SB (3M Company, St.Paul, Minnesota, USA). The animals were then placed in a cage on a warming mat and were allowed to recuperate from the anesthesia. Injury day was considered as day 0. Sham controls were similarly handled and received only the scalp cut under anesthesia.

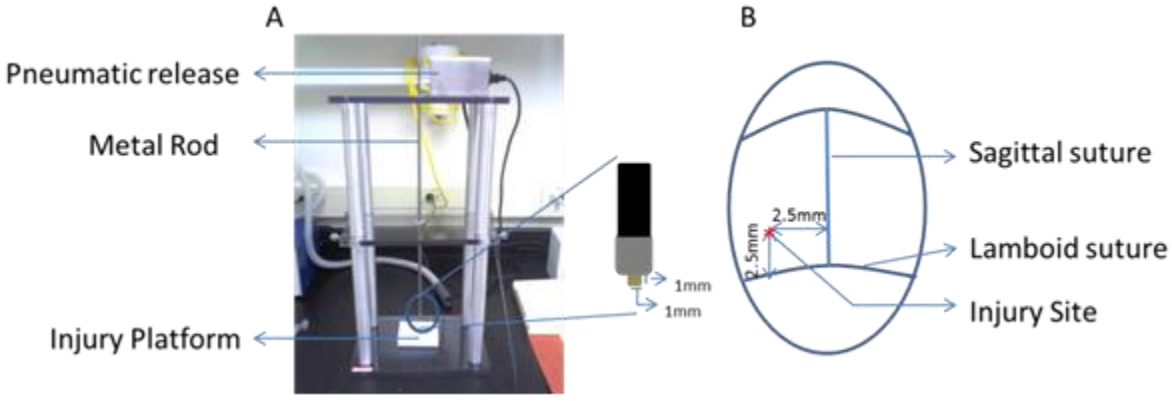

Figure 10: Closed head injury (CHI) device. (A) The CHI device was custom made based on the design of a CHI device described by Flierl et al., 2009. Injury was induced by a metal rod of specific weight (246 g/333 g) falling under gravity. The rod can be set for a specific height using the holding grove made 0.5 cm apart. The rod was released using a pneumatic control operated by a foot pedal. The tip of the rod was fitted with a rubber tip (1 mm in diameter/1 mm in length). (B) Site of injury was selected on the left parietal lobe located 2.5 mm from the sagittal and lamboid suture.

2.2: Study Design for CHI experiments

Identification of serum microRNA signature in acute timepoint for diagnosis of mild traumatic brain injury in a mild closed head injury model: The experiment was designed to evaluate the neurobehavioral alteration in the mice following mTBI over a 30 day period and to evaluate the serum miRNA changes of mice at 3 hr post injury. For behavior study: Multiple smaller cohorts were combined to complete the final analyses. Four different injury groups: 246 g/2 cm (n=32), 246 g/3 cm (n=29), 333 g/2 cm (n=20) and 333 g/3 cm (n=6) were based on the weight of the impact rod and the height of the fall, respectively. These groups are referred to as injury severity 1 (IS1= 246 g/2 cm), injury severity 2 (IS2= 246 g/3 cm), injury severity 3 (IS3= 333 g/2 cm), and injury severity 4 (IS4= 333 g/3 cm). Sham controls (n=42) were included in the experiment to determine the effect of handling and surgery (scalp cut and suture) on the animal's neurobehavioral responses. In the sham group, animals were handled similar to that of the injured groups except that no injury was made after the scalp cut. The skin was sutured back without making the impact trauma and the animals were allowed to recuperate in the cage similar to the injured animals. An additional control of naïve (n=25) animals was included to determine the basal level of normal neurobehavioral responses. The naïve group of animals was kept in their housing room and not taken to the surgery room to avoid any stress related to being in the surgery room. Baseline measurements for NSS-R, OFL, and ASR were taken at 3 days before the injury day (day 0). NSS-R was also measured at day 1 post injury and ASR and OFL were measured at days 1, 14, and 30 post injury. For miRNA study: A separate group of animals for all four injuries as described above in the behavior study, were used in the miRNA study (n=6 each group). In addition, a sham group (n=6) was included to account for miRNA changes that may occur due to the surgical process such as anesthesia, scalp cut, stress of handling the animal, and sutures. MiRNA expression of injured animals was compared with that of the sham to determine the modulation in miRNA expression due to brain injury. For histology study**:** A separate group of four animals for each of the four injury groups (IS1, IS2, IS3 and IS4) as well as for sham group in 3 hr time point were used for histology evaluation study post injury.

Temporal differences in miRNA expression pattern in injury site in brain after mild closed head injury: The experiment was designed to study miRNA expression in brain tissue underlying the injury site at early and delayed time point post injury. For brain miRNA study: Three animals for

each of the four injury groups (IS1, IS2, IS3 and IS4) as well as for sham group in both 24 hr and day 7 time point were used for the miRNA profiling and the validation study. For histology study**:** A separate group of four animals for each of the four injury groups (IS1, IS2, IS3 and IS4) as well as for sham group in both 24 hr and day 7 time point were used for histology evaluation study post injury.

2.3: Controlled cortical impact injury procedure

Animals: Male C57BL/6J mice of 8-10 weeks age (The Jackson Laboratory, Bar Harbor, ME, USA) were used for CCI procedure. The animals were first acclimatized for about a week to the new environment. All the animals were housed in standard rodent containers in a reverse light cycle room with controlled optimum temperature and humidity conditions as well as food and water being provided *ad libitum*.

CCI procedure: CCI injury was administered using a stereotaxic CCI instrument with electromagnetic controller (Impact One, Leica) and an impact tip of 3 mm in diameter. The mice were first placed under anesthesia in a separate anesthesia chamber filled with a mixture of isoflurane and oxygen. After checking for the response to toe pinch using tweezers, the fur on the head was shaven using an electric razor. The mice were maintained under anesthesia by a mixture of isoflurane and oxygen supplied through a nose cone.

After shaving, the scalp was sterilized by applying 10% povidone-iodine (Betadine) solution (Purdue Products L.P., Stamford, CT) followed by 70% ethanol using cotton tipped applicators. A small incision was made in the scalp to expose the skull and using a hand-held trephine a 5.0 mm burr hole was drilled into the skull to expose the dura mater for the injury procedure. The injury consisted of a single contusion of 1mm depth with the injury site lying in the somatosensory cortex (injury coordinates: 0 mm bregma, 2 mm lateral left). The other parameters for the injury were impactor velocity of 1.5 m/s, dwell time of 100 milliseconds and an impact angle of 15º relative to the injury plane. Sham animals were just exposed to craniotomy without the injury and as for the naïve animals they were just exposed to anesthesia minus the craniotomy or injury procedure. Post-surgery, the mice were were housed singularly in a cage placed on a heating pad and monitored till they recovered from anesthesia.

2.4: Blast overpressure injury procedure

Animals: Adult, male Sprague-Dawley rats (250–300 g) were used for this study. Six animals for each experimental and control group were used.

Injury, study design and sample collection: The animals were kept in the end of the expansion chamber of an air-driven shock tube (2.5-ft compression chamber connected to a 15-ft expansion chamber) with the right side ipsilateral to the direction of the blast overpressure injury. Care was taken to prevent secondary and tertiary blast injuries by anesthetizing the animals with isoflurane and placing them in a holder as previously described (Chavko et al., 2006). Two injury groups were used. The short interval injury group (SII), in which animals were exposed to three serial BOP of 120 kPa at an interval of 2 h. The other group is the long interval injury group (LII), for which BOP of 120 kPa was given at an interval of 24 h. From each of these groups, serum and CSF samples were collected at 3 h and 24 h after the last BOP exposure. Blood and cerebrospinal fluid (CSF) samples were collected from the control animals at the same time points sample collection was done for the injured animals.

2.5: Traumatic stress procedure for PTSD animal model

Animals: Male albino Sprague-Dawley rats (n=24), 4-6 weeks old and weighing 76-100 g were used (Taconic Farms, Germantown, NY, USA). The animals were housed in standard rodent containers in a reverse light cycle room with controlled optimum temperature and humidity conditions as well as food and water being provided *ad libitum*.

Study design: The animals were kept for acclimation for a week and then the rats were grouped into two groups of 12 animals each for stress and control. Young animals were used for this study to give sufficient time for simulating PTSD progression as seen in the battlefield scenario. Development of PTSD like symptoms may take at least two weeks after the cessation of stressors in the animal model and hormonal changes occur immediately after stress exposure as compared to the molecular level changes (Servatius et al. 1995). Hence, young animals were used to give sufficient time for studying the molecular level changes like protein or gene expression during PTSD development. Housing conditions, acclimation of rats and the stress protocol were followed as previously described (Jia et al., 2012).

Sample collection: Animals from both the groups of control and post stress were sacrificed immediately (day 0; n=6 each) and day 14 (n=6 each) after the last stress exposure and the samples were collected between 11.00 and 12.00 h. Trunk blood was collected in 15 ml centrifuge tubes (VWR International, Radnor, PA, USA) and was left to clot at room temperature for 30 min for serum extraction. Blood was centrifuged (Allegra 6R centrifuge, Beckman Coulter) at 3500 rpm for 30 min at 4 C and supernatants were harvested in clean tubes. The supernatant was again centrifuged at 3500 rpm for 10 min at 4 °C to pellet down remaining cellular fraction. The serum obtained was aliquoted into 1.5 ml microfuge tubes and stored at - 80 °C until further use. Brain dissection and subsequent collection of amygdala was carried out as described previously (Jia et al., 2012). Amygdala tissues were immediately submerged into RNAlater-RNA stabilization reagent (Qiagen, Valencia, USA) in microfuge tubes and then stored at - 80 °C until further use.

2.6: Behavioral Tests

Neurobehavioral severity scale-revised (NSS-R): A revised form of the neurological severity scale (NSS), initially developed for rats, was modified for mice and measured 3 days before the injury and at day 1 post injury (Grunberg et al., 2007; Sharma et al., 2012). The NSS-R is a specific, continuous sequence of 10 behavioral tests and observations. This measure was originally designed to model a clinical neurological exam conducted in human neurology patients. This particular sensory-motor assessment scale was based on several previous reports and has been modified to increase standardization and sensitivity (Sharma et al., 2012; Shohami et al., 1995; Hamm, 2001; Marti et al., 2005; Yarnell et al., 2013; Xing et al., 2013). Ten tasks assess reflex suppression, general movement, and postural adjustments in response to a challenge. The NSS-R uses a three-point Likert scale, in which a normal, healthy response is assigned a “0”, a partial or compromised response is assigned a “1” and the absence of a response is assigned a “2”. This three-point scale is clear and reliable and allows for greater discrimination based on sensorimotor responses than previous scales that used two-point ratings of each response. The NSS-R has a scoring range of 0-20, with higher scores reflecting greater extent of injury. All personnel were blinded to the treatment groups for the NSS-R measurements, but not to the naïve group. Naïve animals did not exhibit a scalp cut and sutures,

which were otherwise were present on all the sham and injured animals, and therefore were easily identifiable. Animals were evaluated on all 10 tasks as listed in the Table 3.

Table 3: List of tasks performed for determining NSS-R scores.

Activity Test	Score		
	0	1	2
General balance	Balance and walk	Balance/no walk	No balance/fall
Landing	Normal reflex	Partial reflex/unbalanced	No reflex/falls flat
Tail raise	Normal Reflex	Partial reflex/weak	No reflex/limp
Drag	Walking motion	Partial/unilateral	No response/drag
Righting reflex	Instant	Delayed or with effort	No response
Ear reflex	Full response	Partial response	No response
Eye reflex	Blink	Partial response	No response
Sound reflex	Flinch and walk	Flinch and pause/Partial Jump/ Walk ignore	Startle and freeze/ Strong jump
Foot reflex	Turn and bite	Turn/no bite	No response
Tail reflex	Turn and bite	Turn/no bite	No response

Open Field Locomotion (OFL) test: Open field locomotor activity was measured to evaluate the unconditional behavior of the mouse in its environment. OFL test was performed at 3 days prior to injury (baseline; day -3) and days 1, 14, and 30 post injury. Data collected included horizontal activity for general health and gross motor skills; center time as an index of anxiety-related behavior (where less time spent in the center of the cage is interpreted as more anxiety-related behavior); and vertical activity as an index of depression-related behavior (where less vertical activity may indicate less escape behavior which is interpreted as more depression-related behavior) (Bowen et al., 1986; Elliott et al., 2004; Faraday et al., 2003; Grunberg and Bowen, 1985; Morse et al., 1997). Locomotor activity was measured using an Omnitech Electronics Digiscan infrared photocell system (Test box model RXYZCM (16 TAO); Omnitech Electronics, Columbus, Ohio, USA) as described before (Hamilton et al., 2012). One hour activity measurements were obtained during animal's active cycle. Animals were placed singly in a 20 x 20 x 30 cm clear Plexiglas arena covered with a Plexiglas lid with multiple holes to ensure adequate ventilation. A photocell array measured horizontal locomotor activity using 8 pairs of infrared photocells located every 2.5 cm from side-to-side and 16 pairs of infrared photocells located front-to-back in a plane 2 cm above the floor of the arena. A second side-to-side array of 8 pairs of additional photocells located 5.5 cm above the arena floor measured vertical activity. Data were automatically gathered and transmitted to a computer via an Omnitech Model DCM-I-BBU analyzer.

Acoustic startle reflex (ASR) test: Acoustic startle reflex is a characteristic sequence of involuntary, defensive, muscular responses elicited by a sudden, intense acoustic stimulus. Measurement of acoustic startle response with and without prepulse provides information about information processing and attention (Acri et al., 1991; Alain et al., 2008; Swerdlow et al., 1992; Acri et al., 1994; Faraday et al., 1998). ASR was measured 3 days prior to injury (baseline; day -3) and on days 1, 14, and 30 post injury using a Med Associates Acoustic Response Test System (Med Associates, Georgia, Vermont, USA) consisting of weight-sensitive platforms inside individual sound-attenuated chambers. Each mouse was placed individually in a ventilated holding cage that restricted extensive locomotion, but allowed them to turn around and make small movements. Animal movements in response to stimuli were measured as a voltage change by a strain gauge inside each platform. Responses were recorded by an interfaced Nexlink

computer as the maximum response occurring during the no-stimulus periods, during the pre-pulse period, and during the startle period. Startle stimuli ranged from 100 to 110 dB and were white noise bursts of 20 millisecond duration sometimes preceded 100 millisecond by 68, 79, or 90 dB 1 kHz pure tones (pre-pulses). Each stimulus combination was presented six times. The total testing period was about 20 minutes.

Behavior Data Analysis: Analysis of covariance (ANCOVA) and repeated measures analysis of covariance (rmANCOVA) were conducted for each of the behavioral variables, covarying for baseline scores to account for any baseline differences. NSS-R data were analyzed by a change score (1 day post injury – Baseline) (Allison, 1990). OFL data were separated into three subscales: horizontal activity, center time, and vertical activity. The 100 dB ASR data were analyzed. All tests were two tailed using alpha = 0.05. Data for all the behavior analysis is presented as mean +/- standard error mean (SEM).

2.7: Ethics statement for animal experiments

All of the animal experiments were reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Committee on Care And Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86-23, revised 1996).

2.8. MicroRNA profiling procedure

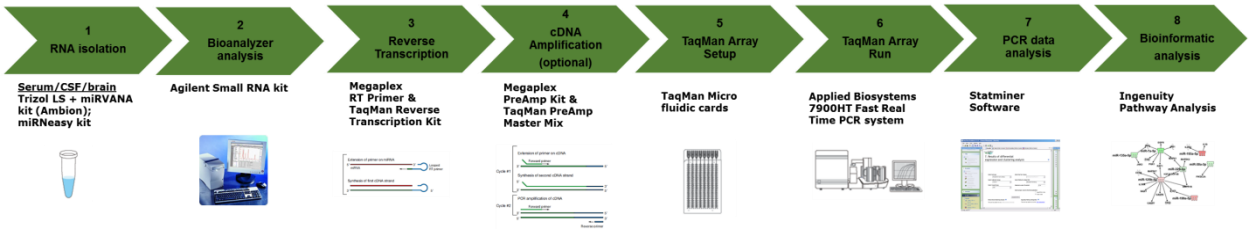

Figure 11: MicroRNA profiling workflow. The general profiling workflow used in this study involved RNA isolation using kits like miRVANA or miRNeasy kits followed by qualitative analysis of RNA using bioanalyzer instrument. The RNA sample is then reverse transcribed to yield cDNA which can be preamplified. The cDNA is then used to run the TaqMan array cards in Real time PCR instrument. The PCR data obtained is analyzed using Statminer software which is followed by various bioinformatic analyses of the expression data like network analysis using Ingenuity Pathway analysis software.

Serum/brain collection and RNA isolation: Mice were sacrificed at three different post-injury time points - 3 hr, 24 hr and 7 days post injury followed by cardiac puncture to collect blood and the brains were harvested simultaneously. Tissue punch biopsy (4 mm) was obtained from the injury site in the left cortex covering the cortex till the base of the brain using a Keyes Tissue Punch- 4 mm (Roboz Surgical Instrument Co., Inc., Gaithersburg, MD, USA) and this was preserved in RNAlater (Qiagen) for later use.

Total RNA including miRNA was isolated from the brain punch tissue using mirVana miRNA isolation kit (Ambion/Life Technologies, Carlsbad, CA, USA) according to manufacturer's protocol. The brain tissue was thawed on ice and weighed in a fresh tube. The tissue was homogenized in 10 volumes of lysis/binding buffer followed by addition of 1/10 volume of miRNA homogenate additive. After vortexing, the homogenate mixture was incubated on ice for 10 min and was mixed with acid-phenol: chloroform solution. Following centrifugation, the upper aqueous phase was collected and mixed with 1.25 volumes of absolute ethanol. The mixture was then passed through a filter column followed by washing and then finally eluted in 50 µl of water.

Total RNA from serum samples was isolated by standard protocol using the miRNeasy serum/plasma kit (Qiagen, Valencia, USA). QIAzol reagent (1 ml) was added to 200 µl of starting volume of serum and vortexed. Chloroform was then added to the mixture and was incubated at room temperature for 3 minutes and centrifuged. Aqueous phase obtained after centrifugation was then mixed with 100 % ethanol. This was followed by loading the mixture in spin column tubes and repeat washes with buffers. Finally, the RNA was eluted with around 14 µl of RNAse free water.

MiRNA expression profiling: RNA quality and miRNA quantity in the total RNA in both the serum and brain RNA was determined was done using the Agilent Small RNA kit (Agilent Technologies, Santa Clara, CA) in Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA). The miRNA concentration in the total RNA was used as the input quantity for the RT reaction (5 ng - brain miRNA). RT reaction mixture consisted of 2 µl Multiscribe Reverse transcriptase, 0.14 µl RNase inhibitor, 0.27 µl 100 mM dNTPs (with dTTP), 1 µl 10X RT buffer, 1.20 µl $MgCl_2$, 1 µl Megaplex RT primers Rodent Pool A/B (v3.0), RNA and nuclease-free water to make up a final volume of 10 µl of final reaction mixture. The RT reaction was carried out on Veriti 96-Well Thermal Cycler (Applied Biosystems/Life Technologies, Carlsbad, California, USA) according to the manufacturer's recommended thermal cycling conditions.

Preamplification of the cDNA product after reverse transcription was done using 12.5 µl TaqMan PreAmp Master Mix, 2.50 µl Megaplex PreAmp primers Rodent Pool A/B (v3.0), 5 µl nuclease-free water and 5 µl of RT product to make up a final volume of 25 µl of final reaction mixture. Preamplification reaction was carried out on a Veriti 96-Well Thermal Cycler (Applied Biosystems/Life Technologies, Carlsbad, CA, USA) according to the following thermal cycling conditions – 95 ºC for 10 min, 55 ºC for 2 min, 72 ºC for 2 min followed by 14 cycles of 95 ºC for 15 sec and 60 ºc for 4 min, followed by a hold at 99.9 ºC for 10 min and a final hold of 4 ºC. The miRNA profiling was done by running the undiluted pre-amplification product in the TaqMan Array Rodent MicroRNA A+B Cards Set v3.0 (Applied Biosystems/Life Technologies, Carlsbad, CA, USA) according to the default thermal cycling conditions in the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems/Life Technologies, Carlsbad, CA, USA).

Real-time PCR data analysis: The real-time PCR data was analyzed using RealTime Statminer software (Integromics). The data were filtered for Ct value<35 and normalized by the global normalization method. Also, the data was adjusted using the Benjamini Hochberg False Discovery Rate (FDR) method and the significant microRNAs were selected based on adjusted p values less than 0.05

2.9: Bioinformatic analysis

CHI brain miRNA expression and CCI miRNA expression study: DIANA mirPath, an online bioinformatics tool was used to predict the possible molecular pathways that can be modulated by the set of microRNAs that are modulated post injury. This basically works by comparing the gene targets of the microRNA dataset obtained from DIANA microT v4.0 database with the KEGG pathway database.

Blast TBI study: For predicting the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of mRNAs that are targeted by miRNAs altered by BOP, we used a DNA intelligent analysis (DIANA) miRPath algorithm, combined with the miRNA target prediction web tools of DIANA microT 4.0, TargetScan 5, and PicTar (Lewis et al., 2005; Papadopoulos et al., 2009). Network analyses of modulated miRNAs were done using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood, City, CA).

Traumatic stress study: Predicted targets of differentially expressed serum and amygdala miRNAs downloaded from miRWalk, a target prediction algorithm, were analyzed. MiRWalk is a combinatorial miRNA-target prediction tool and able to identify both predicted and validated targets (Dweep et al., 2011). Both functional and network analysis of altered miRNA and their gene targets associated with fear responses were performed using Ingenuity Pathway Analysis (IPA) program (Ingenuity Systems Inc, Redwood City, CA).

2.10: Histology

Hematoxylin and eosin (H & E) staining was used to determine the presence of tissue lesion in the injured cortex. Animals were subjected to transfusion procedure using heparinized phosphate buffered saline followed by 10% normal buffered formalin. Following which the brains were fixed in 10% normal buffered formalin. Fixed brain tissues were then processed, paraffin embedded, and 5 micron coronal sections were cut and stained with H&E by standard staining procedures by Histoserv Inc. (Histoserv Inc., Germantown, MD). The brain sections were then scanned in a Nanozoomer instrument (Hamamatsu) and evaluated using the NDP software (Hamamatsu).

Chapter 3: Identification of Serum MicroRNA Signature in Acute Time Point for Diagnosis of Mild Traumatic Brain Injury in a Mild Closed Head Injury Model

3.1: Results

3.1.1: Mortality associated with the initial impact increased with the height of fall and rod weight

CHI was induced using a custom-made weight drop device (Figure 10). Animal mortality immediately after the impact with the falling weight increased with increase in the height of the fall and the weight of the rod. The percent mortality was 6.7 ± 2.29, 41.3 ± 5.4, 33 ± 14.11, and 65.9 ± 11.5 in 246 g/2 cm (IS1), 246 g/3 cm (IS2), 333 g/2 cm (IS3), and 333 g/3 cm (IS4), respectively. No mortality was observed in the injured groups at the later time points in the experiment. Apnea immediately after the impact occurs in rodent model of weight drop injury and resuscitation with oxygen mask and gentle rubbing of the chest cavity improves recovery from the impact (Flierl et al., 2009, Marmarou et al., 1994; Foda et al., 1994). The immediate mortality in the injury groups may have occurred due to the respiratory arrest upon impact (Flierl et al., 2009). The high mortality rate in our study as compared to the previously described similar model may be due to the slight modification to the impactor tip. Specifically, a silicon/ resin covering was used in the previously described model (Flierl et al., 2009), whereas no such covering was used in our system. H&E staining of the brain section at the hippocampus level (site of injury) did not reveal lesion volume in any of the injured groups, indicating mild brain injury in the animals that survived the initial impact of the injury (Figure 12).

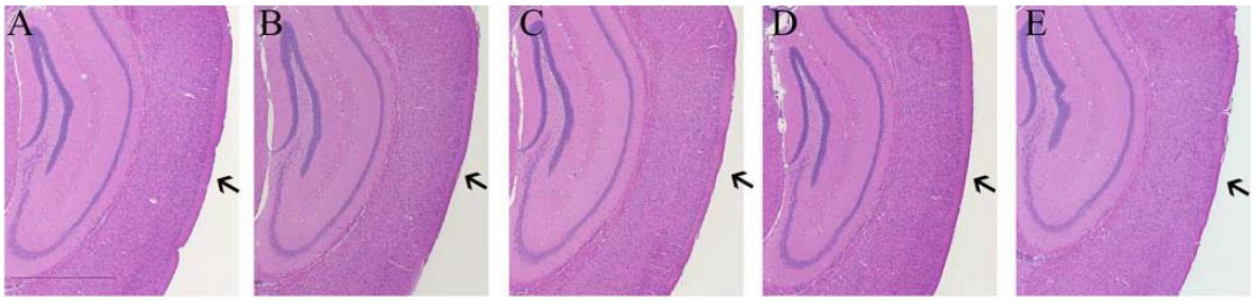

Figure 12: H&E stained sections of the brain. Histological evaluation of the brain tissue at the site of injury (arrow) was done to check for the lesion in the brain tissue following mTBI. No significant difference was observed in brain tissue sections between the sham (A), IS1 (B), IS2 (C), IS3 (D) and IS4 (E). Scale is 1 mm.

3.1.2: NSS-R scores increased with the increase in the fall height and weight

NSS-R scores were determined by measuring behaviors of the naive, sham, and injured animals in a series of 10 tasks to evaluate neurobehavioral effects of acute injury. NSS-R was measured at day - 3 (baseline) and day 1 post injury. Significant increases in NSS-R change scores were observed with increasing severity of the injury (Figure 13). There were significant differences among injury groups, $F(5,92) = 6.04$, $P < .001$, $\eta^2 = .247$. NSS-R change scores and group comparisons are presented in Table 4.

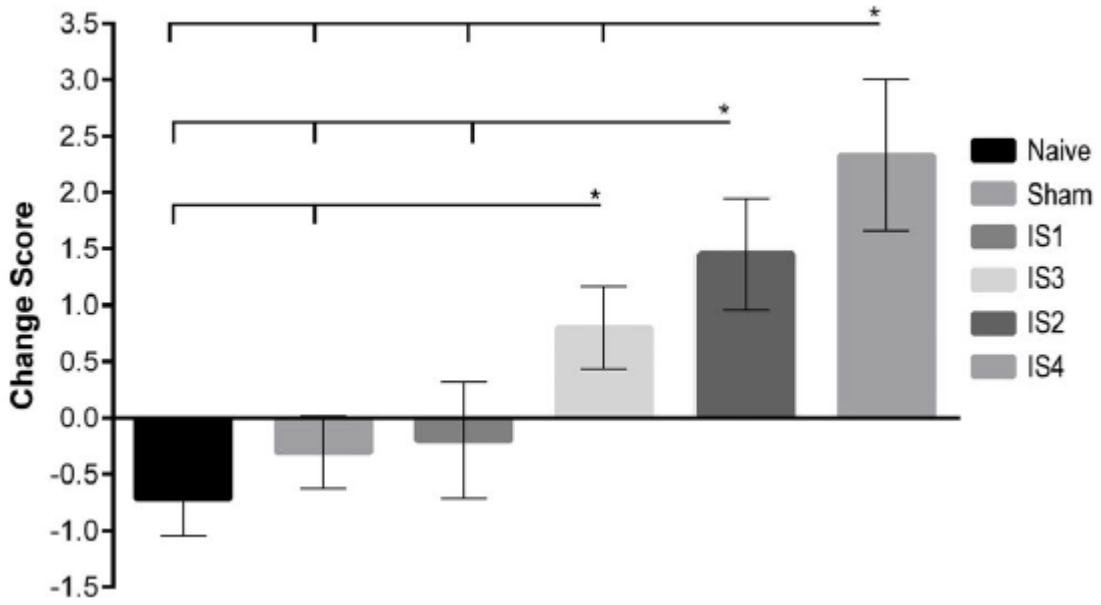

Figure 13: NSS-R for animals at day 1 post injury. Change scores between day 1 post injury and baseline were calculated. A gradual, but significant increase in the NSS-R scores were observed day 1 post injury with the increased severity of the injury within the mild spectrum. As expected, naive and sham groups did not show any increase in their NSS-R scores. No change was observed in the IS1 group. NSS-R score of IS2, IS3, and IS4 groups increased and was the highest among the IS4 group. * P value < 0.05.

Table 4: NSS-R change scores.

Group	Comparison Group	P Value
Naïve (-.720 ± .328)	Sham	0.372
	IS1	0.399
	IS3	0.003*
	IS2	0.000*
	IS4	0.000*
Sham (-.308 ± .322)	Naive	0.372
	IS1	0.860
	IS3	0.025*
	IS2	0.004*
	IS4	0.001*
IS1 (-.200 ± .519)	Naive	0.399
	Sham	0.860
	IS3	0.119
	IS2	0.023*
	IS4	0.004*
IS3 (.800 ± .367)	Naive	0.003*
	Sham	0.025*
	IS1	0.119
	IS2	0.290
	IS4	0.048*
IS2 (1.46 ± .494)	Naive	0.000*
	Sham	0.004*
	IS1	0.023*
	IS3	0.290
	IS4	0.294
IS4 (2.33 ± .670)	Naive	0.000*
	Sham	0.001*
	IS1	0.004*
	IS3	0.048*
	IS2	0.294

NSS-R change scores of the individual groups are given and its significance with the other groups in the study is indicated. Values are expressed as mean ± SEM. * P value < 0.05 (significant).

3.1.3: Open field activity of the animals is reduced following the injury

Horizontal activity of the animals was taken as a measure of the overall health of the animals prior to injury and after the injury. Activity was measured as the number of beam breaks during a 1 hr session. Overall, there was a significant Time x Injury interaction, F(8.33,245.14) =7.69, P,.001, η^2 =.207 (sphericity violated, used Greenhouse-Geisser correction). At 1 day post injury, there were significant differences among injury groups, F(5,149)= 8.46, P,.001, η^2 = .221 (Figure 14 A), such that the activity in IS2, IS3 and IS4 was reduced as compared to the naive, sham, and IS1 groups. No significant differences were observed among the naive, sham, and injury groups at 14 and 30 days post injury. Horizontal activity and comparisons among the groups appear in Table 5.

The time spent in the center of the open field was taken as a measurement of anxiety-related behavior. There was a significant effect of Time, F (2,294) = 16.26, P,.001, η2 =.100 (Table 6). There also was a significant Time x Injury interaction, F (10,294) = 3.45, P,.001, η2= .105. At day 1 post injury, there were significant differences among injury groups, F(5,148) =2.97, P= .014, η2 =.091 (Figure 14 B), such that the center time was significantly reduced in the IS3 group as compared to the naive, sham, IS1, and IS2 groups. At 14 and 30 days post injury, no significant differences between the control and injured groups were observed. Center time of each group and comparisons among groups appear in Table 7.

Vertical activity was taken as a measure of depression-related behavior such that less vertical activity is associated with more depression (i.e., learned helplessness). Overall, there was a significant effect of Time, F(2,294)= 16.20, P,.001, η2= .099 (Table 8). There was also a significant Time x Injury interaction, F(10,294) =4.45, P,.001, η2 = .131 (Figure 14 C). At 1 day post injury, there were significant differences among injury groups, F(5,148) =7.32, p,.001, η2= .198, such that the vertical activity in IS3 and IS4 groups was significantly reduced as compared to naive, sham, IS1, and IS2 groups. No significant differences were observed between naive, sham, and various injury groups at 14 and 30 days post injury. The vertical activity of each group and comparisons among groups appear in Table 9.

The overall negative effect on general health and vertical activity of the mouse was also evaluated as the percent of animals in injury groups that showed reduced activity compared to

the naive group at day 1 post injury. Each group was evaluated for the number of animals that showed activity less than the lower limit of the baseline activity of naive animals. Overall, the more severe the injury, the greater percentage of the animals that showed reduced activity in the open field (Figure 15). To eliminate motor activity loss as a cause of reduced activity, animals were subjected to the rotarod test, which showed no significant differences between the various control and injury groups (Figure 16). No apparent signs of pain such as ruffled fur, hunched posture, altered behavior, vocalization, lethargy, tremors, ataxia etc. that may affect the activity of the injured mice were observed in the animals.

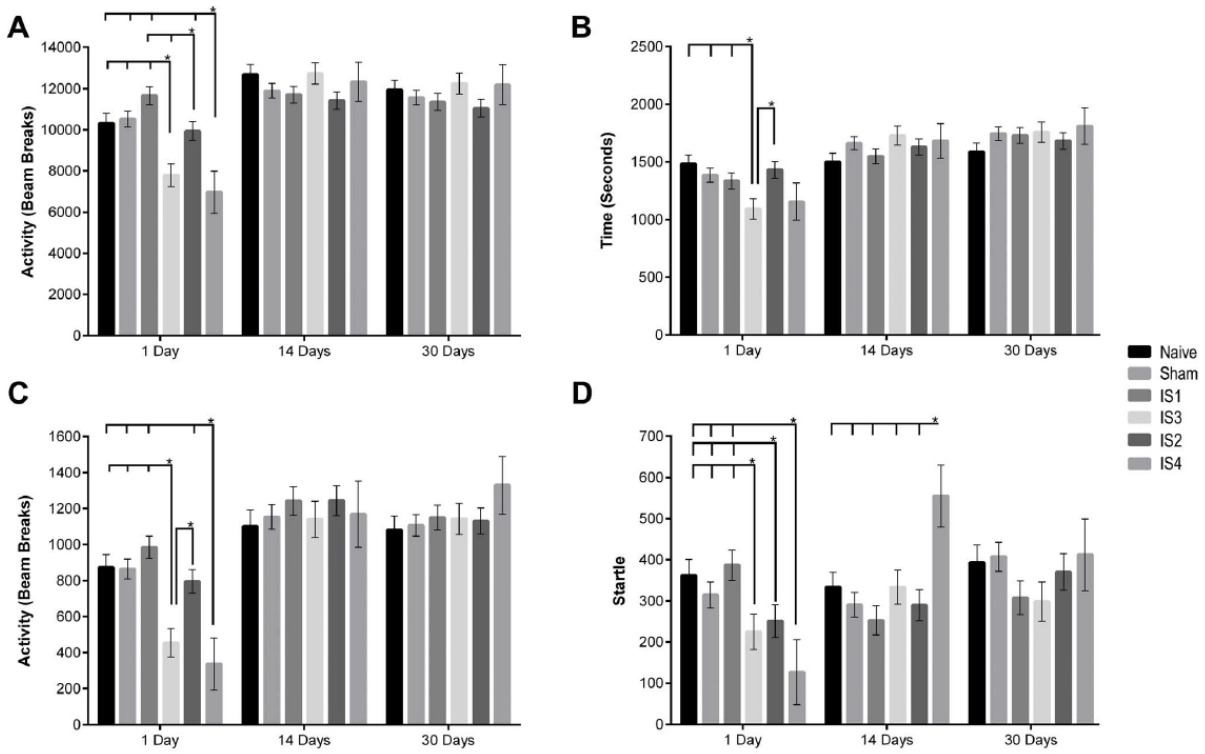

Figure 14: Neurobehavioral Activity. OFL test was conducted to evaluate animals' various activities in an open field as a measurement of behavioral deficits. Overall, day 1 showed significant reductions in the activities of injured animals as compared to the naive and sham controls except for the IS1 group, which was not significantly different from that of naive and sham groups. (A) Horizontal activity was evaluated as an indicator of the overall health. Activity was significantly reduced in the IS3 and IS4 groups. Activity in the IS2 group was significantly reduced as compared to the activity of IS1. Activity of IS1 and IS2 groups were not significantly different from the sham group. (B) Time spent in the center of the open field was evaluated as an indicator of anxiety-like behavior. Center time of the animals in IS3 and IS4 groups was reduced as compared to the 246 g injury groups (i.e., IS1 and IS2), sham and naive animals. The reduction in activity reached significance in the IS3. (C) Vertical activity was measured as an indicator of depression-related behavior. Vertical activity of the injured animals was significantly less than that of the sham and naive animals except the IS1 group. * P value < 0.05. (D) Startle response was measured as an indicator of emotional distress and potential sensory gating impairments. Startle responses of all injured groups, except the IS1 group, were reduced when compared to naive and sham groups at day 1 post injury. There were no differences between groups at days 14 and 30 post injury, except in the IS4 group where the startle response was significantly higher than all other groups at day 14 post injury. Values are expressed as Mean +/- SEM. * P value < 0.05.

Table 5: The horizontal activity in OFL.

Group	Comparison Group	Significance level
Naïve (10379.72 ± 499.03)	Sham	0.770
	IS1	0.070
	IS3	0.001*
	IS2	0.439
	IS4	0.001*
Sham (10564.714 ± 385.01)	Naive	0.770
	IS1	0.080
	IS3	0.000*
	IS2	0.237
	IS4	0.001*
IS1 (11595.69 ± 441.08)	Naive	0.070
	Sham	0.080
	IS3	0.000*
	IS2	0.007*
	IS4	0.000*
IS3 (7762.35 ± 557.93)	Naive	0.001*
	Sham	0.000*
	IS1	0.000*
	IS2	0.004*
	IS4	0.360
IS2 (9855.80 ± 455.55)	Naive	0.439
	Sham	0.237
	IS1	0.007*
	IS3	0.004*
	IS4	0.005*
IS4 (6695.83 ± 1018.63)	Naive	0.001*
	Sham	0.001*
	IS1	0.000*
	IS3	0.360
	IS2	0.005*

The horizontal activity (number of beam breaks) of the individual groups is given and its significance with the other groups in the study is indicated. Values are expressed as mean ± SEM. * P value significant < 0.05.

Table 6: The center time spent over the time period of the study.

Time	Comparison Group	Significance level
Day 1 (1313.54 ± 38.64)	Day 14	0.000*
	Day 30	0.000*
Day 14 (1625.43 ± 36.39)	Day 1	0.000*
	Day 30	0.023*
Day 30 (1718.95 ± 38.13)	Day 1	0.000*
	Day 14	0.023*

The center time of the animals (in seconds) from all groups over the period of the study is given. Values are presented as mean ± SEM. * * P value significant < 0.05.

Table 7: The center time of the animals in OFL.

Group	Comparison Group	Significance level
Naïve (1482.83 ± 79.15)	Sham	0.770
	IS1	0.070
	IS3	0.001*
	IS2	0.439
	IS4	0.001*
Sham (1385.04 ± 61.03)	Naive	0.770
	IS1	0.080
	IS3	0.000*
	IS2	0.237
	IS4	0.001*
IS1 (1335.22 ± 69.96)	Naive	0.070
	Sham	0.080
	IS3	0.000*
	IS2	0.007*
	IS4	0.000*
IS3 (1094.26 ± 88.60)	Naive	0.001*
	Sham	0.000*
	IS1	0.000*
	IS2	0.004*
	IS4	0.360
IS2 (1450.50 ± 72.28)	Naive	0.439
	Sham	0.237
	IS1	0.007*
	IS3	0.004*
	IS4	0.005*
IS4 (1156.08 ± 160.92)	Naive	0.001*
	Sham	0.001*
	IS1	0.000*
	IS3	0.360
	IS2	0.005*

The time spent in the center (in seconds) is given for each of the groups and its comparison with the other groups is given. Values are presented as mean ± SEM. * P value significant < 0.05.

Table 8: The vertical activity over the time period of the study.

Time	Comparison Group	Significance level
Day 1 (718.20 ± 34.65)	Day 14	0.000
	Day 30	0.000
Day 14 (1175.47 ± 44.15)	Day 1	0.000
	Day 30	0.623
Day 30 (1157.07 ± 38.32)	Day 1	0.000
	Day 14	0.623

The vertical activity of the animals (number of beam breaks) from all the groups over the period of the study is given. Values are presented as mean ± SEM. * * P value significant < 0.05.

Table 9: The vertical activity in OFL.

Group	Comparison Group	Significance level
Naïve (871.77 ± 74.96)	Sham	0.933
	IS1	0.262
	IS3	0.000*
	IS2	0.830
	IS4	0.002*
Sham (863.79 ± 57.79)	Naive	0.933
	IS1	0.172
	IS3	0.000*
	IS2	0.877
	IS4	0.001*
IS1 (984.69 ± 66.38)	Naive	0.262
	Sham	0.172
	IS3	0.000*
	IS2	0.159
	IS4	0.000*
IS3 (451.87 ± 83.77)	Naive	0.000*
	Sham	0.000*
	IS1	0.000*
	IS2	0.000*
	IS4	0.484
IS2 (849.94 ± 68.47)	Naive	0.830
	Sham	0.877
	IS1	0.159
	IS3	0.000*
	IS4	0.002*
IS4 (329.18 ± 153.77)	Naive	0.002*
	Sham	0.001*
	IS1	0.000*
	IS3	0.484
	IS2	0.002*

The vertical activity of the animals (number of beam breaks) in each group and its comparison with the other groups for day 1 is given. Values are presented as mean ± SEM. * P value significant < 0.05.

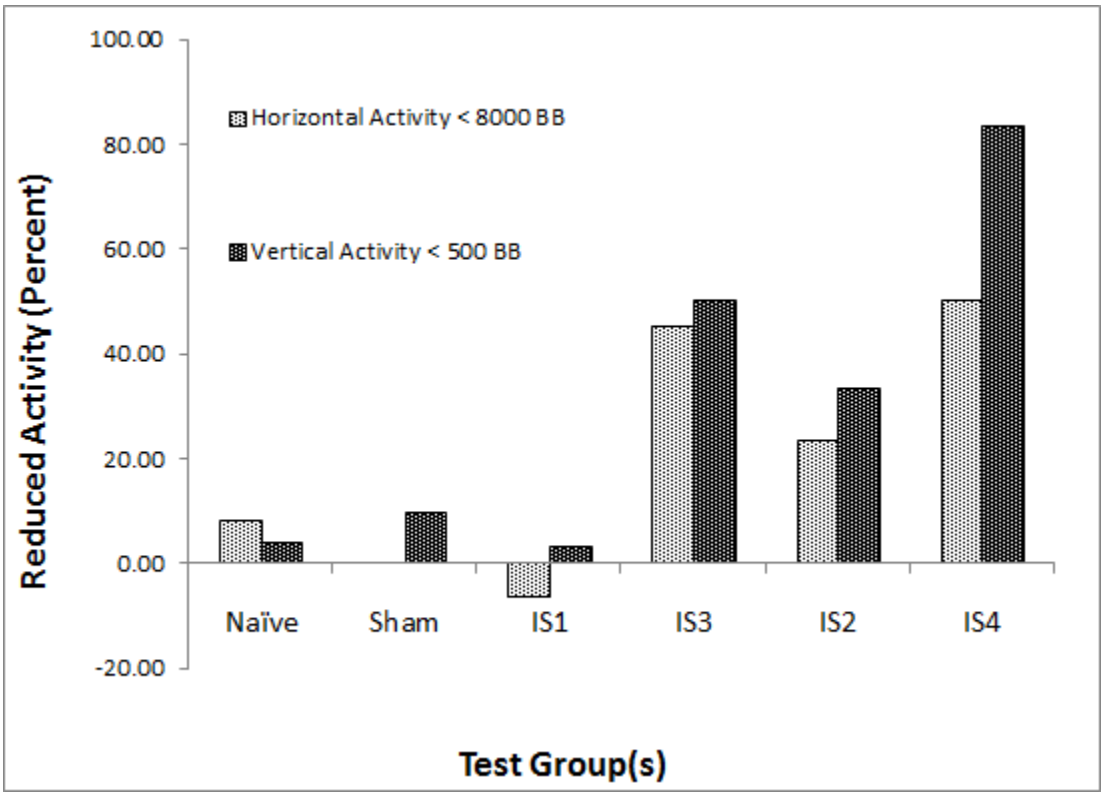

Figure 15: Animal activity in an open field test. Percentage of the animals that show reduced activity within each of the controls and the injury groups was evaluated. The lowest baseline horizontal and vertical activity values (beam breaks) in the naïve group were used as reference numbers. Animals exhibiting activity lower than the reference number were identified and were used to calculate the percentage of the animals within a group that exhibited reduced activity on day 1 post injury ((Number of animals that exhibited reduced activity in a group/total number of the animals in the group)*100). Data showed that as the grade of the injury increased, a higher percentage of the animals showed reduced activity.

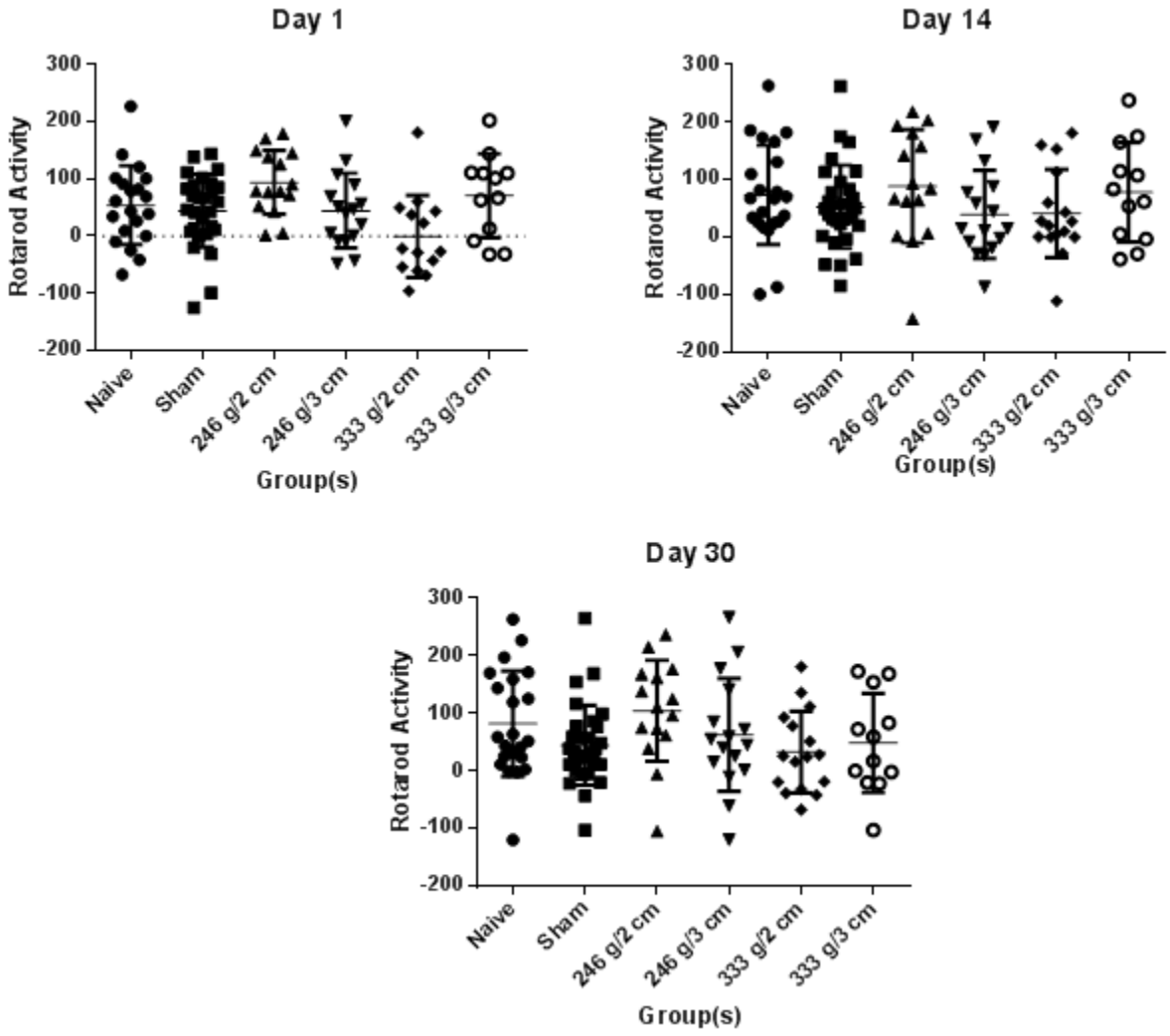

Figure 16: Motor activity post injury. Animals were subjected to the rotarod test to evaluate motor activity deficits post injury using a Med Associates rat rotarod (Med Associates, Inc., St. Albans, VT). Animals were placed on an accelerating (4-40 rpm) rotating rod (7.0 cm diameter) for a maximum period of 5 min and time spent on the rod by each animal was measured. Three attempts of 5 min each were given to each animal and the mean time spent was calculated. Data presented here is change scores between 1 day, 14 day and 30 day post injury and baseline. (Mean time spent at each time point- mean time spent at BL measurement). No significant differences were found in the motor activity of the injured mice compared to the Naïve or Sham groups at any time point measured

3.1.4: Acoustic startle response was reduced in the animals following injury.

Startle response of animals to a 100 dB tone was measured and is presented. Overall, when looking at the responses to a 100 Db tone alone, there was a significant Time x Injury interaction, $F(10,264) = 3.72$, P,.001, $\eta 2 = .123$, such that a lower startle response was observed in the injured animals at day 1 post injury except in the IS4 group, and the response increased by day 14 and 30 post injury (Figure 14 D). At day 1 post injury, there were significant differences among injury groups, $F(5,132) = 3.54$, $P = .005$, $\eta 2 = .118$ (Table 10). On day 14 post injury, there were significant differences among injury groups, $F(5,132) = 2.95$, $P = .015$, $\eta 2 = .100$ (Table 11). No significant differences were observed between naive, sham, and various injury groups at 30 days post injury.

Table 10: Day 1 ASR.

Group	Comparison Group	Significance level
Naïve (362.43 ± 38.31)	Sham	0.338
	IS1	0.650
	IS3	0.019*
	IS2	0.046*
	IS4	0.008*
Sham (314.76 ± 31.50)	Naive	0.338
	IS1	0.141
	IS3	0.096
	IS2	0.213
	IS4	0.028*
IS1 (386.65 ± 36.91)	Naive	0.650
	Sham	0.141
	IS3	0.005*
	IS2	0.014*
	IS4	0.003*
IS3 (225.39 ± 43.18)	Naive	0.019*
	Sham	0.096
	IS1	0.005*
	IS2	0.665
	IS4	0.276
IS2 (251.16 ± 39.72)	Naive	0.046*
	Sham	0.213
	IS1	0.014*
	IS3	0.665
	IS4	0.158
IS4 (127.01 ± 78.41)	Naive	0.008*
	Sham	0.028*
	IS1	0.003*
	IS3	0.276
	IS2	0.158

ASR response of the animals in each groups and its comparison with the other groups is given. Values are presented as mean ± SEM. * P value significant <0.05.

Table 11: Day 14 ASR.

Group	Comparison Group	Significance level
Naïve (333.72 ± 36.72)	Sham	0.365
	IS1	0.114
	IS3	0.998
	IS2	0.408
	IS4	0.009*
Sham (290.52 ± 30.20)	Naive	0.365
	IS1	0.415
	IS3	0.401
	IS2	0.988
	IS4	0.001*
IS1 (252.51 ± 35.38)	Naive	0.114
	Sham	0.415
	IS3	0.138
	IS2	0.477
	IS4	0.000*
IS3 (333.59 ± 41.39)	Naive	0.998
	Sham	0.401
	IS1	0.138
	IS2	0.442
	IS4	0.011*
IS2 (289.78 ± 38.08)	Naive	0.408
	Sham	0.988
	IS1	0.477
	IS3	0.442
	IS4	0.002*
IS4 (554.82 ± 75.15)	Naive	0.009*
	Sham	0.001*
	IS1	0.000*
	IS3	0.011*
	IS2	0.002*

ASR response of the animals in each groups and its comparison with the other groups is given. Values are presented as mean ± SEM. * P value significant <0.05.

3.1.5: Serum miRNAs are modulated in injured animals.

To determine whether miRNAs were modulated after the injury, serum miRNA expression of the injured mice was compared to the serum miRNA expression of the sham mice at 3 hr post injury. The numbers of the miRNA that passed the detection criteria were similar among the injured and sham groups (Figure 17 A). However, the number of significantly modulated miRNAs increased with the increasing grade of injury except in the IS4 injury group where the numbers of significantly modulated miRNA were marginally less than the IS3 injury group (Figure 17 B). 23 miRNAs (14 up- and 9 down- regulated) were significantly modulated in IS1 injury group (Table 12). 53 miRNAs (35 up- and 18 down-regulated) were significantly modulated in IS2 injury group (Table 13). 116 miRNAs (70 up- and 46 downregulated) were significantly modulated in IS3 injury group (Table 14). 106 miRNAs (66 up- and 40 down-regulated) were significantly modulated in IS4 injury group (Table 15). Hierarchical clustering of the DDCt values of the expressed miRNAs showed IS3 and IS4 (i.e., 333 g groups) were similar to each other followed by the association with the IS2. The IS1 group clustered away from the rest of the three injury groups (Figure 18). This observation was consistent with the behavior data where the IS1 group did not show any significant differences from the sham group.

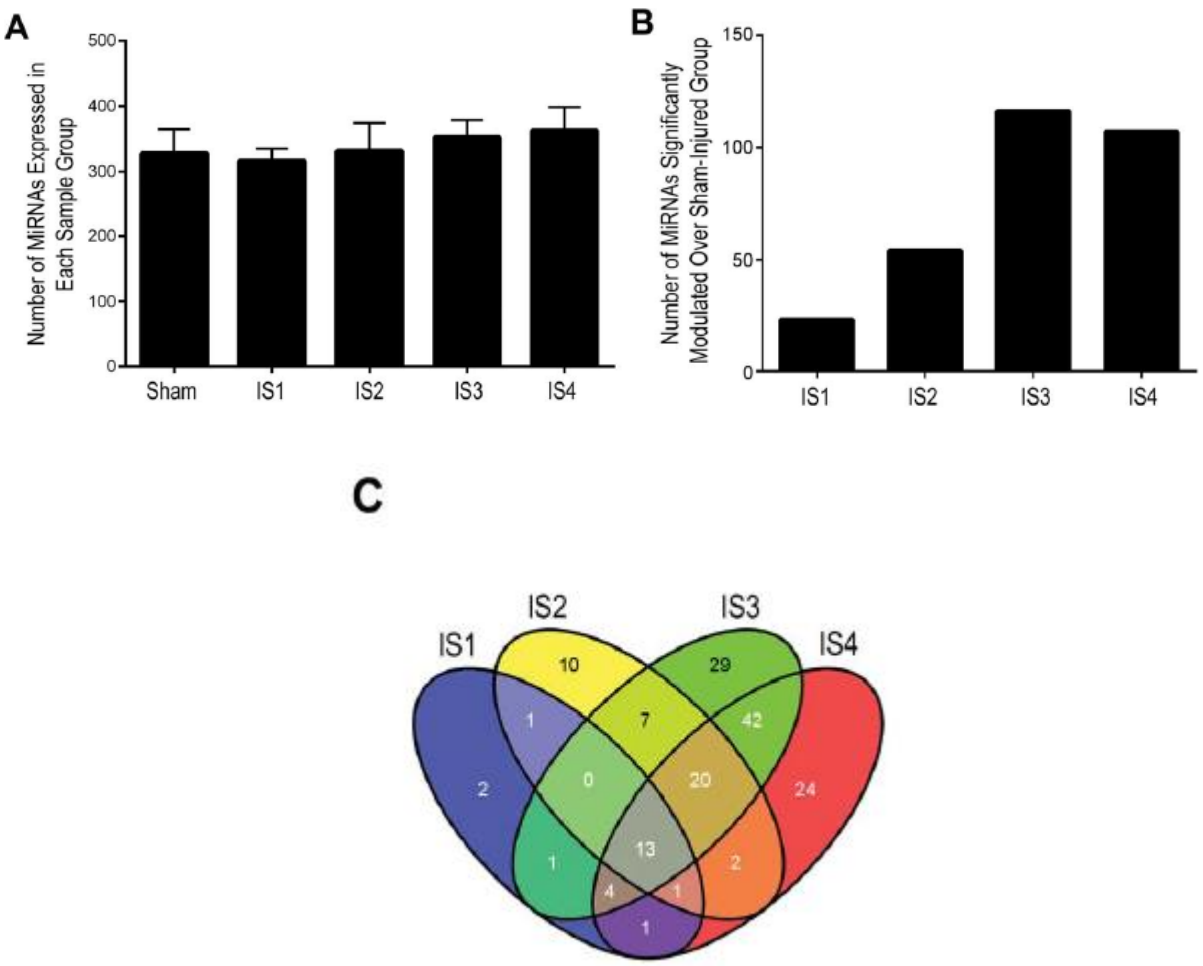

Figure 17: MiRNA expression pattern. (A) MiRNAs that show Ct<36 were considered as expressed. The number of miRNAs expressed ranged from 315.83 - 362.17 with a maximum and minimum numbers detected in IS4 and IS1 respectively. The SD ranged from 19.28243.37, the maximum being in IS2 and the minimum in IS1. The values are presented as Mean 6 SD of the number of miRNAs detected in each group. (B) The number of miRNAs that were significantly modulated ($1.5 fold; P,0.05) following the injury over the time point matched sham controls increased with the height and weight of the free falling metal rod except in the IS3 group where the number of significantly modulated miRNAs was marginally greater than the IS4 group. (C) Overlapping miRNA data analysis for significantly modulated miRNAs in the injury groups was done using the online Venn diagram generation tool (Oliveros (2007).VENNY. An interactive tool for comparing lists with Venn Diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Table 12: Significantly modulated miRNAs in IS1.

S. No.	MiRNA	P Value	Fold Difference(log10 (RQ))
Calibrator not detected			
1	mmu-miR-376a	9.43E-04	1.99
2	mmu-miR-494	1.85E-04	1.94
3	mmu-miR-297a#	3.11E-02	1.56
4	rno-miR-345-3p	4.78E-03	1.08
Valid			
5	hsa-miR-214	1.82E-03	0.58
6	mmu-miR-214	6.37E-03	0.54
7	mmu-miR-337-5p	2.10E-02	0.52
8	mmu-miR-574-3p	2.16E-02	0.51
9	mmu-miR-434-3p	2.16E-02	0.41
10	mmu-miR-671-3p	1.76E-02	0.39
11	mmu-miR-218	4.46E-02	0.36
12	mmu-miR-676	1.58E-02	0.33
13	mmu-miR-199a-3p	3.34E-02	0.32
14	hsa-miR-455	4.71E-02	0.28
15	mmu-miR-322	9.85E-03	-0.34
16	mmu-miR-331-3p	1.99E-02	-0.35
17	hsa-miR-106b#	2.80E-02	-0.4
18	mmu-miR-106b	1.41E-02	-0.47
19	mmu-miR-2138	5.83E-03	-0.61
20	mmu-miR-31	2.16E-02	-0.85
Target not detected			
21	mmu-miR-363	4.59E-02	-1.4
22	mmu-miR-181c	2.86E-02	-1.66
23	rno-miR-196c	4.66E-02	-1.81

Twenty three miRNA were significantly modulated. Of these 14 were up regulated and 9 were down regulated. Values are given as Log10 of the fold change ($2^{-(\text{mean}\Delta\Delta Ct)}$).

Table 13: Significantly modulated miRNAs in IS2.

S.No.	MiRNA	P Value	Fold Difference (log10 (RQ))
Calibrator not detected			
1	mmu-miR-700	2.31E-02	2.21
2	mmu-miR-487b	2.17E-03	1.91
3	mmu-miR-376a	1.11E-03	1.70
4	mmu-miR-125b	4.32E-02	1.23
5	mmu-miR-1932	2.25E-02	1.17
6	mmu-miR-218-1	3.42E-02	1.05
7	mmu-miR-191*	1.79E-02	1.00
Valid			
8	mmu-miR-384-5p	6.70E-03	0.73
9	hsa-miR-214	2.58E-05	0.72
10	mmu-miR-667	5.12E-03	0.69
11	mmu-miR-218	1.82E-04	0.64
12	mmu-miR-214	8.97E-05	0.64
13	mmu-miR-434-3p	3.91E-03	0.61
14	mmu-miR-122	1.59E-03	0.61
15	mmu-miR-34b-3p	3.05E-03	0.60
16	mmu-miR-872*	9.36E-03	0.56
17	mmu-miR-361	8.35E-03	0.55
18	mmu-miR-485-3p	2.64E-02	0.53
19	mmu-miR-574-3p	2.61E-03	0.53
20	hsa-miR-9*	4.56E-02	0.52
21	mmu-miR-337-5p	9.90E-03	0.49
22	mmu-miR-685	3.22E-03	0.48
23	mmu-miR-204	1.44E-02	0.44
24	mmu-miR-376c	2.57E-02	0.43
25	mmu-miR-199a-3p	2.91E-03	0.42
26	mmu-miR-337-3p	2.11E-02	0.40
27	mmu-miR-132	4.27E-02	0.40
28	mmu-miR-671-3p	1.04E-02	0.37
29	mmu-miR-152	1.58E-02	0.36
30	mmu-miR-376b*	1.29E-02	0.34
31	mmu-miR-212	3.85E-02	0.32
32	mmu-miR-511	1.48E-02	0.31
33	mmu-miR-192	2.19E-03	0.30
34	mmu-miR-145	2.81E-02	0.25
35	mmu-miR-146a	3.56E-02	0.24

36	mmu-miR-19b	3.57E-02	-0.25
37	mmu-miR-322	3.22E-02	-0.26
38	hsa-miR-200c	1.20E-02	-0.26
39	mmu-miR-486	2.56E-02	-0.27
40	mmu-miR-1274a	3.52E-02	-0.28
41	hsa-miR-200b	4.62E-02	-0.30
42	mmu-miR-2146	2.90E-02	-0.30
43	mmu-miR-652	1.91E-02	-0.30
44	mmu-miR-18a	2.17E-02	-0.34
45	hsa-miR-106b*	9.68E-03	-0.38
46	mmu-miR-451	1.48E-02	-0.42
47	mmu-miR-106b	7.93E-03	-0.45
48	mmu-miR-26b	3.19E-02	-0.53
49	mmu-miR-31	1.86E-02	-1.03
50	hsa-miR-875-5p	4.14E-02	-3.02
Target not detected			
51	rno-miR-196c	4.67E-02	-1.53
52	mmu-miR-1982.2	3.95E-02	-1.56
53	mmu-miR-7a	8.20E-03	-1.98

Fifty three miRNA were significantly modulated. Of these, 35 were up regulated and 18 were down regulated. Values are given as Log10 of the fold change ($2^{-(\text{mean}\Delta\Delta Ct)}$).

Table 14: Significantly modulated miRNAs in IS3.

S.No	MiRNA	P Value	Fold Difference (log10 (RQ))
Calibrator not detected			
1	mmu-miR-15a*	1.8E-13	4.57
2	mmu-miR-495	1.5E-03	2.87
3	mmu-miR-673	3.0E-02	2.66
4	mmu-miR-433	9.8E-04	2.63
5	mmu-miR-146b	2.7E-02	2.48
6	mmu-miR-702	2.4E-02	2.24
7	mmu-miR-10a	2.6E-02	2.22
8	mmu-miR-700	2.4E-02	2.2
9	mmu-miR-134	1.9E-02	2.09
10	mmu-miR-487b	1.3E-03	2.01
11	mmu-miR-494	7.3E-05	1.96
12	hsa-miR-99b*	7.7E-03	1.87
13	mmu-miR-551b	2.1E-03	1.75
14	mmu-miR-376a	7.1E-04	1.72
15	rno-miR-551B	5.7E-03	1.62
16	mmu-miR-434-5p	2.9E-02	1.61
17	rno-miR-204*	1.7E-02	1.6
18	mmu-miR-467b	1.6E-02	1.6
19	mmu-miR-137	2.7E-02	1.54
20	rno-miR-345-3p	2.0E-03	1.41
21	mmu-miR-547	4.1E-02	1.3
22	mmu-miR-680	4.8E-02	1.14
23	mmu-miR-218-1*	3.0E-02	1.1
24	mmu-miR-345-3p	3.4E-02	1.09
25	mmu-miR-295	2.6E-02	1.09
26	rno-miR-351	6.3E-03	0.89
Valid			
27	mmu-miR-122	2.2E-04	1.24
28	mmu-miR-872	2.6E-02	0.98
29	mmu-miR-667	7.9E-04	0.91
30	mmu-miR-214	2.4E-07	0.88
31	mmu-miR-485-3p	5.1E-04	0.83
32	mmu-miR-365	9.5E-03	0.83

33	mmu-miR-384-5p	9.8E-04	0.78
34	mmu-miR-337-5p	1.0E-05	0.77
35	mmu-miR-193*	7.4E-03	0.74
36	mmu-miR-671-3p	2.1E-05	0.72
37	hsa-miR-214	2.5E-05	0.71
38	mmu-miR-574-3p	4.2E-05	0.71
39	mmu-miR-192	3.5E-03	0.64
40	mmu-miR-434-3p	7.2E-05	0.64
41	mmu-miR-685	3.1E-04	0.63
42	mmu-miR-193	3.5E-02	0.62
43	mmu-miR-872*	2.8E-03	0.6
44	mmu-let-7a*	4.4E-03	0.6
45	mmu-miR-410	4.8E-03	0.58
46	mmu-miR-125b-5p	1.6E-02	0.57
47	mmu-miR-218	1.3E-03	0.54
48	mmu-miR-34b-3p	2.5E-04	0.54
49	mmu-miR-145	1.2E-03	0.53
50	mmu-miR-500	8.2E-05	0.51
51	mmu-miR-132	6.0E-03	0.48
52	mmu-miR-127	3.3E-02	0.46
53	mmu-miR-125a-5p	3.1E-03	0.46
54	mmu-miR-199a-3p	1.4E-03	0.46
55	mmu-miR-706	2.0E-02	0.44
56	hsa-miR-671-5p	1.8E-02	0.44
57	mmu-miR-152	2.4E-03	0.44
58	mmu-miR-1944	4.0E-02	0.42
59	mmu-miR-361	2.7E-02	0.38
60	mmu-miR-376c	8.4E-03	0.37
61	mmu-miR-362-3p	4.0E-02	0.37
62	hsa-miR-744*	7.7E-03	0.36
63	hsa-miR-455	3.0E-02	0.35
64	mmu-miR-31*	2.4E-02	0.35
65	mmu-miR-674*	4.3E-02	0.34

66	mmu-miR-204	4.2E-03	0.32
67	mmu-miR-212	2.4E-02	0.29
68	mmu-miR-532-3p	2.9E-02	0.28
69	mmu-miR-511	2.8E-02	0.26
70	mmu-miR-1839-3p	4.9E-02	0.19
71	mmu-miR-200c	4.7E-02	-0.22
72	mmu-miR-484	2.7E-02	-0.23
73	mmu-miR-18a*	1.6E-03	-0.25
74	hsa-miR-425	4.7E-02	-0.26
75	mmu-miR-1930	3.6E-02	-0.26
76	mmu-miR-301a	4.2E-02	-0.26
77	mmu-miR-2134	3.9E-03	-0.28
78	mmu-miR-19b	1.3E-02	-0.29
79	mmu-miR-222	4.6E-02	-0.31
80	mmu-miR-141	3.9E-02	-0.33
81	hsa-miR-200c	2.1E-03	-0.33
82	mmu-miR-106a	2.0E-02	-0.34
83	mmu-miR-130b	5.5E-03	-0.34
84	mmu-miR-486	1.7E-02	-0.35
85	mmu-miR-17	9.6E-03	-0.39
86	mmu-miR-331-3p	1.3E-02	-0.41
87	mmu-miR-652	7.1E-03	-0.41
88	mmu-let-7d	7.6E-03	-0.41
89	mmu-miR-17*	9.6E-03	-0.43
90	mmu-miR-16	2.7E-02	-0.43
91	hsa-miR-421	6.9E-04	-0.43
92	mmu-miR-345-5p	2.4E-03	-0.43
93	mmu-miR-18a	1.6E-03	-0.43
94	mmu-miR-103	2.1E-02	-0.44
95	mmu-miR-25	4.3E-03	-0.47
96	mmu-miR-93	4.8E-03	-0.47
97	mmu-miR-140	1.7E-02	-0.48
98	mmu-let-7i	1.5E-03	-0.48
99	mmu-miR-20a	8.0E-04	-0.48
100	mmu-miR-20b	2.5E-02	-0.49
101	mmu-miR-340-5p	2.0E-02	-0.5
102	mmu-miR-186*	1.6E-02	-0.51

103	mmu-miR-301b	7.0E-03	-0.54
104	mmu-miR-15b	5.0E-04	-0.54
105	mmu-miR-26a	4.1E-03	-0.57
106	mmu-miR-142-3p	4.2E-03	-0.6
107	mmu-miR-26b	7.4E-03	-0.63
108	hsa-miR-106b*	5.9E-05	-0.64
109	mmu-miR-451	5.0E-05	-0.78
110	mmu-miR-106b	4.2E-05	-0.83
111	mmu-miR-31	2.4E-02	-0.87
112	hsa-miR-875-5p	2.5E-02	-3.16
Target not detected			
113	rno-miR-196c	2.2E-02	-1.39
114	mmu-miR-463	2.2E-03	-1.75
115	mmu-let-7e	3.7E-02	-1.98
116	mmu-miR-363	4.40E-03	-2.95

One hundred and sixteen miRNA were significantly modulated. Of these, 70 were up regulated and 46 were down regulated. Values are given as Log10 of the fold change (2-(mean$\Delta\Delta$Ct)).

Table 15: Significantly modulated miRNAs in IS4.

S.No	MiRNA	P Value	Fold Difference (log10 (RQ))
Calibrator not detected			
1	mmu-miR-15a*	2.14E-13	4.47
2	mmu-miR-129-3p	6.98E-05	3.37
3	hsa-miR-149	7.43E-03	3.33
4	mmu-miR-187	1.92E-03	3.22
5	mmu-miR-433	7.82E-04	2.87
6	mmu-miR-34c	1.08E-04	2.81
7	mmu-miR-146b	2.44E-02	2.52
8	hsa-let-7e*	3.67E-07	2.27
9	mmu-miR-10a	2.49E-02	2.23
10	mmu-miR-134	2.06E-02	2.07
11	mmu-miR-494	1.42E-04	2.02
12	rno-miR-204*	5.97E-03	1.97
13	mmu-miR-383	3.11E-03	1.89
14	mmu-miR-487b	2.96E-03	1.87
15	mmu-miR-376a	4.14E-03	1.68
16	mmu-miR-137	2.01E-02	1.65
17	rno-miR-345-3p	5.67E-04	1.61
18	mmu-miR-381	3.45E-03	1.6
19	mmu-miR-455	3.32E-02	1.59
20	hsa-miR-99b*	1.94E-02	1.58
21	mmu-miR-125b*	3.70E-02	1.28
22	mmu-miR-345-3p	2.48E-02	1.17
23	mmu-miR-218-1*	4.93E-02	0.98
24	rno-miR-351	8.86E-03	0.86
Valid			
25	mmu-miR-122	5.56E-05	1.41
26	mmu-miR-667	9.46E-04	1.19
27	mmu-miR-365	2.39E-03	0.93
28	mmu-miR-34b-3p	6.71E-04	0.91
29	mmu-miR-485-3p	3.61E-04	0.84
30	mmu-miR-214	4.42E-07	0.8
31	mmu-miR-204	1.71E-04	0.77
32	hsa-miR-214	1.03E-05	0.75
33	mmu-miR-193*	1.37E-03	0.74
34	mmu-miR-384-5p	2.60E-02	0.74

35	mmu-miR-125b-5p	2.47E-03	0.7
36	mmu-miR-194	1.78E-03	0.69
37	mmu-miR-127	1.59E-02	0.69
38	mmu-miR-671-3p	1.94E-04	0.69
39	mmu-miR-434-3p	1.61E-03	0.67
40	mmu-miR-199a-3p	1.01E-04	0.62
41	mmu-miR-132	6.76E-03	0.61
42	mmu-miR-410	2.09E-02	0.6
43	mmu-miR-872*	8.59E-03	0.58
44	mmu-miR-192	3.06E-04	0.57
45	mmu-miR-145	1.40E-03	0.56
46	hsa-miR-671-5p	4.68E-03	0.53
47	mmu-miR-574-3p	1.26E-03	0.53
48	mmu-miR-532-5p	1.23E-02	0.52
49	mmu-miR-337-5p	5.90E-03	0.52
50	mmu-miR-375	4.70E-02	0.5
51	mmu-miR-218	7.99E-04	0.5
52	mmu-let-7a*	5.98E-03	0.49
53	hsa-miR-455	2.99E-03	0.49
54	mmu-miR-500	2.66E-03	0.46
55	mmu-miR-1944	1.63E-02	0.45
56	mmu-miR-685	1.30E-03	0.43
57	mmu-miR-148a	1.47E-03	0.42
58	mmu-miR-212	3.16E-02	0.41
59	mmu-miR-224	6.52E-03	0.4
60	mmu-miR-676	2.96E-03	0.4
61	mmu-miR-152	9.64E-03	0.4
62	mmu-miR-200a	4.06E-02	0.39
63	hsa-miR-744*	1.19E-02	0.36
64	mmu-miR-497	9.60E-03	0.35
65	mmu-miR-532-3p	2.04E-02	0.3
66	mmu-miR-133a	4.79E-02	0.27
67	mmu-miR-19b	2.20E-02	20.27
68	mmu-miR-28	1.19E-02	20.27
69	mmu-miR-18a*	1.54E-02	20.30
70	mmu-miR-301a	2.62E-02	20.32
71	mmu-miR-18a	2.96E-02	20.32
72	mmu-miR-15b	7.79E-03	20.34
73	mmu-miR-879*	4.04E-02	20.35
74	mmu-miR-191	2.35E-03	20.35
75	mmu-let-7i	2.49E-02	20.35

76	mmu-miR-222	1.42E-02	20.35
77	mmu-let-7d	1.78E-02	20.36
78	hsa-miR-106b*	1.21E-02	20.36
79	mmu-miR-93	2.02E-02	20.36
80	mmu-miR-106a	2.31E-02	20.37
81	mmu-miR-16*	1.54E-02	20.39
82	mmu-miR-25	3.56E-02	20.39
83	mmu-miR-130b	2.38E-02	20.40
84	mmu-miR-2134	2.10E-02	20.41
85	mmu-miR-17	4.15E-03	20.43
86	mmu-miR-16	3.14E-02	20.43
87	mmu-miR-15a	2.51E-02	20.44
88	rno-miR-148b-5p	1.10E-02	20.45
89	mmu-miR-451	1.04E-02	20.51
90	mmu-miR-340-5p	1.11E-02	20.53
91	mmu-miR-20a	3.68E-03	20.53
92	hsa-miR-421	5.54E-04	20.53
93	mmu-miR-26b	1.48E-02	20.57
94	mmu-miR-1274a	2.25E-02	20.60
95	mmu-miR-186	3.50E-02	20.60
96	mmu-miR-142-3p	4.12E-03	20.61
97	mmu-miR-17*	4.59E-03	20.62
98	mmu-miR-301b	2.02E-03	20.66
99	mmu-miR-26a	1.46E-03	20.66
100	mmu-miR-186*	2.64E-03	20.67
101	mmu-miR-106b	1.92E-03	20.68
102	mmu-miR-31	1.59E-02	20.86
103	hsa-miR-875-5p	1.02E-02	23.75
Target not detected			
104	rno-miR-196c	4.12E-02	21.80
105	mmu-miR-363	1.33E-02	22.38
106	mmu-miR-2138	4.71E-02	22.52

One hundred and six miRNA were significantly modulated. Of these, 66 were up regulated and 40 were down regulated. . Values are given as Log10 of the fold change (2-(mean$\Delta\Delta$Ct)).

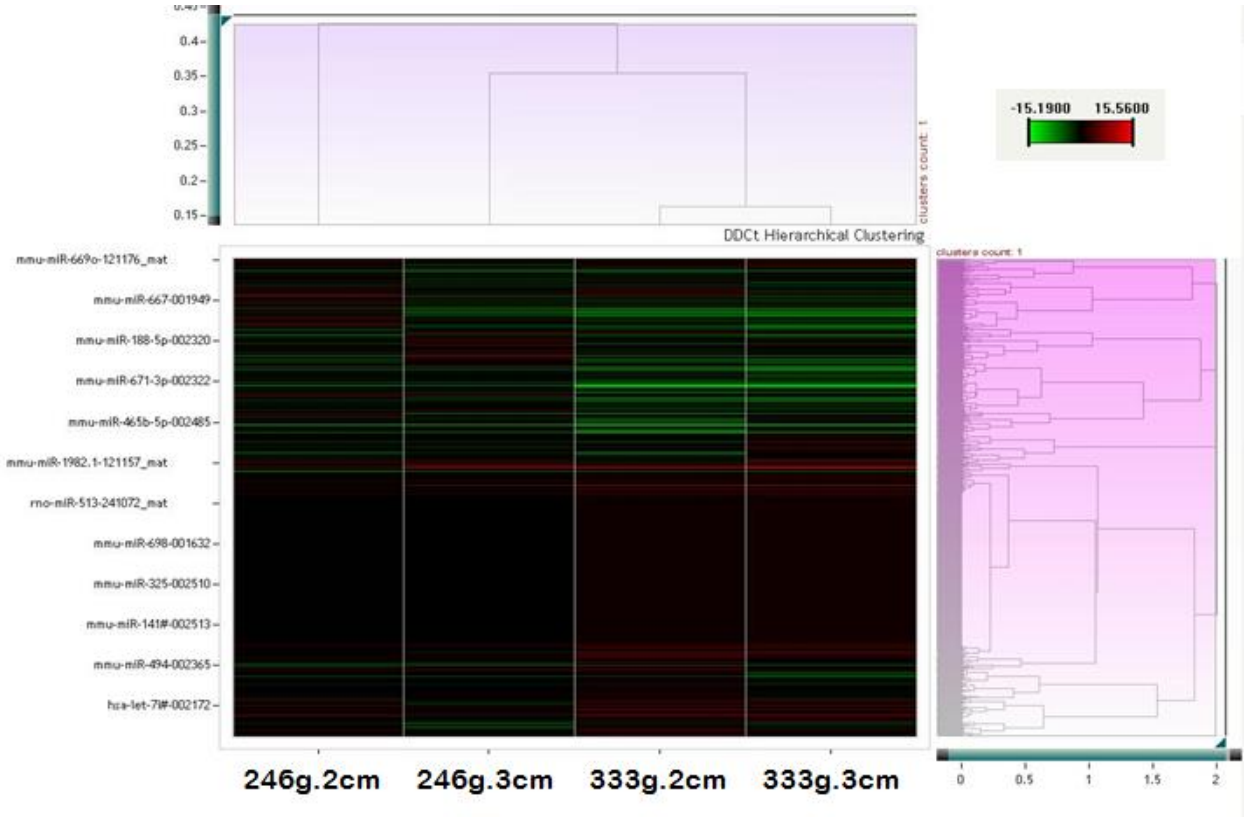

Figure 18: Hierarchical Clustering (HC) of DDCt values. HC of DDCt of the miRNAs calculated over the sham controls showed 333g injury groups clustering together followed by 246 g/3 cm and 246 g/2 cm respectively. HC indicates that more severe of the injuries with in the mild spectrum showed close association.

3.1.6: Common miRNA changes regardless of the mTBI severity

To identify miRNAs that may consistently be present regardless of extent or severity of the injury, significantly modulated miRNAs were compared among the injury groups (Figure 17 C). 13 miRNAs were found to be modulated in all four injury groups and had similar expression patterns. Nine of the 13 miRNAs were up regulated and 4 were down regulated following injury (Table 16). The Ct values of the modulated miRNAs are given in Table 17.

Table 16: Common MiRNA among all injury groups.

S. No.	MiRNA	IS1	IS2	IS3	IS4
1	mmu-miR-376a	1.99	1.7	1.72	1.68
2	hsa-miR-214	0.58	0.72	0.71	0.75
3	mmu-miR-214	0.54	0.64	0.88	0.8
4	mmu-miR-337-5p	0.52	0.49	0.77	0.52
5	mmu-miR-574-3p	0.51	0.53	0.71	0.53
6	mmu-miR-434-3p	0.41	0.61	0.64	0.67
7	mmu-miR-671-3p	0.39	0.37	0.72	0.69
8	mmu-miR-218	0.36	0.64	0.54	0.5
9	mmu-miR-199a-3p	0.32	0.42	0.46	0.62
10	hsa-miR-106b*	-0.4	-0.38	-0.64	-0.36
11	mmu-miR-106b	-0.47	-0.45	-0.83	-0.68
12	mmu-miR-31	-0.85	-1.03	-0.87	-0.86
13	rno-miR-196c	-1.81	-1.53	-1.39	-1.8

Thirteen miRNAs were commonly modulated among all the injury groups. Of these, 9 were up regulated and 4 were down regulated. Values are given as Log10 of the fold change ($2^{-(\text{mean}\Delta\Delta Ct)}$).

Table 17: Average Ct values for the common miRNAs.

MiRNA	IS1	SD	IS2	SD	IS3	SD	IS4	SD	Sham	SD
mmu-miR-574-3p	18.62	2.13	18.37	1.11	16.94	0.61	17.42	1.12	20.37	0.49
hsa-miR-214	19.41	1.92	18.76	1.03	17.97	1.02	17.70	1.05	21.40	0.86
mmu-miR-106b	19.84	2.45	19.61	1.42	20.05	1.11	19.43	1.25	18.37	0.93
mmu-miR-214	21.48	2.57	20.99	1.40	19.36	0.60	19.50	1.09	23.35	0.55
mmu-miR-199a-3p	22.92	2.25	22.40	1.12	21.43	0.91	20.78	1.46	24.04	0.69
mmu-miR-218	23.39	2.58	22.28	1.41	21.78	1.33	21.80	1.43	24.65	0.97
mmu-miR-434-3p	23.73	2.00	22.89	1.73	21.94	0.76	21.73	1.74	25.15	0.86
hsa-miR-106b#	24.28	2.22	24.05	1.47	24.07	1.12	23.02	0.96	23.03	0.78
mmu-miR-337-5p	25.36	2.63	25.28	1.65	23.51	0.92	24.21	1.28	27.14	0.65
mmu-miR-31	25.49	1.50	25.92	1.40	24.55	1.14	24.39	1.19	22.75	3.13
mmu-miR-671-3p	26.91	2.39	26.81	0.93	24.82	1.00	24.81	1.37	28.29	0.61
mmu-miR-376a	30.20	1.06	30.97	1.12	30.08	0.94	30.11	2.93	36.88	3.45
rno-miR-196c	32.20	6.15	31.08	6.97	29.81	5.06	31.04	7.17	26.26	0.95

Average Ct value for each of the miRNA in the injury and the sham control group indicate their abundance in the sample. High expression= Ct ranging from 11-15; Good expression = Ct ranging from 16-20; Moderate expression = Ct ranging from 21-25; Low Expression = Ct ranging from 26-30; and Rare expression = Ct ranging from 31-35.

3.1.7: Brain function specific miRNAs are modulated following mTBI

The miRNAs common among all mTBI groups were analyzed for their combined effect on functional pathways using the DIANA-miRPath v2.0 (Vlachos et al., 2012). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were most significantly predicted to be affected by the combined effect of the modulated miRNAs were identified. Several brain function related pathways, such as axon guidance, gap junction, dopaminergic synapse, cholinergic synapse, long-term potentiation, tight junction, adherens junction, and long-term depression, were found to be targeted by the modulated miRNAs (Figure 19). Specific analysis of the axon guidance pathway, previously reported as affected upon TBI, showed that the number of the miRNAs predicted to be involved in this pathway increased with the severity of the injury (Figure 20).

The combined effect of modulated miRNAs on functional pathways using the DIANA-miRPath v2.0 (Vlachos et al., 2012) was also performed for the significantly modulated miRNAs common among the three injury groups that showed significant change in NSS-R, OFL, and ASR tests (i.e., IS2, IS3, and IS4) (Table 18). The axon guidance pathway, along with several other brain related function pathways, was one of the most affected KEGG Pathway (Figure 19). Unique miRNAs that were modulated only in the IS1 group (Table 18), which did not show any significantly difference in NSS-R and OFL tests, showed long-term potentiation pathways as the most affected pathway by DIANA-miRPath v2.0 analysis (data not shown). Correlation analysis of the modulated miRNAs and their validated targets by the IPA software revealed a direct relation with validated targets of six of the modulated miRNAs to the axon guidance pathway (Figure 20). Because behavioral analysis showed symptoms of depression in the injured animals, a correlation analysis of the modulated miRNAs with depression-related pathways was performed; this showed their direct effect on the depression-related validated targets (Figure 22). The molecular pathway for sensorimotor impairments was also predicted to be targeted by the significantly modulated miRNAs (Figure 22). This correlates with the NSS-R and ASR measurements that also showed sensorimotor impairments. The expression profile of 3 out of 13 commonly modulated miRNAs, miR-199-3p, miR-214, and miR-376a that have been shown to be important in brain related processes and functions was also validated using the singleplex miRNA assay (Life Technologies, Carlsbad, CA) (Figure 23).

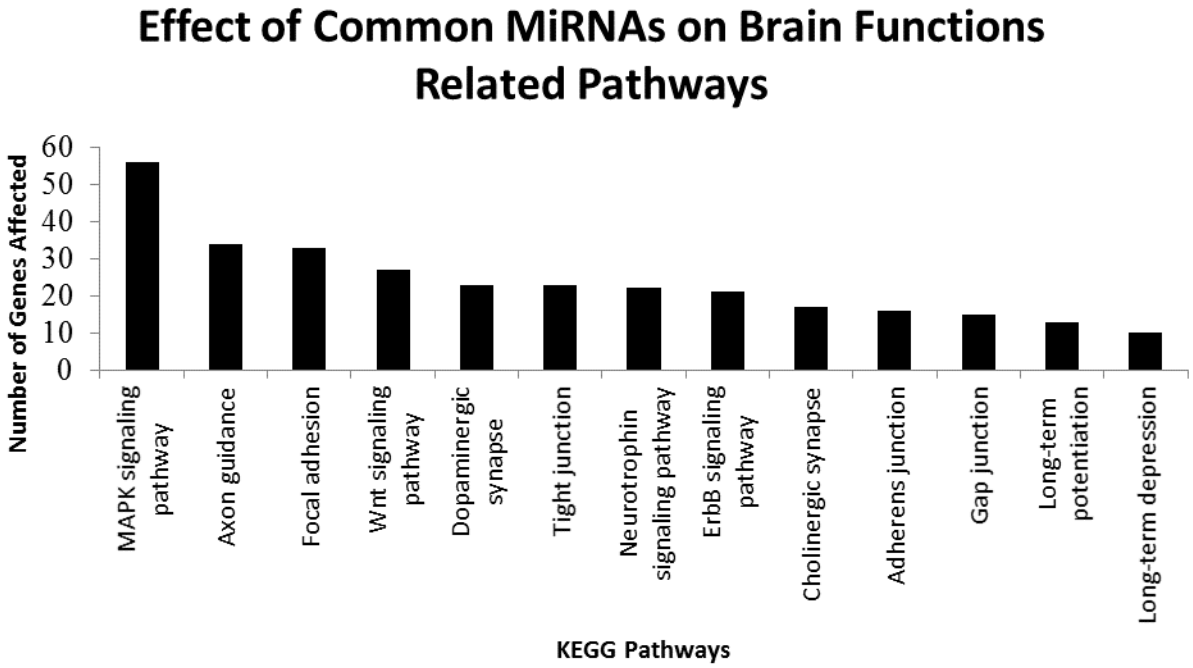

Figure 19: Brain functions related pathways targeted by significantly modulated miRNAs common among four injury groups. Thirteen common miRNAs that were significantly modulated among all four injury groups were analyzed for their combined effect on significant modulation of KEGG pathways using DIANA-miRPath v2.0 (50). Eight of the significantly modulated miRNAs, miR-106b, miR-199a-3p, miR-214, miR-218, miR-31, miR-434-3p, miR-671-3p, miR-574-3p were predicted to significantly modulate several nervous system function and disease related pathways.

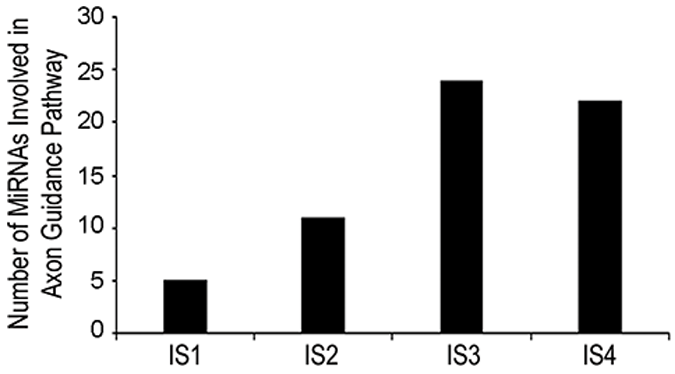

Figure 20: Involvement of significantly modulated miRNA in axon guidance pathway. Quantitative bioinformatics analysis on the number of miRNA involved in the axon guidance pathway was done using IPA. The number of the significantly modulated miRNAs that were predicted to modulate axon guidance pathway increased with the severity of the injury.

Table 18: **MiRNAs present only in the injury groups that demonstrate behavior changes.**

S.No.	MiRNA	IS2	P value	IS3	P value	IS4	P value
1	mmu-miR-487b	1.906	0.002	2.013	0.001	1.865	0.003
2	mmu-miR-218-1*	1.049	0.034	1.105	0.030	0.982	0.049
3	mmu-miR-384-5p	0.731	0.007	0.777	0.001	0.737	0.026
4	mmu-miR-667	0.693	0.005	0.908	0.001	1.194	0.001
5	mmu-miR-122	0.608	0.002	1.240	0.000	1.408	0.000
6	mmu-miR-34b-3p	0.598	0.003	0.537	0.000	0.914	0.001
7	mmu-miR-872*	0.556	0.009	0.603	0.003	0.584	0.009
8	mmu-miR-485-3p	0.534	0.026	0.835	0.001	0.842	0.000
9	mmu-miR-204	0.442	0.014	0.324	0.004	0.769	0.000
10	mmu-miR-132	0.396	0.043	0.480	0.006	0.611	0.007
11	mmu-miR-152	0.361	0.016	0.439	0.002	0.399	0.010
12	mmu-miR-212	0.315	0.039	0.289	0.024	0.410	0.032
13	mmu-miR-192	0.299	0.002	0.644	0.004	0.574	0.000
14	mmu-miR-145	0.247	0.028	0.532	0.001	0.555	0.001
15	mmu-miR-19b	-0.252	0.036	-0.292	0.013	-0.268	0.022
16	mmu-miR-18a	-0.335	0.022	-0.434	0.002	-0.319	0.030
17	mmu-miR-451	-0.418	0.015	-0.777	0.000	-0.513	0.010
18	mmu-miR-26b	-0.529	0.032	-0.635	0.007	-0.574	0.015
19	hsa-miR-875-5p	-3.021	0.041	-3.161	0.025	-3.749	0.010

Nineteen miRNAs were significantly modulated in the injury group that showed significant behavior alterations. Of these 14 were up regulated and 5 were down regulated. Two miRNAs, mmu-miR-487b and mmu-miR-218-1* were expressed only in the injured animals and not in the sham-injured animals *i.e.,* "calibrator not detected". For all other miRNAs Ct value was <36 in both, the injured and the sham-injured groups. Values are given as log10 of the fold change ($2^{-(\text{mean } \Delta\Delta Ct)}$).

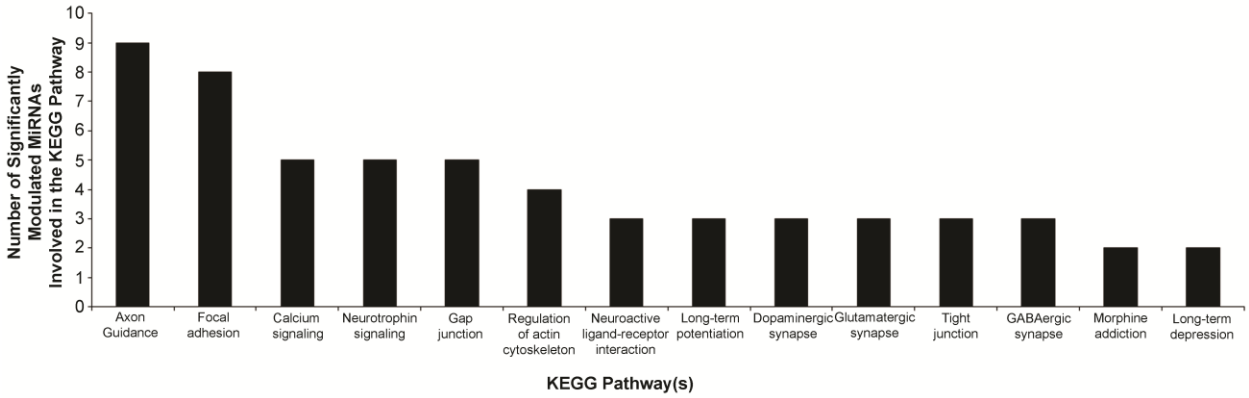

Figure 21: Brain functions related pathway targeted by the significantly modulated miRNAs unique to the injury groups with the neurobehavioral alterations. Nineteen miRNAs that were significantly modulated among all the three injury groups, which demonstrated alteration in the neurobehavioral functions (*i.e.*, IS2, IS3 and IS4) were analyzed for their combined effect on the significant modulation of KEGG pathways using DIANA-miRPath v2.0 software.

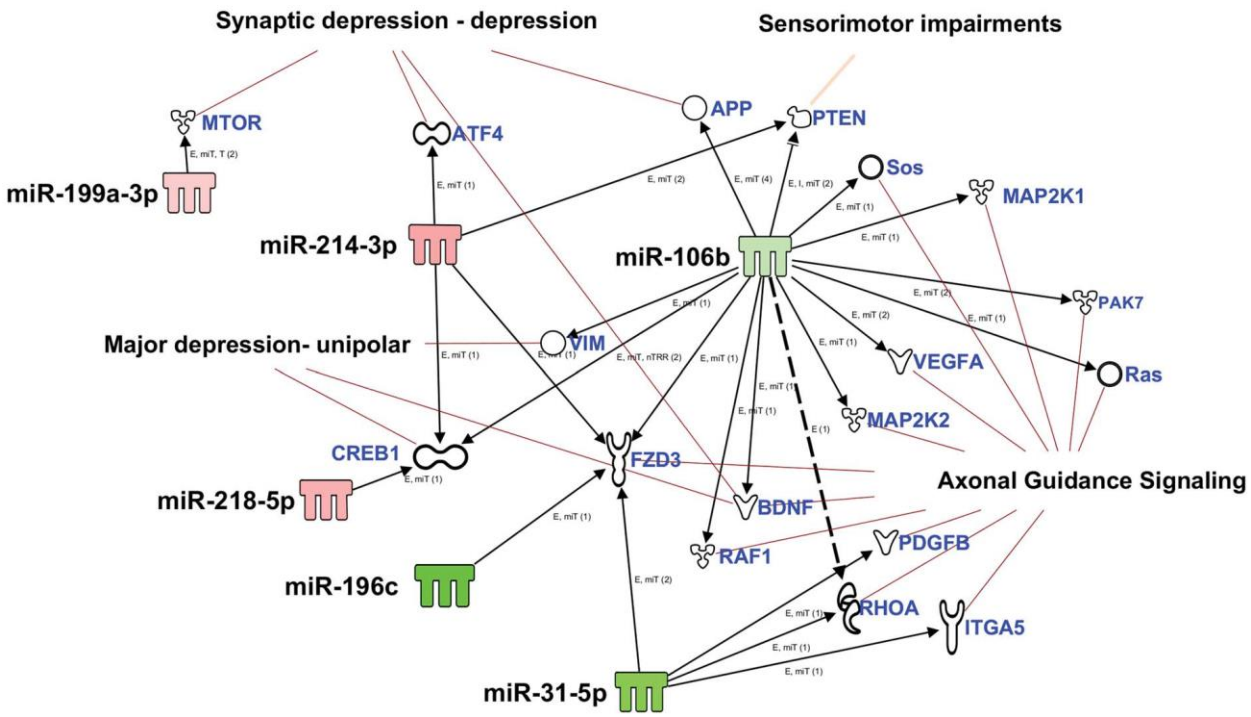

Figure 22: IPA Analysis for effect of significantly modulated miRNAs common among all four injury groups on brain functions related pathways. MiRNAs common to all four injury groups were taken and their targets and pathway analysis in relation to brain related functions were performed. My Pathway tool of IPA software was used to build the custom pathway using miRNAs that show experimentally validated targets by overlaying with 1) depression, 2) sensorimotor function and the 3) axon guidance pathway molecules. Of the 13 common modulated miRNAs, only 6 miRNAs were found to have experimentally validated targets that are predicted to modulate axon guidance, synaptic depression and sensorimotor impairments.

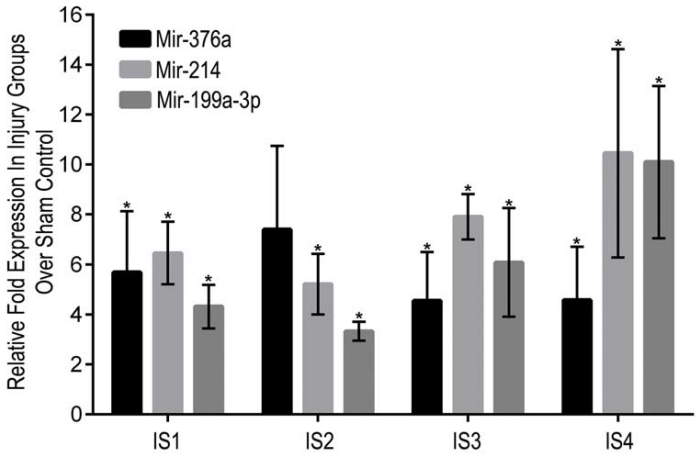

Figure 23: Expression of miRNAs in individual real time PCR assay. The fold upregulation of three miRNAs, miR-376a, miR-214 and miR-199a-3p, in the injury groups over the sham mice was validated using the individual real time PCR assays. Similar to the miRNA arrays, expression of the three miRNAs was found to be up regulated in the injured groups over the sham animals. Data presented is the fold up regulation (+/- SEM; *P<0.05) calculated from the mean DDCt value obtained from three biological samples for each injury group. Statistical significance was calculated using the individual Ct values obtained from the three biological replicates for each injury group.

Table 19: MiRNA present only in the injury groups that do not demonstrate behavior changes.

MiRNA	P value	Ct Status	Fold Change (Log10)
mmu-miR-297a*	0.03	Calibrator not detected	1.55
mmu-miR-181c	0.02	Target not detected	-1.65

Two miRNAs, miR-297a* and miR-181c were found to be significantly modulated in the IS1 injury group, which did not demonstrate any significant neurobehavioral and cognitive changes as compared to the sham injury group. "Calibrator not detected" is the miRNA which is expressed only in the injured animals and not in the un-injured sham animals. "Target not detected" is the miRNA that was expressed only in the un-injured sham animals and not in the injured animals. In such case the Ct value for non-detected miRNA was taken as 40 followed by the fold change and statistical analysis.

3.1.8: Stable miRNA expression in serum

To identify miRNAs that can be used for validating the expression data of the miRNA array, miRNAs that exhibited stable expression in the serum, irrespective of the sham or injury group, were identified. 18 miRNAs were identified that showed stable Ct values across the sham and injury groups with SD <1 (Table 20). One of the miRNAs (miR-16) has been described as stable serum miRNA in other studies (Song et al., 2012). Therefore, miR-16 (Mean Ct =15.5460.91) along with another miRNAs, miR-1937b (Mean Ct =14.9160.90) were taken for use as endogenous controls in the miRNA expression validation experiments. Similar results were obtained with either of the selected stable miRNAs (data not shown) and data presented here used miR-16 as the stable endogenous miRNA. MiR-1937b is no longer considered a miRNA as it is a tRNA-derived small RNA fragment (tRF) (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0009938). Therefore, miR-1937b (like non-coding small nuclear RNA U6), presents a target that is of non-miRNA origin and may be useful as stable endogenous small RNA to validate miRNA expression in serum.

Table 20: Selection of the endogenous control miRNA.

S. No.	Detector	Mean Ct	Median Ct	SD
1	mmu-miR-1937b	14.91	14.81	0.90
2	mmu-miR-486	15.39	15.02	0.92
3	mmu-miR-16	15.54	15.52	0.91
4	mmu-miR-1937c	15.79	15.95	0.78
5	mmu-miR-1274a	15.93	15.64	0.93
6	mmu-miR-106a	17.93	17.94	0.85
7	mmu-miR-17	17.94	17.90	0.84
8	mmu-miR-93	19.73	19.70	0.89
9	mmu-miR-2146	20.44	20.38	0.99
10	hsa-miR-93#	21.03	20.95	0.87
11	mmu-miR-20b	22.59	22.58	0.94
12	hsa-miR-421	23.34	23.00	0.79
13	mmu-miR-18a#	25.04	24.82	0.81
14	mmu-miR-2182	25.64	25.64	0.97
15	mmu-miR-1930	25.92	25.84	0.81
16	rno-miR-148b-5p	27.19	26.98	1.00
17	mmu-miR-29b#	27.36	27.27	0.62
18	mmu-miR-467c	27.61	27.36	0.95

The most stable miRNA were selected based on their Ct values across the samples (n=30). Ct for each miRNA from all the samples, irrespective of the injury or control group, were taken and Ct values with a SD < 1 were selected. Mean Ct were calculated to determine the abundance of miRNAs in the serum samples. To ensure that there are no outliers, median Ct was also calculated. MiRNA that showed similar mean and median Ct values were then selected. MiRNA that show more abundance *i.e.,* Ct<25 were considered for determining a candidate stable endogenous miRNA in validation experiments.

3.2: Discussion

MTBI is a heterogeneous injury that can range from no clinical symptoms to development of post concussive syndrome (PCS) after injury. Rapp and Curly (Rapp and Curly, 2012) describe mTBI as an event that may lead to the development of neurological disorders. The heterogeneous nature of mTBI makes it difficult to diagnose, based exclusively on current behavioral and neurocognitive analyses (Rapp and Curly, 2012). Biochemical assays for brain related proteins in serum, such as GFAP, UCH-L1 and S100b, are either not sensitive or selective for mTBI (Vos etal., 2010, Thelin et al 2013, Papa et al 2012, Papa et al 2010, Brophy et al 2011). Therefore, a more reliable and sensitive molecular assay is needed to accurately diagnose mTBI. In this experiment, acute phase miRNA changes in the serum were measured to determine the feasibility of using miRNA as diagnostic markers of mTBI. Increasing grades of mTBI were induced by a weight drop device by increasing the height and weight of the falling rod. Injuries were characterized by the sensory motor responses designed to model clinical neurological examination and indicated the injuries to be within the mild spectrum of TBI (Grunberg et al 2011, Sharma et al 2012). Consistent with mTBI, histological analysis of the brain showed no gross pathological changes following the injury. Behavior impairments such as depression and loss of interest have been described in patients with mTBI (Bryan et al 2013). Reduced escape behavior, measured in terms of vertical activity of the animal in an open field apparatus, can be a measure of depression-related behavior (Katz et al 1981, Seligman 1967, Overmier and Seligman 1967). Transient, but significant depression-related behavior in terms of reduced vertical activity was observed in the injured animals at day 1 post injury, which recovered over time. A general negative impact on the overall health of the animals was also observed at day 1 post injury, indicated by the reduction in horizontal activity of the injured animals. Sensory gating impairments have also been reported in mTBI (Kumar et al 2005). Increase in the OFL activity of the animals at the later time points of day 14 and day 30 as compared to day 1 activity was interesting. We hypothesize that the naive and sham control animals remember being in the chambers and that the other animals, due to injury, may not remember the chamber as well and spend more time exploring. ASR is a complex behavior that engages higher brain functions and is related to information processing (Acri et al 1991, Swerdlow et al 1992, lee at al 1996, Koch et al 1997). A significantly reduced startle response was observed in animals with injury, such that the greater the injury severity, the less startle response was observed. The parietal lobe, where

the injury was centered, is involved in the sensorimotor information processing (Freund 2003). It is suggested that the right inferior parietal lobe plays a role in auditory signal processing and integration of sensory and motor functions (Acri et al 1991, Freund 2003, Leung and Alain 2011). This observation indicates the diffuse nature of the injury that may also affect central brain functions. Many individuals, who suffer with mTBI do not exhibit neurobehavioral alterations and acute symptoms of mTBI, such as headaches, sleep disturbance and depression or recover spontaneously from the injury (Pogoda et al 2012, Theeler et al 2012, Kiraly et al 2007). A similar observation was made in the current experiment, where the percent of animals' OFL activity in the mildest of mild injury group (i.e., IS1) was similar to sham and naive control groups. Other groups, with a more severe form of the mild injury, however, showed greater reduction in the percent activity than the sham and naive groups.

MiRNA changes in the serum have been suggested as a potential marker of disease and injury (Mayr et al 2013, Allegra et al 2012, Wahid et al 2010). MiRNA modulations in the serum of TBI patients have been reported. Down regulated expression of has-mir-16, and has-mir-92 and increased levels of has-mir-765 correlated with the severe TBI, however, their utility in diagnosing mTBI was limited (Redell et al 2010). Earlier we have also reported the modulation of let-7i in the serum and cerebrospinal fluid of rats following blast injury (Balakathiresan et al 2012). Modulation of miRNA expression in the brain following TBI has been shown before (Redell et al 2009, Lei et al 2009, Balakathiresan et al 2012, Truettner et al 2011). Some of the miRNAs (mir-21, mir-34a, mir-27b, mir-9, mir-874, mir-223, mir-144 and mir-153) that were reported to increase in the brain after moderate to severe TBI (Redell et al., 2011, Let et al 2009, Truettner et al 2011, Liu et al 2014, Sabhirzanov et al., 2014) did not show significant modulation in the serum in our experiment. Mir-497 and mir-149, which were up regulated in IS4 group, were earlier reported to also increase in the brain after TBI (Truettner et al 2011, Bao et al., 2014). Mir-451 and mir-340-5p, which were shown to be up regulated in the brain post TBI, were down regulated in our experiment (Redell et al 2010, Liu et al 2014). Despite the difference in the tissue being analyzed after the TBI, some common targets of modulated miRNAs were observed. Earlier studies found apoptosis as one of the cellular process targeted by the miRNAs modulated in the brain following TBI (Redell et al 2009, Redell et al 2011, Lei et al 2009). Apoptosis was predicted to be affected by the modulated miRNAs in this experiment

as well. BDNF, which was identified as a predicted target of one of the down regulated miRNA mir-106 in this experiment, was also identified as target of the modulated miRNAs following TBI in an earlier study (Redell et al 2009). Two main observations were made in the current experiment. First, the number of miRNAs that were significantly modulated post injury increased with the severity of the injury. Second, thirteen common miRNAs were significantly modulated in all the four injury groups compared to the sham controls. Functional analysis to identify the combined effect of modulated miRNAs showed that eight of the thirteen miRNAs may play a role in CNS related pathways, such as synaptic depression, sensorimotor impairments and the axon guidance pathway (Figure 5). Vimentin (VIM) and phosphatase and tensin homolog (PTEN), targets of the significantly down regulated miRNA mir-106b, expression has been shown to increase in the brain post TBI and has been related to inflammatory cell proliferation and differentiation and neuronal survival and neurite integrity, respectively (Moon et al 2004, Haausmann et al 2001, Wang et al 2013, Goh et al., 2014). Brain-derived neurotrophic factor (BDNF) and Amyloid precursor protein (APP), also targets of mir-106b, have been shown to increase post TBI (Yang et al 1996, van den Heuvel 1998). BDNF has been reported to have a neuroprotective effect post TBI (Ysng et al 1996, Griescbach et al 2002). The role of APP is, however, debated as some studies have shown APP association with neuronal loss and others have shown APP as neuroprotective (Itoh et al 2009, Murakami et al 2008, Corrigan et al., 2014). Axon guidance includes axon outgrowth, repulsion, and attraction, which plays an important role in neuronal functions and axonal regeneration. Axonal injury is prevalent in TBI (Hanell et al 2012). Axon guidance and synaptic plasticity is affected post TBI and positive regulation of axon guidance has been suggested to result in better functional outcomes (Schirmer et al 2013). Elevated levels of axon related proteins, such as neurofilament heavy chain, Tau, S100B and myelin basic protein in the serum, have also been suggested as potential biomarkers of mTBI (Rostami et al., 2012). Two of the significantly up regulated miRNAs that were validated using the singleplex miRNA assay, have been shown to play a role in neuronal differentiation and CNS development. Normal miR- 376a expression has been shown to be involved in the early fetal brain development, whereas accumulation of unedited miR-376a has been linked to neurodevelopmental disorders and increased metastasis potential of gliomas (Goossens et al 2013). MiR-214 has also been shown to enhance neurite outgrowth and its expression is up regulated during the mouse cortical neuron, embryonic stem cells development,

and at the early developmental stages in embryonic retina (Decembrini et al., 2009). However, miR-214 levels have been shown to decrease in the neurons of the dorsal root ganglion after an injury to the sciatic nerve (Zhang et al., 2011). MiR-376a was also up regulated in the serum of rats exposed to blast induced moderate TBI (Balakathiresan et al., 2012). Down regulated mir-106b and up regulated miR-376a and miR-214 along with other modulated miRNAs at the acute stage of injury, may reflect the metabolic active state of neural tissue engaged in the immediate response to injury that involve neuroprotection, synaptic plasticity, axonal damage/regeneration, and neurogenesis. Endogenous neurogenesis and neuronal maturation have been proposed as keys to recovery and as therapeutic targets after TBI.

TBI is often followed by the increase in endogenous neuronal progenitor cells proliferation and migration, neuronal differentiation, and neurogenesis (Rice et al 2003, Zhang et al 2013, Itoh et al., 2005). Proliferating neuroprogenitor cells differentiate into immature neurons post TBI but fail to develop further into the mature neurons (Gao et al., 2013). A small subset of newborn granular neurons, however, has been observed in the brain after TBI, though the formation of these neurons is not significantly enhanced (Bye et al., 2011). Dash and group also reported development of mature neurons in the dentate gyrus of the hippocampus one month after the TBI (Dash et al., 2001). To determine if bioinformatics analysis targeting the axon guidance pathway may be exploited for predicting the severity of the injury, significantly modulated miRNA in each injury groups were analyzed by IPA for their predicted involvement in modulating axon guidance pathway. The number of miRNAs that are predicted to modulate axon guidance pathway related brain functions increased with the severity of the injury, suggesting an enhanced effect of injury on molecular functions associated with the axon guidance and neurogenesis (Figure 5). Long-term studies are needed to evaluate development of chronic pathologies such as axon damage, synapse and blood brain barrier functions in the brain following injury in this model of mTBI. However, the quantitative bioinformatics analysis of the modulated miRNAs relating to their potential CNS involvement can be helpful to identify the severity of the injury at early time points after the injury.

3.3: Conclusion

In conclusion, a cohort of 13 serum miRNAs identified in this study, may be used as acute biomarkers of mTBI regardless of any neurobehavioral or cognitive alterations following the injury. Future studies will be needed to validate this cohort of 13 miRNAs as acute biomarkers of mTBI in the other animal models of injury and in the human mTBI. In addition, further behavioral studies will be needed to identify the long-term cognitive deficits and/or sleep disorders in the mTBI model described in this study. Such long-term studies will also be able to determine the prognostic value of the miRNAs identified in this study as acute biomarkers of mTBI.

Chapter 4: Temporal Differences in MiRNA Expression Pattern in Brain post Mild CHI and in Brain/Serum post Mild CCI Injury

4.1: Results

4.1.1: Absence of visible brain tissue damage post mild closed head injury in mice

Weight drop model is one of the standard animal models of traumatic brain injury used to study the pathophysiology as well as the post-injury complications like behavioral deficits and brain inflammation. After inducing the injury, the brains were examined by H & E staining which showed an absence of any visible tissue lesions at the injury site in the brain among all the four injury groups and in both the two different post injury time points – 24 hr and Day 7 suggesting that all the four different injury severities (246 g/2 cm, 246 g/3 cm, 333 g/2 cm and 333 g/3 cm) might fall under the mild injury category (Figure 24).

Previously we have shown in chapter 3 that this weight drop model of traumatic brain injury induces transient behavioral deficits indicated by an increase in the neurological severity scale-revised (NSS-R) and also a significant reduction in the various parameters like vertical activity and horizontal activity as part of the open field locomotion activity, acoustic startle response at 24 hr post injury (Sharma et al., 2014). The absence of gross pathological changes as seen in histological examination along with the transient behavioral deficits shows that indeed the injury might be similar to a mild closed head injury as seen in human cases. In order to understand the miRNA(s) alterations post injury in the brain tissues, we selected both the early time point of 24 hr and the delayed time point of day 7.

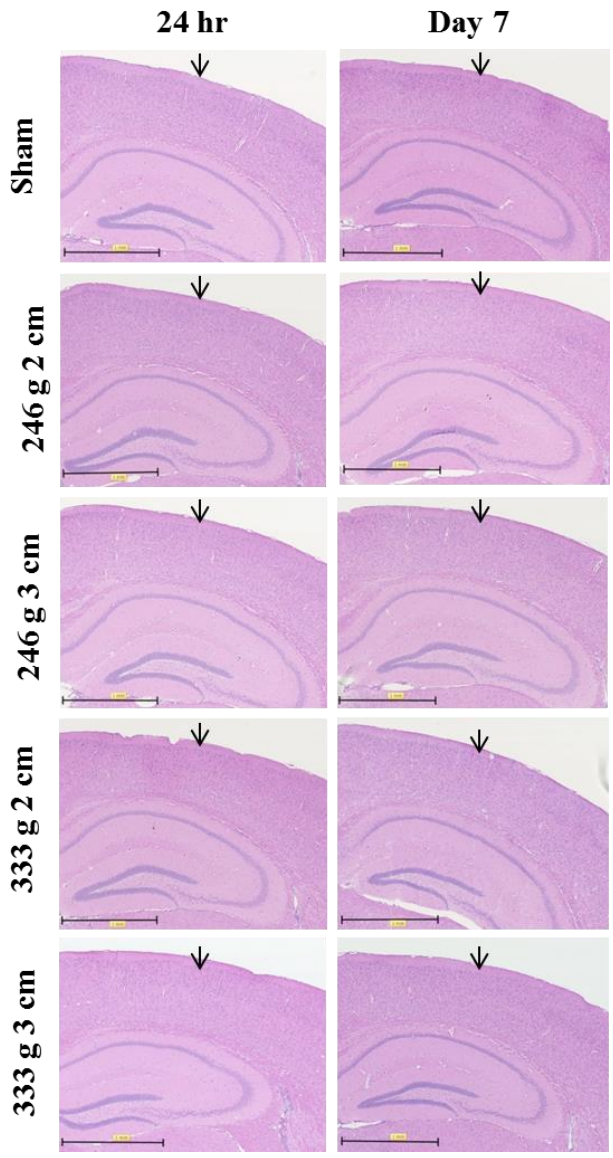

Figure 24: Absence of tissue damage in mild closed head injury. H & E staining of coronal brain sections at both the post injury time points for all the four injury groups indicating an absence of tissue lesions at the site of injury indicated by the black arrow. Each of the image is representative of a collection of n=4 for each of the injury groups as well as sham groups.

4.1.2: Clustering analysis and miRNA distribution graph shows difference in miRNA expression between early and delayed time point post injury post mild CHI

Animals (n=3/group) were subjected to CHI with two different fall heights of 2 cm and 3 cm with two different rods of 246 g and 333 g weight thus recreating a mild head injury with four different grades of injury severity. The animals were sacrificed at 24 hr and day 7 following CHI. Total RNA was extracted from the brain tissue underlying the injury site followed by miRNA expression profiling using the TLDA platform.

Clustering analysis performed on the ddCt values of the miRNA expression data shows a difference among the grouping between the four injury groups at both 24 hr and day 7 time point. In the 24 hr time point, both the 246 g/2 cm and 246 g/3 cm is clustering together and the other two injury groups – 333 g/2 cm and 333 g/3 cm get grouped separately. In day 7 time point, the 246 g/2 cm and 333 g/2 cm come closer together while there is clustering of the 246 g/3 cm and 333 g/3 cm injury groups indicating a clear difference in miRNA expression due to difference in post injury time point (Figure 25). Based on the criteria of adj. p value < 0.05 and fold change > 1.5, the number of significantly modulated miRNAs compared to the controls (including 'calibrator not detected' and 'target not detected') were calculated and this showed an increase in the total number of miRNAs at 24 hr post injury compared to the day 7 time point in all the injury groups except that of 333 g/ 3 cm injury group (Figure 26). Within the 24 hr time point, the 246 g/2 cm was found to have the highest number of miRNAs compared to the other three groups and in case of the delayed time point of day 7, the 333 g/3 cm was found to have the highest expression of miRNAs among the injury groups.

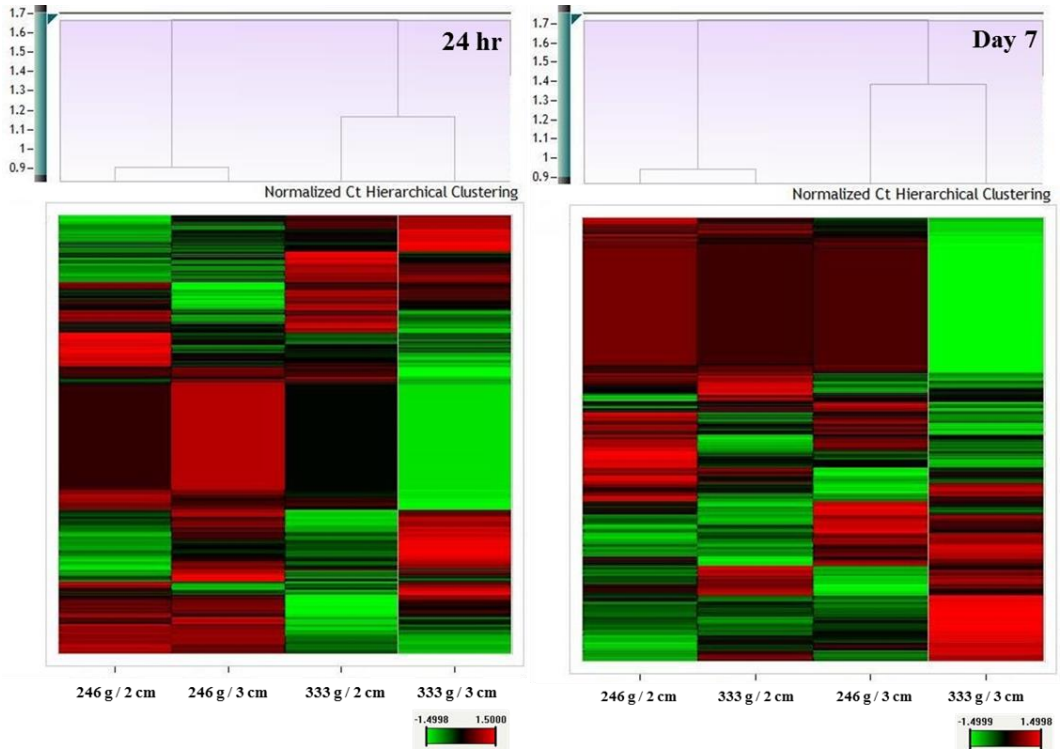

Figure 25: Hierarchical clustering of miRNAs shows the difference in the miRNA expression between the 24 hr and day 7 time point. The clustering is done using complete linkage method together with Pearson correlation measure based on the ddCt values normalized using z-score normalization method. In both the heat maps, each row represents an individual miRNA and each of the columns representing a single injury group (n=3) normalized to the sham control group (n=3). The pattern of the group wise clustering is found to be different in both the time points. In the 24 hr time point, both the 246 g/2 cm and 246 g/3 cm is clustering together and the other two injury groups – 333 g/2 cm and 333 g/3 cm get grouped together. While the day 7 time point shows a entirely different pattern with both the 246 g/2 cm and 333 g/2 cm coming together along with clustering of the 246 g/3 cm and 333 g/3 cm injury groups indicating a clear difference in miRNA expression due to difference in post injury time point.

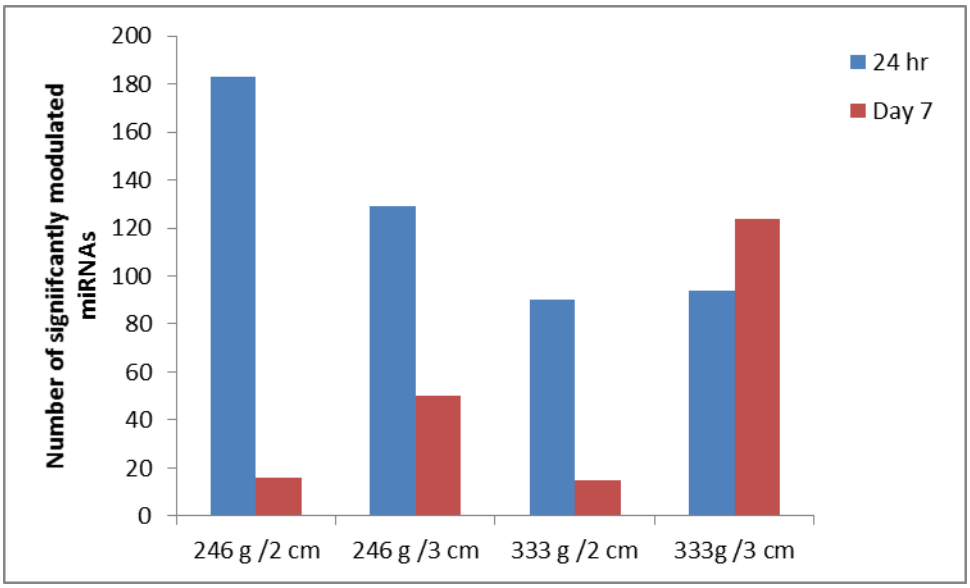

Figure 26: Difference in miRNA expression based on time point and injury severity. The number of significantly modulated brain microRNAs (including 'calibrator not detected' and 'target not detected' candidates) compared to the sham control group is shown. The miRNA expression graph shows a general trend of higher expression at day 7 compared to the 24 hr timepoint except for the 333 g/3 cm injury group.

4.1.3: Venn diagrams of upregulated and downregulated miRNAs

Venn diagrams were drawn to show the number of upregulated and downregulated miRNAs distributed among the four different injury groups. The distribution of the both the upregulated and downregulated miRNAs was also shown in the form of a bar graph (Figure 27A)

Upregulated miRNAs in both the early time point (24 hr) and the delayed time point (day 7) among the four different injury groups were shown in Figure 27B. In the early 24 hr timepoint, 88 miRNAs were upregulated in 246 g/2 cm injury group, 46 in 246 g/3 cm, 28 in the 333 g/2 cm group and about 33 miRNA in the 333 g/3 cm group. Five of the miRNAs - mmu-miR-296-5p, hsa-miR-378, mmu-miR-485-3p, hsa-miR-154* and hsa-miR-149 were upregulated in all the four injury groups in the 24 hr time point. In the case of the delayed time point (day 7), 4 miRNAs were upregulated in 246 g/2 cm injury group, 16 in 246 g/3 cm, 4 in the 333 g/2 cm group and about 60 miRNA in the 333 g/3 cm group. None of the upregulated miRNAs were commonly expressed in all the four injury groups in this time point.

A similar venn diagram was constructed to show the distribution for the downregulated miRNAs in the different groups time point wise. The Venn diagram in Figure 27C shows that the number of downregulated miRNAs in all the four groups is lower in the day 7 time point compared to the 24 hr time point. In the early 24 hr timepoint, 83 miRNAs were downregulated in 246 g/2 cm injury group, 58 in 246 g/3 cm, 45 in the 333 g/2 cm group and about 43 miRNA in the 333 g/3 cm group. Among the four different combinations of venn diagrams, the largest overlap was observed for the downregulated miRNAs in the 24 hr time point with about 16 miRNAs - mmu-miR-704, mmu-miR-222, mmu-miR-150, mmu-miR-1306, mmu-miR-449a, mmu-miR-493, mmu-miR-191, mmu-miR-484, mmu-miR-138*, mmu-miR-186, mmu-miR-503*, mmu-miR-155, mmu-miR-1932, mmu-miR-1948, mmu-miR-125b* and mmu-miR-872 found to be common between the four injury groups.

In the case of the delayed time point (day 7), 5 miRNAs were upregulated in 246 g/2 cm injury group, 25 in 246 g/3 cm, 4 in the 333 g/2 cm group and about 45 miRNA in the 333 g/3 cm group. Two miRNAs - mmu-miR-18a and mmu-miR-369-3p were commonly downregulated in all the four injury groups in this time point. The list of significantly modulated miRNAs for both the time points and all the four injury groups has been shown in the Tables 21-28.

Based on the injury severity, the miRNA expression was compared between the four injury groups (Figure 4A). Interestingly, the least severe of all the four injury groups - 246 g/2 cm indicates a high number of modulated miRNAs in 24 hr compared to the other three injury groups but this is found to drastically change at the day 7 time point. In case of the 333 g/3 cm (the more severe of the four injury groups), the number of modulated miRNAs increases from the 24 hr time point to the day 7 time point and has the highest number of miRNAs among all the four injury groups at the delayed day 7 time point.

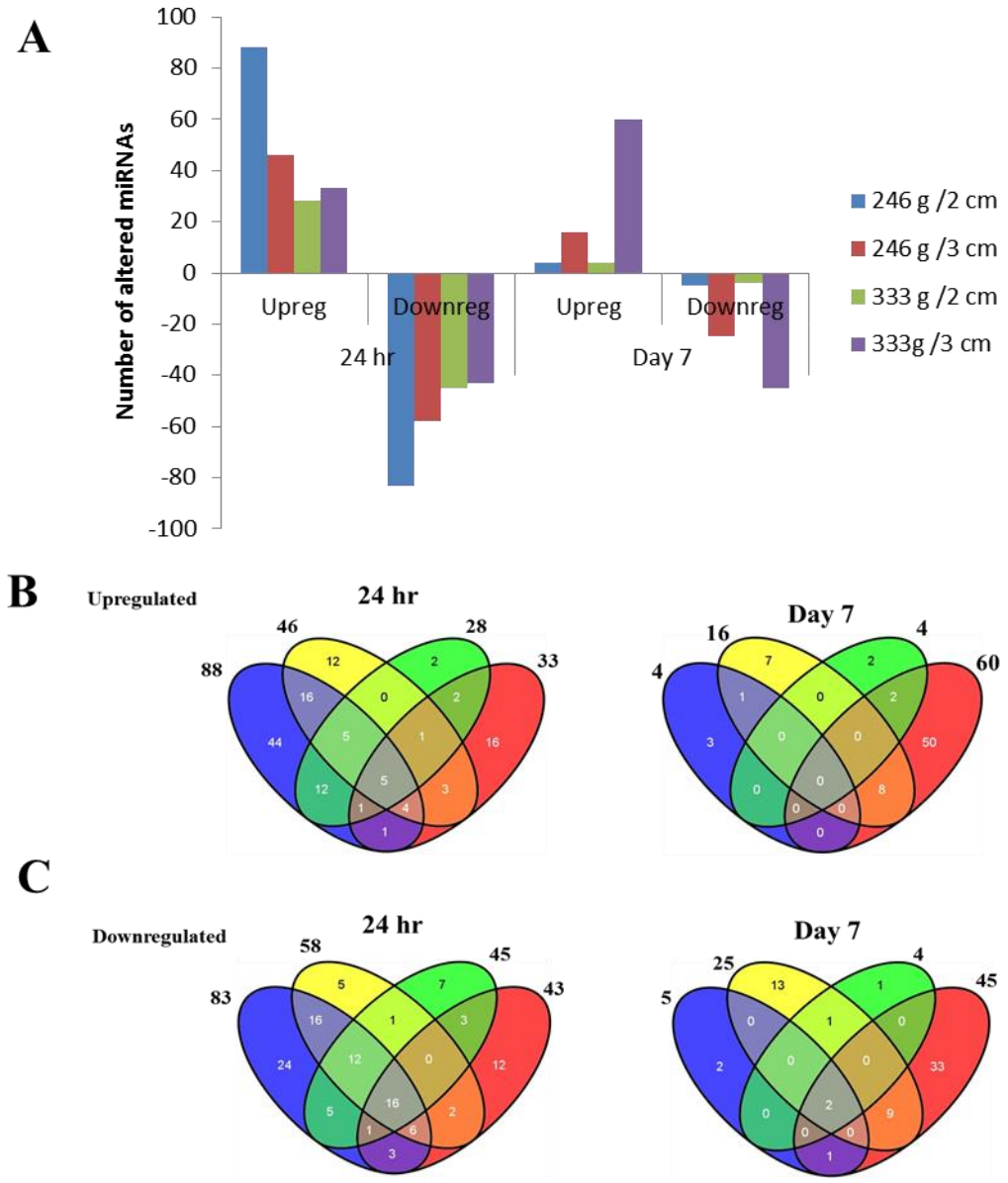

Figure 27: MiRNA expression plot and Venn diagrams showing distribution of upregulated and downregulated miRNAs among the time points and injury groups. a. MiRNA distribution graph, b. Upregulated miRNAs, c. Downregulated miRNAs

Table 21: Significantly modulated microRNAs in 246 g/2 cm injury group at 24 hr post injury.

S.No	MicroRNA	adj. P Value	Fold Change (Log10RQ)
246 g_2 cm _24 hr			
Valid			
1	mmu-miR-369-3p	3.54E-02	1.74
2	mmu-miR-504	5.72E-03	1.34
3	rno-miR-224	1.12E-02	1.03
4	mmu-miR-296-5p	1.28E-03	1.00
5	mmu-miR-455	1.23E-02	0.80
6	hsa-miR-378	9.84E-03	0.79
7	mmu-miR-339-5p	1.05E-02	0.79
8	mmu-miR-224	2.10E-03	0.77
9	mmu-miR-221	2.10E-03	0.76
10	mmu-miR-328	2.10E-03	0.76
11	mmu-miR-18a	3.81E-02	0.76
12	mmu-miR-292-3p	2.10E-03	0.73
13	mmu-miR-369-5p	1.79E-02	0.70
14	hsa-miR-106b*	4.17E-02	0.66
15	mmu-miR-135a	7.70E-03	0.63
16	mmu-miR-127	2.10E-03	0.62
17	mmu-miR-7a	4.17E-02	0.59
18	mmu-miR-20a	8.27E-03	0.53
19	mmu-miR-362-3p	2.10E-03	0.51
20	mmu-miR-598	2.10E-03	0.51
21	mmu-miR-154	7.32E-03	0.51
22	mmu-miR-485-3p	3.86E-03	0.51
23	mmu-miR-324-5p	2.34E-03	0.50
24	hsa-miR-671-5p	2.95E-02	0.50
25	mmu-miR-190	2.97E-02	0.48
26	mmu-miR-671-3p	2.10E-03	0.48
27	mmu-miR-345	3.94E-02	0.48
28	mmu-miR-540-5p	2.10E-03	0.47
29	mmu-miR-18a*	1.84E-02	0.46
30	mmu-miR-28	2.10E-03	0.46
31	mmu-let-7a	8.69E-03	0.45

32	mmu-miR-668	1.28E-02	0.45
33	mmu-miR-30b	3.36E-03	0.44
34	mmu-miR-34c	5.32E-03	0.44
35	mmu-miR-216b	2.05E-02	0.43
36	mmu-miR-148a	2.10E-03	0.43
37	mmu-miR-423-5p	2.15E-03	0.43
38	hsa-miR-154*	4.34E-02	0.43
39	hsa-miR-206	3.28E-02	0.43
40	mmu-miR-298	2.10E-03	0.42
41	mmu-miR-30c	4.50E-03	0.41
42	mmu-miR-665	4.06E-02	0.40
43	mmu-miR-300	2.10E-03	0.40
44	mmu-miR-351	1.71E-02	0.40
45	mmu-miR-26b	4.02E-03	0.40
46	mmu-miR-376a*	4.69E-02	0.40
47	mmu-miR-19a	6.29E-03	0.39
48	mmu-miR-27a	8.85E-03	0.39
49	mmu-miR-335-5p	2.80E-02	0.38
50	mmu-miR-25	1.01E-02	0.38
51	mmu-miR-879*	4.74E-02	0.37
52	mmu-miR-375	1.30E-02	0.37
53	mmu-miR-744	3.17E-03	0.37
54	hsa-miR-149	2.72E-02	0.36
55	mmu-miR-582-5p	7.88E-03	0.36
56	mmu-miR-19b	3.44E-02	0.36
57	mmu-miR-99b	1.62E-02	0.35
58	mmu-let-7c	1.33E-02	0.35
59	mmu-miR-125b-5p	1.27E-02	0.35
60	mmu-miR-551b	1.01E-02	0.34
61	mmu-miR-448	1.15E-02	0.33
62	mmu-miR-142-3p	2.06E-02	0.32
63	mmu-miR-9	1.71E-02	0.32
64	rno-miR-758	1.14E-02	0.31
65	mmu-miR-106b	4.76E-03	0.31
66	mmu-miR-652	3.20E-02	0.31
67	mmu-miR-501-3p	4.45E-02	0.30
68	mmu-miR-543	1.65E-02	0.29
69	mmu-miR-488	3.20E-02	0.29
70	mmu-miR-497	8.54E-03	0.28
71	rno-miR-219-2-3p	8.21E-03	0.28
72	mmu-miR-325	8.96E-03	0.28

73	mmu-miR-376c	1.67E-02	0.28
74	mmu-miR-301a	1.34E-02	0.27
75	mmu-miR-130a	3.34E-02	0.27
76	mmu-miR-26a	4.53E-02	0.27
77	mmu-miR-30d	2.25E-02	0.26
78	mmu-let-7b	1.54E-02	0.26
79	mmu-miR-27b	3.71E-02	0.25
80	mmu-miR-361	3.21E-02	0.25
81	mmu-miR-340-3p	4.52E-02	0.24
82	mmu-miR-152	1.76E-02	0.23
83	mmu-miR-30a	1.89E-02	0.23
84	mmu-miR-500	2.88E-02	0.21
85	mmu-miR-451	2.41E-02	0.19
86	mmu-miR-331-3p	4.18E-02	0.18
87	mmu-miR-434-3p	4.91E-02	0.18
88	mmu-miR-491	4.69E-02	0.17
89	hsa-miR-30e-3p	4.65E-02	-0.17
90	mmu-miR-34b-3p	4.71E-02	-0.18
91	mmu-miR-542-5p	4.06E-02	-0.18
92	mmu-miR-192	4.69E-02	-0.18
93	rno-miR-7*	4.01E-02	-0.19
94	mmu-miR-28*	2.88E-02	-0.21
95	hsa-miR-30a-3p	4.54E-02	-0.22
96	mmu-miR-370	4.72E-02	-0.23
97	mmu-miR-194	4.62E-02	-0.24
98	rno-miR-339-3p	1.80E-02	-0.24
99	hsa-miR-93*	3.97E-02	-0.24
100	rno-miR-7a*	3.47E-02	-0.25
101	mmu-miR-297a*	2.25E-02	-0.25
102	hsa-let-7i*	4.63E-02	-0.26
103	mmu-miR-322*	3.24E-02	-0.26
104	mmu-miR-431	3.15E-02	-0.27
105	mmu-miR-187	2.46E-02	-0.27
106	rno-miR-381	1.22E-02	-0.29
107	mmu-miR-669m-3p	2.00E-02	-0.29
108	hsa-miR-190b	3.11E-02	-0.30
109	mmu-miR-494	4.16E-02	-0.30
110	mmu-miR-503	2.02E-02	-0.33
111	mmu-miR-301b	1.43E-02	-0.33
112	mmu-miR-409-3p	6.95E-03	-0.33
113	mmu-miR-215	3.08E-02	-0.34

114	mmu-miR-151-3p	9.84E-03	-0.34
115	mmu-miR-16	2.99E-02	-0.37
116	mmu-miR-676	4.42E-03	-0.37
117	rno-miR-146B	1.01E-02	-0.38
118	mmu-miR-704	8.63E-03	-0.40
119	hsa-miR-183*	3.60E-02	-0.42
120	mmu-miR-541	4.72E-03	-0.44
121	mmu-miR-376a	7.86E-03	-0.45
122	rno-miR-344-5p	1.65E-02	-0.45
123	mmu-miR-331-5p	1.30E-02	-0.45
124	mmu-miR-669H-5P	1.11E-02	-0.45
125	mmu-miR-770-3p	2.10E-03	-0.46
126	mmu-miR-433	2.10E-03	-0.47
127	mmu-miR-134	1.23E-02	-0.48
128	mmu-miR-450a-5p	1.65E-02	-0.49
129	mmu-miR-222	2.56E-03	-0.49
130	mmu-miR-125a-3p	2.56E-03	-0.51
131	mmu-miR-320	2.10E-03	-0.51
132	hsa-miR-412	2.31E-03	-0.52
133	hsa-miR-421	1.27E-02	-0.55
134	mmu-miR-150	4.09E-03	-0.56
135	mmu-miR-1306	2.10E-03	-0.57
136	mmu-miR-666-5p	1.20E-02	-0.57
137	mmu-miR-133a	5.27E-03	-0.58
138	mmu-miR-223	8.22E-03	-0.61
139	mmu-miR-449a	2.10E-03	-0.63
140	rno-miR-598-5p	2.10E-03	-0.64
141	mmu-miR-872*	2.10E-03	-0.64
142	mmu-miR-802	4.18E-02	-0.65
143	mmu-miR-429	2.10E-03	-0.65
144	mmu-miR-146a	2.10E-03	-0.65
145	mmu-miR-493	5.71E-03	-0.66
146	hsa-miR-140-3p	2.10E-03	-0.67
147	hsa-miR-338-5P	2.10E-03	-0.68
148	mmu-miR-212	4.50E-03	-0.68
149	mmu-miR-191	3.97E-03	-0.68
150	rno-miR-125b*	3.92E-03	-0.70
151	mmu-miR-877*	2.10E-03	-0.71
152	mmu-miR-574-3p	1.05E-02	-0.72
153	mmu-miR-146b	2.10E-03	-0.73
154	mmu-miR-484	1.69E-03	-0.77

155	mmu-miR-138*	2.10E-03	-0.78
156	mmu-miR-186	2.10E-03	-0.79
157	mmu-miR-503*	2.10E-03	-0.85
158	mmu-miR-342-3p	1.19E-03	-0.85
159	mmu-miR-383	2.10E-03	-0.86
160	hsa-miR-425	2.10E-03	-0.86
161	mmu-miR-155	1.19E-03	-0.90
162	mmu-miR-339-3p	2.10E-03	-0.90
163	mmu-miR-667	2.10E-03	-0.94
164	mmu-miR-1932	2.10E-03	-1.00
165	mmu-miR-1948	2.10E-03	-1.02
166	mmu-miR-125b*	2.10E-03	-1.02
167	hsa-miR-200b	3.79E-03	-1.04
168	mmu-miR-1894-3p	1.33E-02	-1.12
169	mmu-miR-34c*	2.10E-03	-1.19
170	hsa-miR-99b*	2.10E-03	-1.27
171	mmu-miR-872	2.10E-03	-1.69
Calibrator not detected			
172	mmu-miR-702	6.25E-05	5.13
173	mmu-miR-874	7.22E-06	4.88
174	mmu-miR-466J	1.52E-04	4.33
175	hsa-miR-30d*	9.46E-05	4.32
176	hsa-miR-431*	1.79E-04	3.95
177	hsa-miR-299-5p	1.06E-04	3.47
178	mmu-miR-488*	1.79E-04	3.33
179	mmu-miR-669o-5p	2.32E-04	2.62
Target not detected			
180	mmu-miR-196b	1.52E-04	-3.73
181	rno-miR-207	4.92E-02	-4.83
182	mmu-miR-680	7.22E-06	-5.64
183	mmu-miR-465a-5p	2.21E-07	-7.34

Table 22: Significantly modulated microRNAs in 246 g/3 cm injury group at 24 hr post injury.

S.No	MicroRNA	adj. P Value	Fold Change (Log10 RQ)
246 g_3 cm _24 hr			
Valid			
1	mmu-miR-1894-3p	8.81E-03	1.38
2	mmu-miR-504	4.05E-03	1.20
3	mmu-miR-217	3.13E-02	1.01
4	rno-miR-224	1.77E-02	0.96
5	mmu-miR-296-5p	2.17E-03	0.86
6	hsa-miR-378	1.59E-03	0.81
7	hsa-miR-106b*	2.37E-02	0.78
8	mmu-miR-455	1.61E-02	0.75
9	mmu-miR-224	1.59E-03	0.74
10	mmu-miR-328	5.48E-03	0.73
11	mmu-miR-339-5p	2.44E-02	0.72
12	mmu-miR-369-5p	2.29E-02	0.67
13	hsa-miR-200c	1.56E-02	0.64
14	mmu-miR-1982-3p	8.66E-03	0.64
15	mmu-miR-376a*	1.63E-02	0.60
16	hsa-miR-206	1.46E-02	0.59
17	mmu-miR-669C	3.55E-02	0.58
18	mmu-miR-18a*	1.57E-02	0.58
19	mmu-miR-485-3p	4.23E-03	0.56
20	mmu-miR-292-3p	7.06E-03	0.53
21	mmu-miR-423-5p	1.28E-02	0.53
22	mmu-miR-345	2.77E-03	0.53
23	mmu-miR-351	1.27E-02	0.51
24	hsa-miR-671-5p	9.85E-03	0.50
25	mmu-miR-221	2.76E-02	0.49
26	mmu-miR-193*	4.34E-03	0.48
27	mmu-miR-127	1.43E-02	0.48
28	mmu-miR-669n	4.25E-02	0.47
29	mmu-miR-326	1.06E-02	0.47
30	mmu-miR-1839-5p	1.15E-02	0.44
31	hsa-miR-149	9.85E-03	0.43
32	hsa-miR-22	1.52E-02	0.42

33	mmu-miR-375	1.72E-02	0.41
34	mmu-miR-184	1.52E-02	0.41
35	mmu-miR-671-3p	1.34E-02	0.40
36	hsa-miR-154*	2.55E-02	0.38
37	mmu-miR-24-2*	3.41E-02	0.37
38	rno-miR-148b-5p	7.72E-03	0.37
39	mmu-miR-879*	4.45E-02	0.37
40	mmu-miR-598	2.55E-02	0.35
41	hsa-miR-143	3.00E-02	0.34
42	hsa-miR-338	2.48E-02	0.33
43	mmu-miR-540-5p	2.76E-02	0.32
44	hsa-miR-29a*	1.70E-02	0.31
45	mmu-miR-448	1.85E-02	0.30
46	mmu-miR-300	2.55E-02	0.28
47	mmu-miR-467b	2.87E-02	-0.23
48	mmu-miR-34b-3p	2.29E-02	-0.25
49	rno-miR-381	1.49E-02	-0.27
50	mmu-miR-384-5p	2.70E-02	-0.28
51	mmu-miR-409-3p	1.55E-02	-0.30
52	mmu-miR-200b	2.29E-02	-0.31
53	mmu-miR-106a	1.59E-02	-0.32
54	mmu-miR-1306	4.01E-02	-0.33
55	mmu-miR-151-3p	1.05E-02	-0.33
56	mmu-miR-376a	1.34E-02	-0.34
57	mmu-miR-338-3p	2.07E-02	-0.35
58	rno-miR-339-3p	1.34E-02	-0.36
59	mmu-miR-547	1.72E-02	-0.36
60	mmu-miR-433	6.72E-03	-0.37
61	mmu-miR-153	1.73E-02	-0.37
62	mmu-miR-676	1.84E-02	-0.40
63	mmu-miR-125a-3p	3.60E-03	-0.41
64	mmu-miR-301b	1.49E-02	-0.41
65	mmu-miR-138*	4.47E-02	-0.44
66	mmu-miR-320	3.75E-03	-0.46
67	hsa-miR-338-5P	3.22E-02	-0.46
68	mmu-miR-134	2.28E-02	-0.47
69	mmu-miR-16	1.13E-02	-0.48
70	mmu-miR-704	5.41E-03	-0.48
71	mmu-miR-877*	6.40E-03	-0.50
72	hsa-miR-421	1.35E-02	-0.51
73	mmu-miR-331-5p	2.29E-02	-0.53

74	mmu-miR-150	6.70E-03	-0.54
75	mmu-miR-449a	2.48E-02	-0.54
76	mmu-miR-503	9.90E-03	-0.55
77	mmu-miR-223	2.16E-03	-0.56
78	mmu-miR-222	5.91E-03	-0.56
79	hsa-miR-140-3p	5.38E-03	-0.58
80	mmu-miR-146a	6.77E-03	-0.59
81	mmu-miR-1932	9.51E-03	-0.60
82	rno-miR-598-5p	8.22E-03	-0.63
83	mmu-miR-212	1.66E-02	-0.64
84	mmu-miR-450a-5p	6.47E-03	-0.64
85	mmu-miR-133a	5.69E-03	-0.65
86	mmu-miR-125b*	1.47E-02	-0.67
87	mmu-miR-429	2.70E-03	-0.69
88	mmu-miR-802	1.17E-02	-0.71
89	mmu-miR-493	9.87E-03	-0.73
90	mmu-miR-342-3p	6.11E-03	-0.74
91	mmu-miR-146b	6.05E-03	-0.75
92	mmu-miR-186	5.69E-03	-0.76
93	mmu-miR-484	6.73E-03	-0.77
94	mmu-miR-503*	3.99E-03	-0.77
95	mmu-miR-191	2.14E-03	-0.78
96	mmu-miR-667	1.59E-03	-0.81
97	mmu-miR-383	6.75E-03	-0.84
98	hsa-miR-200b	4.76E-03	-0.89
99	mmu-miR-339-3p	6.16E-03	-0.93
100	mmu-miR-155	1.59E-03	-0.93
101	mmu-miR-1948	1.59E-03	-0.96
102	hsa-miR-200a*	8.65E-03	-0.98
103	mmu-miR-34c*	4.72E-03	-1.02
104	mmu-miR-872	1.59E-03	-1.78
Calibrator not detected			
105	hsa-miR-485-5p	1.26E-06	6.01
106	mmu-miR-702	1.70E-06	5.36
107	mmu-miR-874	6.80E-06	4.86
108	hsa-miR-124*	1.40E-05	4.86
109	hsa-miR-30d*	6.17E-06	4.54
110	mmu-miR-466J	5.85E-06	4.33
111	hsa-miR-431*	9.18E-06	4.24
112	mmu-miR-488*	2.76E-05	3.50
113	hsa-miR-299-5p	1.22E-04	3.46

114	mmu-miR-669l-5p	7.13E-05	2.99
115	mmu-miR-669o-5p	2.73E-05	2.72
116	mmu-miR-96	1.59E-04	2.20
117	mmu-miR-465a-3p	1.59E-03	2.05
118	mmu-miR-193	4.71E-02	1.83
119	mmu-miR-1941-5p	4.60E-02	1.73
120	mmu-miR-1953	3.48E-03	1.65
121	mmu-miR-878-3p	6.23E-04	1.39
122	rno-miR-653	1.99E-03	1.29
123	mmu-miR-293	4.45E-02	1.12
124	hsa-miR-653	2.42E-03	1.05
Target not detected			
125	mmu-miR-680	1.62E-02	-4.50
126	mmu-miR-2183	4.65E-07	-5.58
127	rno-miR-207	4.41E-06	-6.72
128	mmu-miR-465a-5p	2.43E-07	-7.42
129	mmu-miR-196b	7.13E-05	-3.82

Table 23: Significantly modulated microRNAs in 333 g/2 cm injury group at 24 hr post injury.

S.No	MicroRNA	adj. P Value	Fold Change (Log10 RQ)
333 g_2 cm _24 hr			
Valid			
1	mmu-miR-296-5p	1.57E-02	0.76
2	mmu-miR-292-3p	5.68E-03	0.72
3	hsa-miR-378	6.18E-03	0.55
4	hsa-miR-200c	7.47E-03	0.51
5	mmu-miR-216b	2.23E-02	0.48
6	mmu-miR-582-5p	1.58E-02	0.48
7	mmu-miR-190	4.07E-02	0.47
8	mmu-miR-331-3p	5.68E-03	0.47
9	mmu-miR-485-3p	9.50E-03	0.47
10	hsa-miR-149	4.53E-02	0.44
11	hsa-let-7i*	6.35E-03	0.43
12	mmu-miR-127	4.00E-02	0.42
13	mmu-miR-328	4.66E-02	0.42
14	mmu-miR-30b	2.49E-02	0.40
15	mmu-miR-154	3.47E-02	0.40
16	mmu-miR-671-3p	1.33E-02	0.40
17	hsa-miR-154*	4.57E-02	0.38
18	mmu-miR-130a	3.09E-02	0.37
19	rno-miR-758	2.70E-02	0.37
20	mmu-miR-491	1.99E-02	0.36
21	rno-miR-551B	4.06E-02	0.34
22	mmu-miR-224	2.57E-02	0.34
23	mmu-miR-551b	4.26E-02	0.34
24	mmu-miR-362-3p	3.19E-02	0.33
25	mmu-miR-30c	4.00E-02	0.31
26	mmu-miR-434-3p	2.68E-02	0.31
27	mmu-miR-465a-5p	4.00E-02	0.27
28	mmu-miR-140	3.51E-02	0.24
29	mmu-miR-409-3p	2.94E-02	-0.24
30	mmu-miR-146a	4.57E-02	-0.28
31	mmu-miR-1930-5p	2.86E-02	-0.29

32	mmu-miR-187	1.88E-02	-0.32
33	mmu-miR-412	3.79E-02	-0.32
34	hsa-miR-412	4.01E-02	-0.34
35	mmu-miR-673-3p	2.14E-02	-0.37
36	mmu-miR-222	1.61E-02	-0.38
37	mmu-miR-28*	2.67E-02	-0.38
38	mmu-miR-133a	4.00E-02	-0.41
39	mmu-miR-668	3.65E-02	-0.42
40	hsa-miR-22	3.01E-02	-0.42
41	mmu-miR-150	2.06E-02	-0.42
42	hsa-miR-338-5P	2.49E-02	-0.44
43	mmu-miR-877*	1.20E-02	-0.45
44	rno-miR-351	5.68E-03	-0.46
45	mmu-miR-1306	5.68E-03	-0.46
46	hsa-miR-140-3p	3.13E-02	-0.47
47	mmu-miR-146b	1.71E-02	-0.48
48	mmu-miR-449a	1.07E-02	-0.48
49	mmu-miR-214	2.08E-02	-0.48
50	mmu-miR-212	4.88E-02	-0.49
51	mmu-miR-339-3p	2.96E-02	-0.50
52	mmu-miR-667	6.02E-03	-0.51
53	mmu-miR-200a	9.14E-03	-0.52
54	mmu-miR-704	9.51E-03	-0.52
55	mmu-miR-669D	1.46E-02	-0.53
56	mmu-miR-574-3p	3.79E-02	-0.55
57	mmu-miR-186	7.11E-03	-0.56
58	mmu-miR-191	1.40E-02	-0.59
59	mmu-miR-138*	5.68E-03	-0.61
60	mmu-miR-503*	8.23E-03	-0.64
61	mmu-miR-493	1.21E-02	-0.65
62	mmu-miR-484	5.68E-03	-0.67
63	mmu-miR-1932	9.35E-03	-0.67
64	mmu-miR-34c*	1.98E-02	-0.67
65	hsa-miR-183*	5.68E-03	-0.68
66	hsa-miR-200b	4.03E-02	-0.68
67	rno-miR-219-1-3p	5.68E-03	-0.74
68	mmu-miR-125b*	5.68E-03	-0.77
69	mmu-miR-155	5.68E-03	-0.88
70	mmu-miR-1948	5.68E-03	-0.97
71	hsa-miR-99b*	5.68E-03	-1.03
72	hsa-miR-200a*	5.68E-03	-1.36

73	mmu-miR-872	5.68E-03	-1.72
Calibrator not detected			
74	mmu-miR-702	6.85E-06	5.63
75	hsa-miR-485-5p	7.33E-06	5.40
76	hsa-miR-124*	6.85E-06	4.86
77	hsa-miR-30d*	4.15E-05	4.37
78	hsa-miR-431*	1.32E-05	4.18
79	mmu-miR-466J	2.30E-05	4.14
80	hsa-miR-28-3p	2.34E-05	3.83
81	hsa-miR-30c-1*	2.04E-04	3.50
82	mmu-miR-488*	2.89E-05	3.29
83	mmu-miR-10b	5.49E-05	3.21
84	mmu-miR-670	6.33E-03	3.16
85	hsa-miR-299-5p	5.19E-04	3.15
86	mmu-miR-669o-5p	1.53E-04	3.00
87	mmu-miR-293	1.21E-02	1.86
88	mmu-miR-465a-3p	3.63E-03	1.86
Target not detected			
89	mmu-miR-1961	5.19E-04	-4.34
90	mmu-miR-680	6.85E-06	-5.61

Table 24: Significantly modulated microRNAs in 333 g/3 cm injury group at 24 hr post injury.

S.No	MicroRNA	adj. P Value	Fold Change (Log10RQ)
333g_3 cm _24 hr			
Valid			
1	hsa-miR-200c	4.91E-02	2.03
2	rno-miR-224	4.91E-02	1.17
3	mmu-miR-182	4.91E-02	0.75
4	hsa-miR-149	4.91E-02	0.58
5	hsa-miR-27a*	4.91E-02	0.57
6	mmu-miR-296-5p	4.91E-02	0.53
7	mmu-miR-1982-3p	4.91E-02	0.53
8	hsa-miR-378	4.91E-02	0.52
9	hsa-miR-671-5p	4.91E-02	0.51
10	hsa-miR-154*	4.91E-02	0.51
11	hsa-miR-189	4.91E-02	0.48
12	mmu-miR-300	3.09E-02	0.48
13	rno-miR-551B	4.91E-02	0.47
14	mmu-miR-485-3p	4.91E-02	0.46
15	hsa-miR-151-5P	4.91E-02	0.45
16	mmu-miR-376b*	4.91E-02	0.42
17	mmu-miR-184	4.91E-02	0.38
18	hsa-let-7i*	4.91E-02	0.38
19	hsa-miR-455	4.91E-02	0.37
20	mmu-miR-676*	4.91E-02	0.37
21	hsa-miR-223	4.91E-02	0.37

22	hsa-miR-213	4.91E-02	0.37
23	hsa-miR-136*	4.91E-02	0.36
24	mmu-miR-345	4.91E-02	0.33
25	rno-miR-664	4.91E-02	0.33
26	mmu-let-7g*	4.91E-02	0.31
27	mmu-miR-30b	4.91E-02	0.31
28	hsa-miR-411*	4.91E-02	0.30
29	mmu-miR-532-5p	4.91E-02	0.29
30	rno-miR-148b-5p	4.91E-02	0.29
31	mmu-miR-487b	4.91E-02	0.27
32	hsa-miR-93*	4.91E-02	0.23
33	mmu-miR-744	4.91E-02	0.23
34	mmu-miR-192	4.91E-02	-0.23
35	mmu-miR-128a	4.91E-02	-0.25
36	mmu-miR-101a	4.91E-02	-0.27
37	rno-miR-339-3p	4.91E-02	-0.28
38	mmu-let-7e	4.91E-02	-0.30
39	mmu-miR-34b-3p	4.91E-02	-0.30
40	mmu-miR-451	4.91E-02	-0.30
41	mmu-miR-322	4.91E-02	-0.31
42	mmu-miR-106a	4.91E-02	-0.33
43	mmu-miR-704	4.91E-02	-0.35
44	mmu-miR-153	4.91E-02	-0.36
45	mmu-miR-320	4.91E-02	-0.37
46	rno-miR-351	4.91E-02	-0.37

47	mmu-miR-376a	4.91E-02	-0.38
48	mmu-miR-676	4.91E-02	-0.38
49	mmu-miR-1306	4.91E-02	-0.38
50	mmu-miR-222	4.91E-02	-0.38
51	mmu-miR-150	4.91E-02	-0.39
52	mmu-miR-351	4.91E-02	-0.40
53	mmu-miR-490	4.91E-02	-0.40
54	mmu-miR-342-5p	4.91E-02	-0.41
55	mmu-miR-770-3p	4.91E-02	-0.41
56	mmu-miR-1932	4.91E-02	-0.42
57	mmu-miR-503*	4.91E-02	-0.42
58	mmu-miR-138*	4.91E-02	-0.44
59	mmu-miR-380-3p	4.91E-02	-0.44
60	mmu-miR-449a	4.91E-02	-0.45
61	mmu-miR-186	4.91E-02	-0.46
62	mmu-miR-191	4.91E-02	-0.46
63	mmu-miR-669D	4.91E-02	-0.47
64	mmu-miR-484	4.91E-02	-0.52
65	mmu-miR-1948	4.91E-02	-0.53
66	hsa-miR-99b*	4.91E-02	-0.58
67	mmu-miR-155	3.18E-02	-0.62
68	mmu-miR-493	4.91E-02	-0.63
69	mmu-let-7a	3.11E-02	-0.65
70	mmu-miR-450a-5p	4.91E-02	-0.70
71	mmu-miR-467e	4.91E-02	-0.70

72	mmu-miR-125b*	4.91E-02	-0.70
73	rno-miR-219-1-3p	2.77E-02	-0.73
74	rno-miR-196c	4.89E-02	-0.75
75	mmu-miR-1894-3p	4.91E-02	-1.16
76	mmu-miR-872	1.16E-02	-1.78
Calibrator not detected			
77	hsa-miR-485-5p	1.13E-05	5.62
78	hsa-miR-124*	1.13E-05	4.85
79	hsa-miR-30d*	1.13E-05	4.58
80	hsa-miR-431*	1.13E-05	4.17
81	mmu-miR-466J	1.13E-05	4.15
82	hsa-miR-28-3p	1.26E-05	3.92
83	hsa-miR-30c-1*	1.95E-05	3.57
84	hsa-miR-299-5p	1.32E-05	3.51
85	mmu-miR-669l-5p	2.15E-04	3.40
86	mmu-miR-488*	1.94E-05	3.38
87	mmu-miR-669o-5p	9.18E-04	3.05
88	mmu-miR-293	4.91E-02	2.05
89	mmu-miR-1953	4.91E-02	1.42
Target not detected			
90	mmu-miR-196b	3.14E-04	-3.56
91	mmu-miR-2183	4.91E-02	-4.00
92	mmu-miR-1961	8.93E-04	-4.21
93	mmu-miR-680	1.30E-05	-5.47
94	rno-miR-207	1.30E-05	-6.47

Table 25: Significantly modulated microRNAs in 246 g/2 cm injury group at day 7 post injury.

S.No	MicroRNA	adj. P Value	Fold Change (Log10RQ)
246 g_2 cm _Day 7			
Valid			
1	mmu-miR-125b-3p	1.62E-02	0.94
2	rno-miR-224	4.12E-02	0.69
3	mmu-miR-489	4.79E-02	0.64
4	mmu-miR-494	3.27E-02	0.48
5	mmu-miR-467b	4.36E-02	-0.43
6	mmu-miR-668	2.93E-02	-0.45
7	mmu-miR-18a	4.12E-02	-0.66
8	rno-miR-632	2.39E-03	-0.70
9	mmu-miR-369-3p	1.99E-03	-1.85
Calibrator not detected			
10	mmu-miR-465a-5p	2.79E-06	7.66
11	mmu-miR-466a-3p	2.69E-05	4.85
12	mmu-miR-466J	6.23E-06	4.76
13	rno-miR-760-5p	2.12E-05	4.38
14	mmu-miR-423-5p	1.49E-05	4.34
15	mmu-miR-302d	2.54E-04	3.96
Target not detected			
16	mmu-miR-1961	4.89E-04	-4.45

Table 26: Significantly modulated microRNAs in 246 g/3 cm injury group at day 7 post injury.

S.No	MicroRNA	adj. P Value	Fold Change (Log10RQ)
246 g_3 cm _Day 7			
Valid			
1	mmu-miR-872	1.95E-04	1.26
2	rno-miR-190b	3.37E-02	1.15
3	mmu-miR-184	2.72E-02	1.04
4	mmu-miR-181c	1.18E-02	0.77
5	mmu-miR-489	4.91E-02	0.66
6	mmu-miR-770-3p	1.40E-02	0.61
7	mmu-miR-676	4.91E-02	0.42
8	mmu-miR-380-3p	3.70E-02	0.40
9	mmu-miR-292-3p	1.94E-02	0.33
10	mmu-miR-433	2.61E-02	0.33
11	mmu-miR-107	4.03E-02	0.30
12	mmu-miR-667	4.83E-02	0.29
13	mmu-miR-7b	3.42E-02	0.28
14	mmu-miR-320	3.34E-02	0.28
15	mmu-miR-1943-5p	2.98E-02	0.27
16	mmu-miR-125a-3p	4.49E-02	0.27
17	mmu-miR-31*	3.64E-02	-0.21
18	rno-miR-379*	2.72E-02	-0.22
19	mmu-miR-24-2*	4.91E-02	-0.22
20	rno-miR-148b-5p	3.50E-02	-0.25
21	mmu-let-7c-1*	3.42E-02	-0.25
22	mmu-miR-676*	3.64E-02	-0.25
23	mmu-miR-101b	3.64E-02	-0.25
24	hsa-miR-213	2.98E-02	-0.26
25	mmu-miR-300*	2.61E-02	-0.27
26	mmu-miR-300	3.64E-02	-0.30
27	mmu-miR-16*	4.91E-02	-0.30
28	hsa-miR-154*	1.46E-02	-0.34
29	mmu-miR-15a*	1.07E-02	-0.34
30	hsa-miR-338	3.74E-02	-0.34
31	hsa-miR-22	2.72E-02	-0.37
32	hsa-miR-455	2.72E-02	-0.38

33	mmu-miR-337	2.40E-02	-0.39
34	hsa-miR-30d*	4.91E-02	-0.42
35	mmu-miR-466g	6.90E-03	-0.54
36	mmu-miR-467f	2.40E-02	-0.64
37	mmu-miR-690	5.17E-03	-0.66
38	mmu-miR-18a	6.94E-03	-0.66
39	mmu-miR-704	1.45E-02	-0.79
40	mmu-miR-369-3p	5.17E-03	-1.44
41	mmu-miR-193	9.00E-04	-1.81
Calibrator not detected			
42	mmu-miR-694	3.13E-03	8.20
43	mmu-miR-465a-5p	1.30E-06	7.63
44	mmu-miR-466a-3p	1.63E-06	4.71
45	mmu-miR-423-5p	8.14E-06	4.69
46	mmu-miR-466J	1.63E-06	4.50
47	rno-miR-760-5p	1.63E-06	4.40
48	mmu-miR-302d	8.30E-06	3.80
Target not detected			
49	mmu-miR-1961	3.51E-04	-4.35
50	rno-miR-207	1.30E-06	-5.90

Table 27: Significantly modulated microRNAs in 333 g/2 cm injury group at day 7 post injury.

S.No	MicroRNA	adj. P Value	Fold Change (Log10RQ)
333g _2 cm_Day 7			
Valid			
1	mmu-miR-542-3p	6.25E-04	1.94
2	mmu-miR-450a-5p	1.94E-02	0.72
3	mmu-miR-215	8.42E-03	0.65
4	mmu-miR-582-5p	1.00E-02	0.52
5	mmu-miR-18a	3.15E-02	-0.62
6	mmu-miR-542-5p	8.42E-03	-0.83
7	mmu-miR-193	2.05E-02	-1.62
8	mmu-miR-369-3p	1.13E-03	-2.32
Calibrator not detected			
9	mmu-miR-465a-5p	1.03E-06	7.75
10	mmu-miR-743a	8.51E-06	6.61
11	mmu-miR-466a-3p	3.87E-06	5.16
12	rno-miR-760-5p	6.62E-06	5.05
13	mmu-miR-466J	4.29E-05	4.98
14	mmu-miR-423-5p	1.00E-05	4.60
15	mmu-miR-302d	5.48E-05	3.38

Table 28: Significantly modulated microRNAs in 333 g/3 cm injury group at day 7 post injury.

S.No	MicroRNA	adj. P Value	Fold Change (Log10RQ)
333g _3 cm_Day 7			
Valid			
1	mmu-miR-542-3p	7.76E-03	1.42
2	mmu-miR-184	1.43E-03	1.33
3	mmu-miR-574-3p	2.03E-04	1.04
4	mmu-miR-667	1.32E-04	1.01
5	mmu-miR-770-3p	2.11E-03	0.97
6	mmu-miR-202-3p	7.77E-03	0.94
7	hsa-miR-99b*	8.88E-04	0.84
8	mmu-miR-877*	3.84E-03	0.79
9	mmu-miR-339-3p	9.80E-04	0.76
10	mmu-miR-802	5.15E-03	0.75
11	mmu-miR-215	2.15E-02	0.70
12	mmu-miR-1932	7.31E-03	0.65
13	mmu-miR-383	7.29E-04	0.65
14	rno-miR-204*	1.55E-02	0.64
15	mmu-miR-342-3p	1.55E-03	0.61
16	mmu-miR-370	4.52E-03	0.61
17	mmu-miR-146a	3.70E-04	0.59
18	mmu-miR-186	1.08E-03	0.59
19	mmu-miR-142-5p	1.41E-02	0.59
20	hsa-miR-140-3p	7.25E-03	0.58
21	mmu-miR-134	7.76E-03	0.57
22	mmu-miR-191	1.13E-03	0.57
23	mmu-miR-409-3p	1.06E-03	0.57
24	mmu-miR-7b	1.17E-03	0.56
25	mmu-miR-433	7.76E-03	0.55
26	mmu-miR-676	3.33E-03	0.55
27	mmu-miR-503*	1.63E-03	0.54
28	mmu-miR-146b	7.49E-04	0.53
29	rno-miR-598-5p	1.07E-03	0.52
30	mmu-miR-150	6.28E-03	0.49
31	mmu-miR-487b	7.42E-03	0.48
32	mmu-miR-151-3p	3.90E-03	0.46
33	mmu-miR-212	4.52E-03	0.45
34	hsa-miR-421	6.37E-03	0.45
35	mmu-miR-133a	5.47E-03	0.45

36	mmu-miR-155	4.74E-03	0.45
37	mmu-miR-672	4.32E-02	0.44
38	mmu-miR-323-3p	1.04E-02	0.42
39	mmu-miR-455	2.69E-02	0.41
40	mmu-miR-320	2.54E-02	0.40
41	mmu-miR-223	6.28E-03	0.39
42	mmu-miR-125a-3p	1.98E-02	0.39
43	mmu-miR-429	3.61E-02	0.39
44	mmu-miR-434-5p	4.71E-02	0.37
45	mmu-miR-744	1.15E-02	0.35
46	mmu-miR-331-5p	1.55E-02	0.35
47	mmu-miR-125a-5p	8.44E-03	0.35
48	mmu-miR-222	7.78E-03	0.35
49	mmu-miR-28*	3.61E-02	0.33
50	mmu-miR-139-3p	1.69E-02	0.33
51	mmu-miR-32	1.15E-02	0.32
52	mmu-miR-1948	1.27E-02	0.32
53	mmu-let-7d	4.05E-03	0.31
54	mmu-miR-24	8.93E-03	0.31
55	mmu-miR-345-5p	2.19E-02	0.30
56	mmu-miR-16	1.71E-02	0.28
57	mmu-let-7g	1.70E-02	0.28
58	mmu-miR-99a	3.52E-02	0.26
59	mmu-miR-543	2.74E-02	0.25
60	mmu-miR-384-5p	2.92E-02	0.21
61	mmu-miR-23b	3.85E-02	-0.17
62	mmu-miR-15b	1.93E-02	-0.19
63	mmu-miR-376c	3.29E-02	-0.21
64	mmu-miR-99b	3.11E-02	-0.22
65	mmu-miR-598	1.30E-02	-0.22
66	mmu-miR-329	2.04E-02	-0.24
67	mmu-miR-135a	3.61E-02	-0.25
68	mmu-miR-106b	2.55E-02	-0.27
69	mmu-miR-30d	3.29E-02	-0.29
70	mmu-miR-27a	3.74E-03	-0.32
71	mmu-miR-219	1.95E-03	-0.33
72	mmu-miR-133b	2.55E-02	-0.33
73	mmu-miR-186*	1.15E-02	-0.34
74	rno-miR-224	6.93E-03	-0.34
75	mmu-miR-29b	3.52E-02	-0.34
76	mmu-miR-337	2.25E-02	-0.35

77	mmu-miR-451	6.65E-03	-0.35
78	mmu-miR-7a	3.21E-03	-0.36
79	mmu-miR-448	2.29E-02	-0.37
80	mmu-miR-190	4.10E-03	-0.38
81	mmu-miR-381	7.31E-03	-0.40
82	mmu-miR-712	4.77E-03	-0.44
83	hsa-miR-29b-2*	4.66E-02	-0.46
84	hsa-miR-154*	2.39E-02	-0.47
85	hsa-miR-136*	8.68E-03	-0.48
86	mmu-miR-706	7.07E-03	-0.49
87	mmu-miR-322	3.07E-03	-0.51
88	hsa-miR-455	1.04E-02	-0.51
89	mmu-miR-466g	2.96E-03	-0.54
90	mmu-miR-467f	5.18E-03	-0.56
91	mmu-miR-504	4.33E-03	-0.57
92	rno-miR-148b-5p	1.01E-02	-0.57
93	hsa-miR-338	2.20E-03	-0.58
94	mmu-miR-30b*	6.23E-03	-0.60
95	hsa-miR-378	1.69E-02	-0.64
96	mmu-miR-467b	6.76E-03	-0.64
97	mmu-miR-879*	7.70E-03	-0.65
98	mmu-miR-704	3.07E-03	-0.71
99	mmu-miR-21*	3.11E-02	-0.72
100	rno-miR-551b	1.41E-03	-0.73
101	mmu-miR-18a	2.03E-04	-0.92
102	mmu-miR-449b	4.32E-02	-0.95
103	hsa-miR-106b*	4.83E-03	-1.03
104	mmu-miR-369-3p	2.03E-04	-2.06
105	mmu-miR-690	2.35E-04	-2.22
Calibrator not detected			
106	mmu-miR-465a-5p	2.25E-06	7.84
107	rno-miR-760-5p	1.90E-04	4.96
108	mmu-miR-466a-3p	4.38E-05	4.95
109	mmu-miR-423-5p	4.51E-06	4.83
110	mmu-miR-466J	1.74E-05	4.66
Target not detected			
111	mmu-miR-193	4.48E-03	-1.64
112	mmu-miR-494	4.29E-05	-2.43
113	mmu-miR-1928	4.38E-02	-2.50
114	mmu-miR-1947-5p	2.55E-06	-2.62
115	mmu-miR-669o-5p	2.03E-04	-2.72

116	mmu-miR-542-5p	8.11E-06	-3.18
117	mmu-miR-1949	5.79E-05	-3.21
118	mmu-miR-1961	2.03E-04	-3.56
119	hsa-miR-431*	1.79E-06	-3.76
120	mmu-miR-702	5.14E-06	-4.95
121	mmu-miR-696	8.11E-06	-5.04
122	rno-miR-207	2.25E-06	-5.11
123	mmu-miR-374-5p	2.55E-06	-5.92
124	hsa-miR-935	1.79E-06	-6.33

4.1.4: Pathway Analysis of significantly altered brain miRNAs post TBI

The diversity seen in the miRNA expression data may also reflect a concomitant difference in the activation of cellular/biological pathways or mechanisms based on the time point in the injured brain and this was examined by functional pathway analysis which was performed using the DIANA mirPATH online tool with the micro-T database. As we were primarily interested in the temporal pattern only the common pathways between both the four injury cohorts for both the time points post injury were studied. Also, the pathway analysis was done separately for the upregulated and downregulated miRNAs.

The neurologically relevant pathways based on previous literature were focused on further analysis. For the 24 hr time point, pathway analysis indicated the PI3K-Akt signaling pathway, axon guidance, MAPK signaling pathway and regulation of actin cytoskeleton were found to be commonly targeted by the upregulated miRNAs, while downregulated miRNAs were predicted to target PI3K-Akt signaling pathway, axon guidance and neurotrophin signaling pathways among all the four injury groups. A similar comparison for the day 7 time point showed Wnt signaling pathway and axon guidance to be commonly targeted by the upregulated miRNAs whereas axon guidance and MAPK signaling pathway being a target of the downregulated miRNAs (Table 29-32). Axon guidance was found to be common for all the four injury groups in both the early and delayed time points. Both the upregulated and downregulated miRNAs was predicted to play a role in the axon guidance pathway in all the injury groups across both the time points shown in a sample pathway diagram for 246g/ 2cm (Figure 28 & Figure 29). The number and the names of the different gene targets that are a part of thes axon guidance pathway in the different groups have been mentioned in Table 33.

Table 29: Top ten pathways for up regulated miRNAs of 24 hr time point determined by DIANA mirPATH analysis.

A. Top ten pathways for 246 g/2 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Pathways in cancer	7.76E-54	188	72	122.29
2	PI3K-Akt signaling pathway	5.75E-51	197	71	115.68
3	MAPK signaling pathway	2.99E-42	160	73	95.61
4	Regulation of actin cytoskeleton	1.06E-35	131	71	80.53
5	Endocytosis	1.13E-35	126	73	80.47
6	Focal adhesion	2.42E-31	121	72	70.50
7	HTLV-I infection	4.19E-30	145	73	67.64
8	Wnt signaling pathway	1.10E-29	104	71	66.68
9	Protein processing in endoplasmic reticulum	1.38E-28	98	63	64.15
10	Axon guidance	7.84E-27	90	69	60.11

B. Top ten pathways for 246 g/3 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Axon guidance	8.67E-23	60	33	50.80
2	Endocytosis	1.52E-22	82	33	50.24
3	Regulation of actin cytoskeleton	9.28E-18	78	36	39.22
4	PI3K-Akt signaling pathway	1.73E-17	109	36	38.60
5	Wnt signaling pathway	5.53E-17	61	30	37.43
6	MAPK signaling pathway	5.16E-16	87	32	35.20
7	Ubiquitin mediated proteolysis	4.37E-15	52	30	33.06
8	Long-term potentiation	9.37E-12	28	23	25.39
9	Prostate cancer	1.79E-11	33	24	24.74
10	Calcium signaling pathway	1.79E-11	60	30	24.74

C. Top ten pathways for 333 g/2 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Axon guidance	7.69E-40	64	21	90.06
2	Ubiquitin mediated proteolysis	1.36E-23	54	20	52.65
3	Wnt signaling pathway	1.12E-16	57	20	36.73
4	Endocytosis	1.19E-16	69	24	36.66
5	Regulation of actin cytoskeleton	2.35E-16	69	23	35.99
6	Transcriptional misregulation in cancer	2.05E-15	63	21	33.82
7	Prion diseases	8.99E-14	10	10	30.04
8	PI3K-Akt signaling pathway	8.99E-14	94	22	30.04
9	Dopaminergic synapse	3.78E-11	44	19	24.00
10	MAPK signaling pathway	9.43E-11	73	23	23.08

D. Top ten pathways for 333 g/3 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Axon guidance	1.46E-25	54	22	57.19
2	Transcriptional misregulation in cancer	3.33E-22	64	22	49.45
3	Ubiquitin mediated proteolysis	2.14E-21	53	19	47.60
4	Prostate cancer	3.41E-19	37	19	42.52
5	ErbB signaling pathway	3.13E-18	35	18	40.30
6	MAPK signaling pathway	4.04E-16	79	24	35.44
7	Regulation of actin cytoskeleton	2.04E-15	69	22	33.83
8	Focal adhesion	1.02E-14	63	24	32.22
9	PI3K-Akt signaling pathway	2.53E-13	94	23	29.01
10	Dorso-ventral axis formation	1.24E-11	13	12	25.11

Table 30: Top ten pathways for down regulated miRNAs of 24 hr time point determined by DIANA mirPATH analysis.

A. Top ten pathways for 246 g/2 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Pathways in cancer	1.41E-67	173	65	153.93
2	Focal adhesion	2.27E-50	105	57	114.31
3	Wnt signaling pathway	1.30E-48	93	60	110.26
4	PI3K-Akt signaling pathway	1.31E-47	161	64	107.95
5	Axon guidance	6.74E-47	83	58	106.31
6	Regulation of actin cytoskeleton	4.46E-36	106	65	81.40
7	Neurotrophin signaling pathway	3.88E-34	69	54	76.93
8	Endocytosis	5.32E-31	105	54	69.71
9	T cell receptor signaling pathway	2.42E-29	60	54	65.89
10	TGF-beta signaling pathway	1.15E-27	50	42	62.03

B. Top ten pathways for 246 g/3 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Axon guidance	1.40E-51	85	47	117.10
2	Wnt signaling pathway	1.02E-49	87	43	112.81
3	Focal adhesion	3.90E-47	100	44	106.86
4	Regulation of actin cytoskeleton	1.53E-38	104	48	87.07
5	Neurotrophin signaling pathway	6.75E-38	69	43	85.59
6	Pathways in cancer	1.77E-37	154	48	84.63
7	T cell receptor signaling pathway	4.90E-32	59	40	72.09
8	Endocytosis	7.99E-30	100	41	67.00
9	TGF-beta signaling pathway	1.11E-29	49	35	66.67
10	PI3K-Akt signaling pathway	3.41E-29	143	47	65.55

C. Top ten pathways for 333 g/2 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	ErbB signaling pathway	8.32E-33	45	31	73.87
2	Axon guidance	2.35E-31	68	35	70.53
3	T cell receptor signaling pathway	1.89E-28	52	31	63.83
4	MAPK signaling pathway	1.89E-28	104	36	63.83
5	Wnt signaling pathway	7.20E-28	73	35	62.50
6	Neurotrophin signaling pathway	1.47E-26	57	33	59.48
7	Pathways in cancer	1.47E-26	127	36	59.48
8	PI3K-Akt signaling pathway	1.82E-25	126	35	56.97
9	Prostate cancer	2.36E-24	42	30	54.40
10	mTOR signaling pathway	5.18E-24	34	24	53.62

D. Top ten pathways for 333 g/3 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Axon guidance	1.88E-42	70	29	96.08
2	MAPK signaling pathway	3.33E-36	110	31	81.69
3	PI3K-Akt signaling pathway	5.00E-36	138	33	81.28
4	Pathways in cancer	7.66E-33	133	32	73.95
5	Neurotrophin signaling pathway	3.08E-29	60	26	65.65
6	Fc gamma R-mediated phagocytosis	1.03E-27	45	25	62.14
7	T cell receptor signaling pathway	2.68E-27	52	25	61.19
8	Focal adhesion	3.76E-25	83	29	56.24
9	ErbB signaling pathway	1.35E-23	44	27	52.66
10	mTOR signaling pathway	4.52E-23	34	20	51.45

Table 31: Top ten pathways for upregulated miRNAs of day 7 time point determined by DIANA mirPATH analysis.

A. Top ten pathways for 246 g/2 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Proximal tubule bicarbonate reclamation	2.29E-09	7	3	19.89
2	Mucin type O-Glycan biosynthesis	2.68E-08	4	2	17.43
3	D-Glutamine and D-glutamate metabolism	6.41E-08	3	2	16.56
4	Protein processing in endoplasmic reticulum	5.50E-07	18	3	14.41
5	Circadian rhythm	1.13E-05	7	2	11.39
6	Endocrine and other factor-regulated calcium reabsorption	9.24E-05	7	3	9.29
7	Wnt signaling pathway	0.0002049	16	3	8.49
8	Axon guidance	0.0002558	13	3	8.27
9	Gap junction	0.0003418	7	3	7.98
10	Ubiquitin mediated proteolysis	0.0013469	13	3	6.61

B. Top ten pathways for 246 g/3 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Axon guidance	3.31E-17	38	14	37.95
2	Ubiquitin mediated proteolysis	7.47E-13	37	12	27.92
3	mTOR signaling pathway	1.70E-11	21	10	24.80
4	Pathways in cancer	3.11E-09	64	13	19.59
5	Adherens junction	1.26E-08	21	12	18.19
6	PI3K-Akt signaling pathway	1.31E-08	61	13	18.15
7	Melanoma	4.67E-08	20	10	16.88
8	Wnt signaling pathway	5.21E-08	35	13	16.77
9	Fc gamma R-mediated phagocytosis	6.40E-08	22	10	16.56
10	Transcriptional misregulation in cancer	1.76E-07	38	11	15.55

C. Top ten pathways for 333 g/2 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Wnt signaling pathway	2.58E-06	14	3	12.87
2	Axon guidance	5.23E-06	11	2	12.16
3	Fc gamma R-mediated phagocytosis	5.23E-06	10	2	12.16
4	Long-term depression	5.23E-06	6	3	12.16
5	ErbB signaling pathway	1.17E-05	10	3	11.36
6	Regulation of actin cytoskeleton	2.49E-05	16	4	10.60
7	Focal adhesion	0.0001139	14	2	9.08
8	Dopaminergic synapse	0.0002298	11	3	8.38
9	Peroxisome	0.0005112	7	2	7.58
10	Taurine and hypotaurine metabolism	0.0005128	2	1	7.58

D. Top ten pathways for 333 g/3 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Axon guidance	9.33E-47	79	47	105.99
2	Wnt signaling pathway	1.81E-39	81	47	89.21
3	Focal adhesion	1.02E-38	98	47	87.48
4	MAPK signaling pathway	1.37E-37	122	48	84.88
5	Neurotrophin signaling pathway	1.37E-34	67	41	77.97
6	Regulation of actin cytoskeleton	4.09E-32	104	48	72.27
7	Pathways in cancer	2.95E-31	152	48	70.30
8	ErbB signaling pathway	4.26E-29	54	44	65.33
9	PI3K-Akt signaling pathway	2.55E-28	145	50	63.53
10	Insulin signaling pathway	1.94E-26	67	42	59.21

Table 32: Top ten pathways for down regulated miRNAs of day 7 time point determined by DIANA mirPATH analysis.

A. Top ten pathways for 246 g/2 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Axon guidance	1.47E-13	22	4	29.55
2	Endocytosis	2.56E-07	21	4	15.18
3	Transcriptional misregulation in cancer	1.86E-06	18	3	13.19
4	TGF-beta signaling pathway	1.84E-05	11	4	10.90
5	Regulation of actin cytoskeleton	9.60E-05	19	4	9.25
6	MAPK signaling pathway	0.0001642	19	4	8.71
7	Circadian rhythm	0.0002604	6	3	8.25
8	Primary bile acid biosynthesis	0.0002904	2	1	8.14
9	ErbB signaling pathway	0.0007176	9	4	7.24
10	Glycosphingolipid biosynthesis - globo series	0.0010686	3	2	6.84

B. Top ten pathways for 246 g/3 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Axon guidance	2.42E-25	55	23	56.68
2	Ubiquitin mediated proteolysis	2.58E-20	55	21	45.10
3	Endocytosis	3.16E-20	73	21	44.90
4	Pathways in cancer	4.06E-16	99	23	35.44
5	MAPK signaling pathway	8.06E-16	78	22	34.75
6	ErbB signaling pathway	9.43E-16	34	18	34.60
7	Transcriptional misregulation in cancer	3.67E-15	59	23	33.24
8	Wnt signaling pathway	4.28E-14	54	21	30.78
9	Prostate cancer	7.38E-13	32	20	27.93
10	Focal adhesion	1.04E-12	61	22	27.59

C. Top ten pathways for 333 g/2 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	Transcriptional misregulation in cancer	5.07E-10	20	3	21.40
2	Axon guidance	9.85E-10	19	3	20.74
3	MAPK signaling pathway	1.19E-06	21	3	13.64
4	Circadian rhythm	6.37E-06	7	3	11.96
5	Acute myeloid leukemia	7.57E-06	8	3	11.79
6	Pathways in cancer	4.73E-05	23	3	9.96
7	Endocytosis	9.44E-05	18	3	9.27
8	Primary bile acid biosynthesis	0.0002051	2	1	8.49
9	mTOR signaling pathway	0.0003151	8	3	8.06
10	ErbB signaling pathway	0.0004062	9	3	7.81

D. Top ten pathways for 333 g/3 cm

S.No	KEGG pathway	p-value	No. of genes	No.of miRNAs	-ln (p-value)
1	MAPK signaling pathway	1.23E-55	132	36	126.43
2	Focal adhesion	1.74E-45	102	36	103.06
3	Axon guidance	2.78E-42	85	36	95.69
4	Pathways in cancer	3.69E-39	161	38	88.49
5	PI3K-Akt signaling pathway	1.55E-38	159	37	87.06
6	Transcriptional misregulation in cancer	2.94E-38	95	37	86.42
7	Ubiquitin mediated proteolysis	1.69E-34	74	34	77.76
8	Wnt signaling pathway	2.39E-33	84	38	75.11
9	Insulin signaling pathway	1.20E-31	70	33	71.20
10	Osteoclast differentiation	4.26E-30	65	30	67.63

Table 33: Gene data for axon guidance pathway common between all the injury groups.

Injury group	Up/ down	No. of genes	Gene list
246 g/ 2 cm/24 hr	Up	90	Rhod/Sema3d/Epha5/Mapk1/Ephb4/Gsk3b/Efna4/Ppp3cc/Efnb2/Srgap2/Epha1/Sema4f/Gnai3/Sema6a/Pak3/Gnai1/Sema4b/Robo1/Ppp3r2/Ptk2/Plxna4/Plxna2/Dpysl5/Pak6/Rock1/Nck2/Sema3e/Nck1/Nfatc3/Plxna1/Sema4d/Rhoa/Cfl2/Sema7a/Sema3c/Slit2/Ppp3cb/Rgs3/Nfatc1/Arhgef12/Cxcr4/Sema5b/Unc5d/Kras/Mapk3/Sema6b/Gnai2/Ntng1/Unc5b/Sema4c/Nras/Rac1/Rock2/Nrp1/Plxnc1/Dpysl2/Epha3/Rasa1/Sema5a/Efna3/Robo2/Epha4/Ephb2/Ntn1/Chp1/Unc5a/Srgap1/Srgap3/Srgap3/Ppp3r1/Cdk5/Nfat5/Efnb3/Nfatc4/Itgb1/Ppp3ca/Epha7/Epha2/Sema3a/Cxcl12/Pak7/Sema6d/Pak2/Nfatc2/Met/Cdc42/Unc5c/Sema4g/Efna1/Limk2
	Down	83	Sema3d/Epha5/Mapk1/Gsk3b/Efna4/Efnb2/Lrrc4c/Gnai3/Sema6a/Pak3/Cfl1/Gnai1/Sema4b/Robo1/Ppp3r2/Ptk2/Plxna4/Plxna2/Dpysl5/Rock1/Nck2/Efna2/Sema3g/Ngef/Nck1/Nfatc3/Plxna1/Sema4d/Ablim1/Rhoa/Cfl2/Sema3c/Slit2/Ppp3cb/Rgs3/Ablim3/Arhgef12/Cxcr4/Sema5b/Unc5d/Kras/Gnai2/Slit1/Ntng1/4930544G11Rik/Sema4c/Nras/Rac1/Rock2/Nrp1/Plxnc1/Dpysl2/Epha3/Rasa1/Efnb1/Sema5a/Sema3f/Robo2/Slit3/Epha4/Ephb2/Chp1/Ephb1/Fyn/Srgap3/Ppp3r1/Nfat5/Rac2/Itgb1/Ephb3/Ppp3ca/Epha7/Sema3a/Rnd1/Pak7/Sema6d/Pak2/Met/Cdc42/Unc5c/Sema4g/Efna1
246 g/3 cm/24 hr	Up	80	Rhod/Ephb4/Efna4/Efnb2/Epha8/Srgap2/Epha1/Sema4f/Lrrc4c/Gnai3/Sema6a/Pak3/Robo1/Ppp3r2/Plxna4/Plxna2/Dpysl5/Pak6/Rock1/Nck2/Sema3e/Nfatc3/Sema4d/Rhoa/Cfl2/Sema3c/Arhgef12/Cxcr4/Sema5b/Kras/Ntng1/Sema4c/Nrp1/Plxnc1/Dpysl2/Epha3/Rasa1/Sema5a/Robo2/Slit3/Epha4/Unc5a/Fyn/Srgap3/Ppp3r1/Nfat5/Itgb1/Plxnb3/Ephb3/Epha7/Epha2/Sema3a/Cxcl12/Sema6d/Nfatc2/Unc5c/Sema4g/Efna1/Limk2
	Down	85	Epha5/Mapk1/Ephb4/Gsk3b/Efna4/Efnb2/Srgap2/Lrrc4c/Gnai3/Sema6a/Pak3/Cfl1/Gnai1/Sema4b/Robo1/Ppp3r2/Ptk2/Plxna4/Plxna2/Dpysl5/Rock1/Nck2/Efna2/Sema3g/Ngef/Nck1/Nfatc3/Plxna1/Rhoa/Cfl2/Sema7a/Sema3c/Slit2/Ppp3cb/Rgs3/Ablim3/Abl1/Arhgef12/Cxcr4/Sema5b/Unc5d/Kras/Sema6b/Gnai2/Ntng1/4930544G11Rik/Sema4c/Nras/Rac1/Rock2/Nrp1/Plxnc1/Dpysl2/Rasa1/Efnb1/Sema5a/Efna3/Sema3f/Robo2/Slit3/Epha4/Ephb2/Chp1/Unc5a/Ephb1/Fyn/Srgap1/Srgap3/Ppp3r1/Nfat5/Itgb1/Plxnb3/Ephb3/Ppp3ca/Epha7/Sema3a/Pak7/Sema6d/Pak2/Nfatc2/Met/Cdc42/Unc5c/Sema4g/Efna1
333 g/2 cm/24 hr	Up	64	Ephb4/Gsk3b/Efnb2/Sema4f/Gnai3/Sema6a/Gnai1/Robo1/Plxna4/Plxna2/Dpysl5/Pak6/Rock1/Nck2/Nck1/Nfatc3/Plxna1/Rhoa/Cfl2/Sema7a/Sema3c/Nfatc1/Arhgef12/Cxcr4/Unc5d/Kras/Sema6b/Gnai2/Ntng1/Unc5b/Sema4c/Rock2/Nrp1/Plxnc1/Dpysl2/Epha3/Rasa1/Sema5a/Efna3/Robo2/Epha4/Ephb2/Chp1/Unc5a/Fyn/Srgap3/Ppp3r1/Cdk5/Nfat5/Nfatc4/Itgb1/Ppp3ca/Epha7/Epha2/Sema3a/Cxcl12/Pak7/Sema6d/Pak2/Nfa

			tc2/Met/Unc5c/Sema4g/Efna1
	Down	68	Sema3d/Epha5/Mapk1/Gsk3b/Efna4/Efnb2/Srgap2/Sema4f/Lrrc4c/Gnai3/Sema6a/Pak3/Cfl1/Sema4b/Robo1/Ppp3r2/Ptk2/Plxna4/Plxna2/Dpysl5/Rock1/Nck2/Efna2/Sema3g/Ngef/Nck1/Nfatc3/Plxna1/Sema4d/Cfl2/Ppp3cb/Rgs3/Arhgef12/Unc5d/Kras/Gnai2/Ntng1/4930544G11Rik/Sema4c/Nras/Nrp1/Plxnc1/Dpysl2/Rasa1/Efnb1/Sema5a/Slit3/Epha4/Ephb2/Ephb1/Fyn/Srgap3/Ppp3r1/Nfat5/Ephb3/Ppp3ca/Epha7/Epha2/Sema3a/Cxcl12/Sema6d/Pak2/Nfatc2/Met/Cdc42/Unc5c/Sema4g/Efna1
333 g/3 cm/24 hr	Up	54	Epha5/Mapk1/L1cam/Efnb2/Srgap2/Gnai3/Sema6a/Pak3/Robo1/Ptk2/Plxna4/Plxna2/Dpysl5/Pak6/Nck2/Sema3e/Nfatc3/Ablim1/Rhoa/Cfl2/Sema3c/Cxcr4/Unc5d/Kras/Sema6b/Gnai2/Ntng1/Rac1/Plxnc1/Dpysl2/Epha3/Rasa1/Efna3/Robo2/Slit3/Epha4/Ephb2/Unc5a/Ephb1/Fyn/Srgap1/Srgap3/Ppp3r1/Nfat5/Itgb1/Ppp3ca/Epha2/Sema3a/Pak7/Sema6d/Pak2/Nfatc2/Unc5c/Efna1
	Down	70	Epha5/Mapk1/Ephb4/Gsk3b/Efnb2/Sema4f/Gnai3/Sema6a/Cfl1/Sema4b/Ppp3r2/Ptk2/Plxna4/Plxna2/Dpysl5/Rock1/Nck2/Efna2/Sema3g/Ngef/Nck1/Nfatc3/Sema4d/Cfl2/Sema7a/Rgs3/Arhgef12/Sema5b/Kras/Mapk3/Gnai2/Ntng1/4930544G11Rik/Plxnb1/Sema4c/Nras/Rac1/Rock2/Plxnc1/Dpysl2/Rasa1/Efnb1/Sema5a/Efna3/Robo2/Epha4/Ephb2/Unc5a/Ephb1/Fyn/Srgap1/Ppp3r1/Nfat5/Rac2/Itgb1/Ephb3/Ppp3ca/Epha7/Sema3a/Cxcl12/Pak7/Sema6d/Pak2/Nfatc2/Met/Cdc42/Unc5c/Sema4g/Efna1/Limk2
246 g/2 cm/Day 7	Up	13	Epha5/Efnb2/Lrrc4c/Dpysl5/Rock1/Arhgef12/Unc5d/Nrp1/Dpysl2/Nfat5/Epha2/Rnd1/Pak7
	Down	22	Sema3d/Epha5/Efnb2/Lrrc4c/Ppp3r2/Plxna2/Nck2/Sema3e/Cfl2/Rgs3/Arhgef12/Cxcr4/Unc5d/Plxnc1/Sema5a/Robo2/Epha4/Ntn1/Fyn/Nfat5/Epha7/Cdc42
246 g/3 cm/Day 7	Up	38	Rhod/Epha5/Mapk1/Srgap2/Sema6a/Ppp3r2/Plxna2/Dpysl5/Rock1/Nck1/Nfatc3/Rgs3/Arhgef12/Cxcr4/Unc5d/Kras/Rac1/Plxnc1/Epha3/Rasa1/Sema5a/Robo2/Epha4/Unc5a/Srgap1/Ppp3r1/Nfat5/Efnb3/Rac2/Itgb1/Ephb3/Epha7/Sema3a/Rnd1/Pak7/Unc5c/Sema4g/Efna1
	Down	55	Sema3d/Epha5/Gsk3b/Efnb2/Gnai3/Sema6a/Pak3/Gnai1/Robo1/Ppp3r2/Plxna4/Plxna2/Dpysl5/Nck2/Sema3e/Nfatc3/Rhoa/Cfl2/Sema3c/Ppp3cb/Rgs3/Arhgef12/Cxcr4/Unc5d/Kras/Ntng1/Rac1/Rock2/Plxnc1/Epha3/Rasa1/Sema5a/Efna3/Robo2/Slit3/Epha4/Ntn1/Chp1/Fyn/Srgap1/Srgap3/Ppp3r1/Nfat5/Itgb1/Plxnb3/Ephb3/Epha7/Sema3a/Cxcl12/Pak7/Sema6d/Pak2/Met/Cdc42/Unc5c/
333 g/2 cm/Day 7	Up	11	Gsk3b/Gnai3/Pak3/Arhgef12/Rock2/Plxnc1/Robo2/Srgap3/Cxcl12/Pak2/Unc5c
	Down	19	Sema3d/Epha5/Efnb2/Ppp3r2/Plxna2/Nck2/Sema3e/Cfl2/Rgs3/Arhgef12/Unc5d/Plxnc1/Sema5a/Robo2/Ntn1/Fyn/Nfat5/Epha7/Cdc42

	Up	79	Rhod/Sema3d/Epha5/Mapk1/Gsk3b/Efna4/Efnb2/Srgap2/Sema4f/Gnai3/Sema6a/Pak3/Gnai1/Sema4b/Robo1/Ppp3r2/Ptk2/Plxna4/Plxna2/Dpysl5/Pak6/Rock1/Nck2/Efna2/Sema3g/Ngef/Sema3e/Nck1/Nfatc3/Plxna1/Sema4d/Rhoa/Cfl2/Sema7a/Slit2/Ppp3cb/Abl1/Arhgef12/Sema5b/Ntn3/Unc5d/Kras/Sema6b/Gnai2/Ntng1/4930544G11Rik/Sema4c/Nras/Rac1/Rock2/Nrp1/Plxnc1/Epha3/Rasa1/Sema5a/Efna3/Sema3f/Robo2/Epha4/Ephb2/Ephb1/Fyn/Srgap3/Ppp3r1/Nfat5/Rac2/Itgb1/Ppp3ca/Epha7/Sema3a/Pak7/Sema6d/Pak2/Cdc42/Unc5c/Sema4g/Efna1/Sema4a/Limk2
333 g/3 cm/Day 7	Down	85	Rhod/Sema3d/Epha5/Mapk1/Gsk3b/Efna4/Efnb2/Srgap2/Epha1/Sema4f/Gnai3/Sema6a/Gnai1/Sema4b/Robo1/Ppp3r2/Ptk2/Plxna4/Plxna2/Dpysl5/Pak6/Rock1/Nck2/Sema3e/Nck1/Nfatc3/Rhoa/Cfl2/Sema7a/Sema3c/Slit2/Ppp3cb/Rgs3/Nfatc1/Ablim3/Arhgef12/Sema5b/Unc5d/Kras/Mapk3/Sema6b/Gnai2/Ntng1/Unc5b/Sema4c/Nras/Rock2/Plxnc1/Dpysl2/Epha3/Rasa1/Efnb1/Sema5a/Efna3/Robo2/Slit3/Epha4/Ephb2/Ntn1/Chp1/Unc5a/Ephb1/Fyn/Srgap1/Srgap3/Ppp3r1/Nfat5/Nfatc4/Itgb1/Plxnb3/Ephb3/Ppp3ca/Epha7/Epha2/Sema3a/Cxcl12/Pak7/Sema6d/Pak2/Nfatc2/Met/Cdc42/Unc5c/Sema4g/Limk2

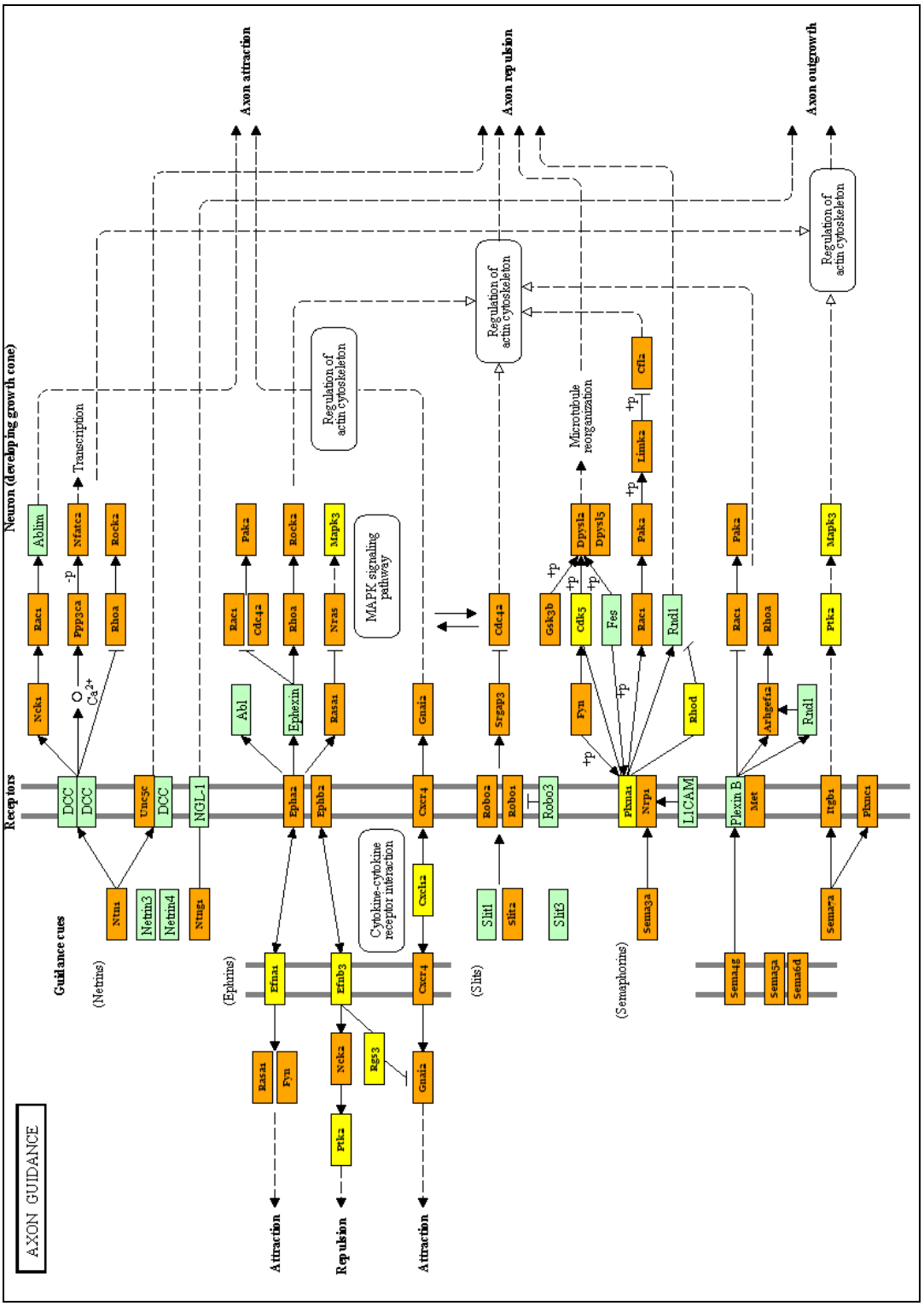

Figure 28: Pathway diagram shown only for upregulated miRNAs of 246/2 cm/24 hr cohort.

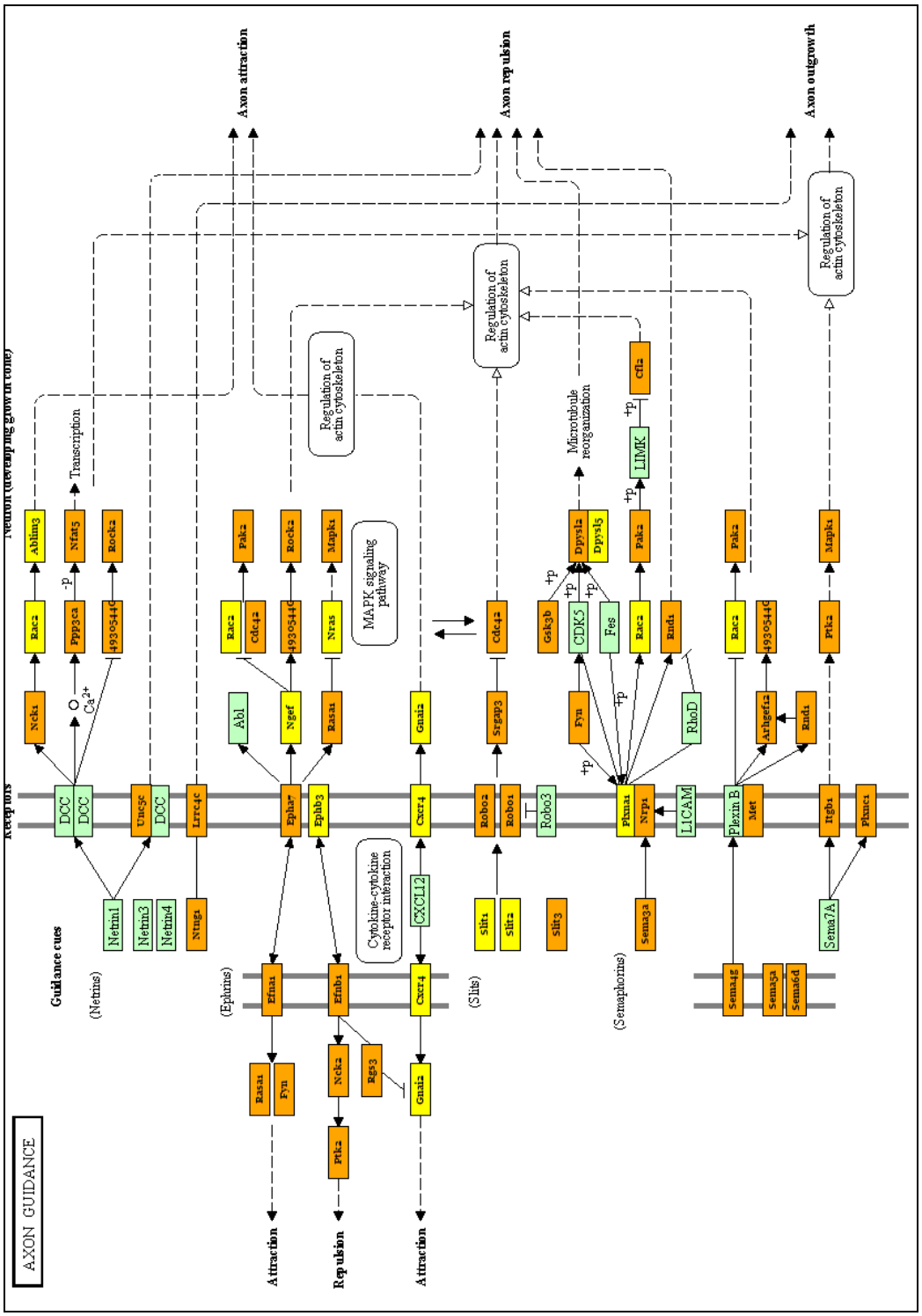

Figure 29: Pathway diagram shown only for down regulated miRNAs of 246/2 cm/24 hr cohort.

4.1.5: Validation of selected miRNA candidates

Two different miRNAs which were found to be up regulated in the 24 hr time point in the TLDA miRNA expression data was selected for further validation with the standard single tube PCR assay. Similar expression between the validation PCR and the global miRNA expression data can help in reinforcing confidence on the miRNA profiling data. The pattern of expression in both of the miRNAs – miR-296 and miR-154* were found to be similar as in the TLDA data and also this was tested in the same set of samples in which the original miRNA profiling was performed. In validation assays for both the miRNAs, U6 snRNA was used as endogenous normalization control. The validation data shows that there was a up regulation of around 2-3 fold difference for miR-296 and around 3-5 fold up regulation for the other miRNA – miR-154* (Figure 30).

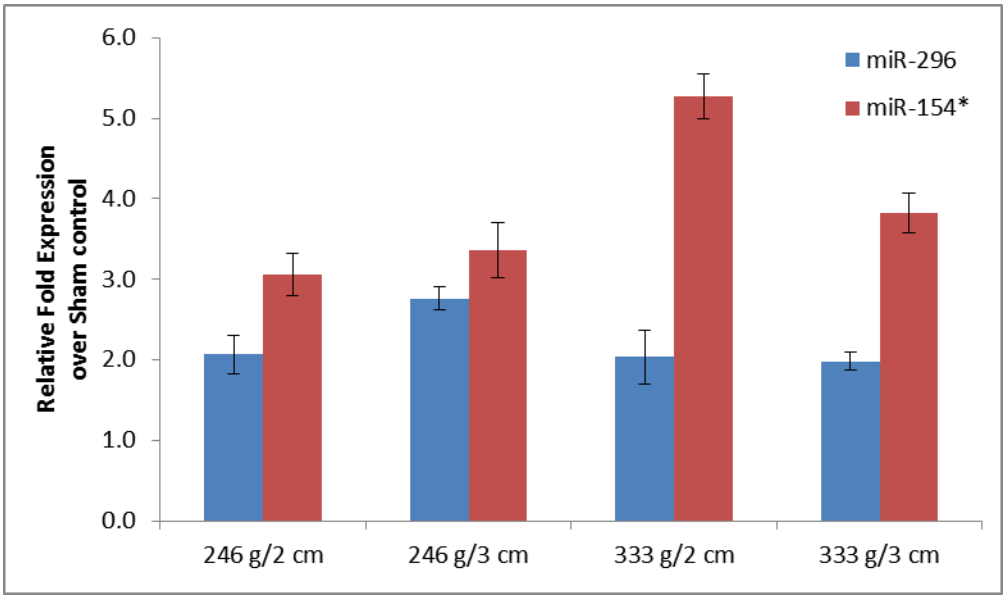

Figure 30: Validation of miR-296 and miR-154* in 24 hr brain samples using individual real time PCR assay. Both the miRNAs - miR-296 and miR-154* were found to be upregulated compared to sham samples and display a similar trend of expression as observed in TLDA data.

4.1.6: Differential expression patterns in brain and serum miRNA profiles post mild CCI indicated by clustering analysis and miRNA distribution graph

The highly reproducible nature of the injury and a better control over the injury parameters in the controlled cortical impact injury model makes it a widely used one as compared to other animal models of traumatic brain injury. Earlier studies have shown that the 1 mm depth CCI injury used in this study has been described as of mild severity due to absence of any gross pathology (Mierzwa et al., 2014, Radomski et al., 2013).

Animals (n=5/group) were subjected to mild CCI and were sacrificed at three different time points-3 h, 24 h and day 4. Total RNA was extracted from the serum as well as the brain tissue underlying the injury site and was used for the miRNA expression profiling done using the TaqMan array platform. Temporal changes in miRNA expression in both serum and brain were studied and also compared between each other. The clustering analysis clearly shows a difference in the miRNA expression between the serum and the brain (Figure 31). The main difference being observed is the difference in the clustering of the three time points. In the brain, the early time points of 3 h and 24 h is clustering together while in serum the 24 h and day 4 samples group together indicating a difference in the miRNA expression in both serum and brain post injury.

The miRNA distribution graph shows that the difference seen in the clustering analysis between serum and brain miRNA expression among the three time points is also evident in the number of miRNAs significantly modulated time point wise (Figure 32). In brain, there is a peak in the miRNA expression at 24 h which is nearly double than that of the 3 h count but this decreases at the day 4 time point. In the serum, a higher number of differentially expressed miRNAs is observed in 3 h time point compared to the 24 h and day 4 time point.

Based on the criteria of p value<0.05 and fold change>2, the number of significantly modulated miRNAs compared to the naïve controls were calculated and the expression data shows about 93, 43 and 64 miRNAs were differentially expressed in the serum at 3 h, 24 h and day 4 time points respectively (Table 34-36). Similarly, the expression data for the brain samples indicates differential expression of about 64, 174 and 83 miRNAs in the 3 h, 24 h and day 4 time points respectively (Table 37-39).

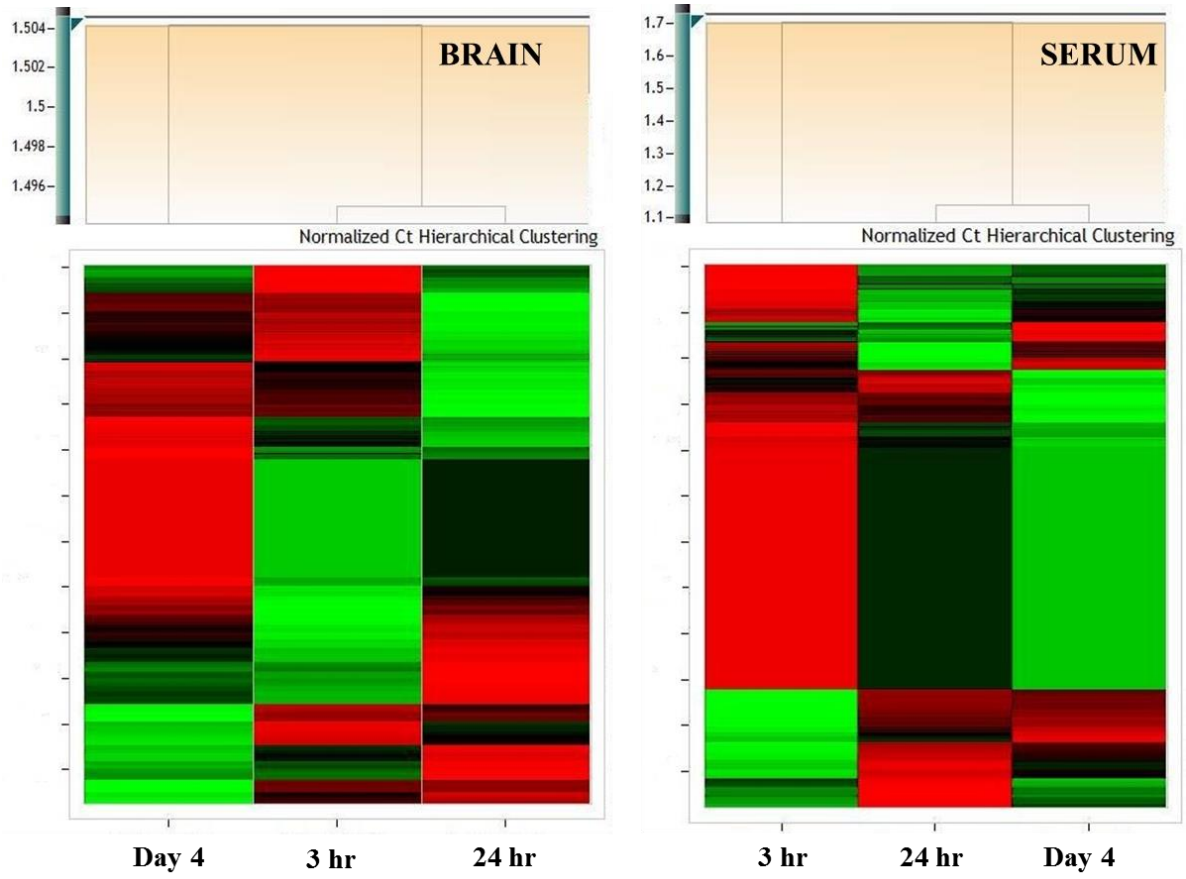

Figure 31: Hierarchical clustering of miRNAs shows the difference in the miRNA expression between serum and brain. The clustering is done using complete linkage method together with Pearson correlation measure based on the ddCt values normalized using z-score normalization method. In both the heat maps, each row represents an individual miRNA and each of the columns representing a single injury group (n=5) normalized to the sham control group (n=5). The pattern of the group wise clustering is found to be different in both the serum and brain. In the brain, the early time points of 3 h and 24 h is clustering together while in serum , a different pattern of miRNA expression emerges where the 24 h and day 4 samples group together indicating a difference in the miRNA expression in both serum and brain post injury.

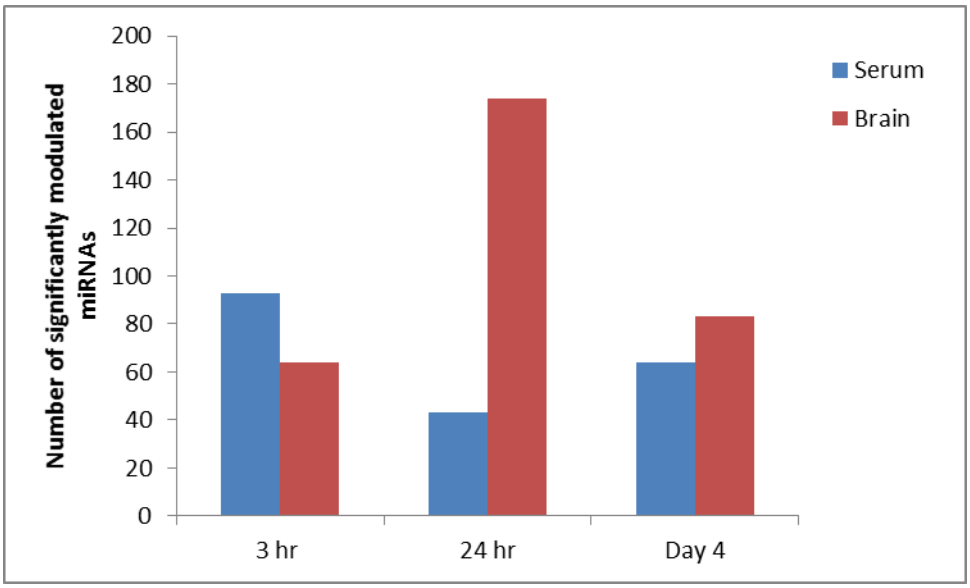

Figure 32: Changes in miRNA expression based on time point in serum and brain. The number of significantly modulated brain microRNAs (including 'calibrator not detected' and 'target not detected' candidates) compared to the naive control group is shown. The miRNA expression graph shows a peak of miRNA expression at 24 h post injury in the brain.

Table 34: Significantly modulated microRNAs in serum at 3 h post injury post mild CCI injury

S.No	MicroRNA	P Value	Fold change (Log10RQ)
Valid			
1	mmu-miR-376c	4.48E-06	1.48
2	hsa-miR-206	9.13E-06	1.44
3	hsa-miR-214	3.56E-04	1.07
4	mmu-miR-214	1.90E-03	0.96
5	mmu-miR-205	1.63E-02	0.86
6	mmu-miR-193b	3.30E-04	0.82
7	mmu-miR-146a	1.94E-03	0.76
8	mmu-miR-574-3p	2.04E-03	0.74
9	mmu-miR-133a	6.88E-05	0.72
10	mmu-miR-184	3.76E-02	0.69
11	mmu-miR-322*	2.55E-02	0.62
12	mmu-miR-186	1.65E-02	0.61
13	hsa-miR-30e-3p	1.18E-02	0.60
14	hsa-miR-223	5.83E-03	0.57
15	mmu-miR-126-3p	2.85E-02	0.56
16	mmu-miR-434-3p	9.31E-03	0.54
17	mmu-miR-199a-3p	7.19E-04	0.53
18	mmu-miR-204	8.97E-03	0.52
19	mmu-miR-410	9.49E-03	0.51
20	hsa-miR-30a-3p	2.18E-02	0.49
21	mmu-miR-31	3.73E-02	0.48
22	mmu-miR-24	3.50E-04	0.46
23	mmu-miR-146b	0.020282031	0.46
24	mmu-miR-342-3p	3.96E-02	0.43
25	mmu-miR-384-5p	2.90E-02	0.42
26	mmu-miR-218	3.37E-03	0.36
27	mmu-miR-125a-5p	0.032329847	0.36
28	mmu-miR-222	0.03462904	0.34
29	mmu-miR-152	1.33E-03	0.34
30	mmu-miR-350	2.09E-02	-0.30
31	mmu-miR-712	4.50E-02	-0.31
32	mmu-miR-2146	5.14E-03	-0.31
33	mmu-miR-345	1.95E-02	-0.32
34	mmu-miR-451	2.28E-02	-0.33

35	rno-miR-224	0.022218841	-0.33
36	mmu-miR-93	1.07E-02	-0.34
37	mmu-miR-150	2.99E-02	-0.34
38	hsa-miR-671-5p	1.10E-02	-0.35
39	mmu-miR-30b	1.35E-02	-0.36
40	mmu-let-7c	5.69E-03	-0.36
41	mmu-miR-20b	1.79E-02	-0.36
42	mmu-miR-128a	4.13E-02	-0.36
43	mmu-miR-467c	3.01E-02	-0.40
44	mmu-miR-362-3p	1.26E-02	-0.40
45	mmu-let-7b	4.82E-03	-0.40
46	mmu-miR-339-5p	2.61E-02	-0.42
47	mmu-miR-25	1.36E-02	-0.43
48	mmu-miR-361	1.49E-02	-0.45
49	mmu-miR-143	3.57E-03	-0.46
50	mmu-miR-148a	1.53E-02	-0.47
51	mmu-miR-10a	3.46E-03	-0.48
52	mmu-miR-224	1.45E-02	-0.49
53	mmu-miR-1944	2.19E-02	-0.50
54	mmu-miR-423-5p	3.72E-02	-0.51
55	mmu-miR-328	1.28E-02	-0.51
56	mmu-miR-100	6.82E-04	-0.54
57	mmu-miR-141	2.08E-02	-0.58
58	mmu-miR-29b	1.25E-02	-0.59
59	mmu-miR-669a	2.74E-02	-0.59
60	mmu-let-7d	7.99E-03	-0.60
61	mmu-miR-99b	1.10E-04	-0.61
62	mmu-miR-142-5p	1.36E-02	-0.65
63	mmu-miR-15b	1.02E-03	-0.75
64	mmu-miR-331-5p	5.80E-03	-0.75
65	mmu-miR-296-5p	1.40E-03	-0.88
66	mmu-miR-1928	1.88E-02	-1.15
67	mmu-miR-194	6.42E-07	-2.76
Calibrator not detected			
68	hsa-miR-149	1.37E-02	4.01
69	hsa-miR-214*	2.82E-03	3.00
70	hsa-miR-22	1.13E-02	3.39
71	hsa-miR-29a*	5.78E-04	3.59
72	hsa-miR-425	4.88E-04	3.75

73	hsa-miR-744*	1.52E-03	2.73
74	hsa-miR-9*	1.34E-02	2.08
75	hsa-miR-99b*	1.18E-04	3.28
76	mmu-let-7a*	1.37E-02	2.25
77	mmu-miR-125b*	1.31E-07	2.96
78	mmu-miR-134	1.18E-03	3.85
79	mmu-miR-200a	8.84E-04	4.96
80	mmu-miR-329	2.77E-02	2.18
81	mmu-miR-337-3p	1.31E-02	1.91
82	mmu-miR-376a	5.80E-04	1.93
83	mmu-miR-376b*	2.13E-03	2.74
84	mmu-miR-382	4.71E-02	2.55
85	mmu-miR-433	1.36E-03	3.04
86	mmu-miR-465b-5p	1.02E-02	2.52
87	mmu-miR-487b	1.83E-03	2.38
88	mmu-miR-676*	7.86E-05	3.30
89	mmu-miR-700	4.66E-03	3.05
90	rno-miR-204*	1.71E-07	3.83
Target not detected			
91	mmu-let-7e	3.92E-02	-3.19
92	mmu-miR-381	6.88E-03	-2.71
93	mmu-miR-542-3p	2.78E-02	-1.88

Table 35: Significantly modulated microRNAs in serum at 24 h post injury post mild CCI injury

S.No	MicroRNA	P Value	Fold change (Log10RQ)
Valid			
1	mmu-miR-322*	1.04E-02	0.67
2	mmu-miR-376c	1.65E-03	0.66
3	hsa-miR-223	1.03E-03	0.61
4	mmu-miR-337	2.03E-03	0.59
5	hsa-miR-200c	4.15E-02	0.54
6	rno-miR-7*	2.47E-02	0.48
7	mmu-miR-101b	2.55E-02	0.46
8	hsa-miR-93*	6.56E-03	0.41
9	mmu-miR-186	3.02E-03	0.40
10	mmu-miR-1274a	2.87E-02	0.38
11	mmu-miR-126-3p	8.37E-03	0.32
12	mmu-miR-26a	0.034728065	0.31
13	rno-miR-351	3.55E-02	0.30
14	mmu-miR-19b	1.40E-02	0.30
15	mmu-miR-29c	0.034664154	-0.29
16	mmu-miR-345	5.08E-03	-0.30
17	mmu-miR-674*	3.83E-02	-0.32
18	mmu-miR-135a	4.60E-02	-0.33
19	mmu-miR-148a	1.11E-02	-0.34
20	mmu-miR-20b	3.63E-02	-0.34
21	mmu-miR-339-3p	4.79E-02	-0.35
22	mmu-miR-361	7.91E-04	-0.35
23	mmu-miR-10a	6.45E-03	-0.36
24	mmu-miR-486	1.11E-02	-0.38
25	mmu-miR-296-5p	3.23E-03	-0.40
26	mmu-miR-331-5p	2.64E-02	-0.41
27	mmu-miR-199b	2.65E-02	-0.41
28	mmu-miR-29b*	2.16E-03	-0.41
29	mmu-miR-423-5p	1.87E-03	-0.46
30	mmu-let-7d	9.86E-03	-0.53
31	mmu-miR-365	2.30E-04	-0.61
32	mmu-miR-141	0.001014915	-0.73
Calibrator not detected			
33	hsa-miR-29a*	5.39E-04	3.57

34	hsa-miR-340	4.88E-02	1.98
35	hsa-miR-9*	7.26E-03	2.40
36	mmu-let-7a*	5.74E-03	2.61
37	mmu-miR-136	3.57E-02	1.53
38	mmu-miR-449b	8.82E-03	1.96
39	mmu-miR-467b	4.90E-02	1.69
Target not detected			
40	hsa-miR-30d*	1.65E-02	-2.90
41	mmu-miR-194	3.21E-04	-5.58
42	mmu-miR-363	8.94E-03	-3.49
43	mmu-miR-381	1.66E-02	-2.32

Table 36: Significantly modulated microRNAs in serum at day 4 post injury post mild CCI injury

S.No	MicroRNA	P Value	Fold change (Log10RQ)
Valid			
1	mmu-miR-322*	4.52E-03	0.94
2	mmu-miR-155	7.08E-03	0.87
3	mmu-miR-146a	5.91E-05	0.75
4	mmu-miR-376c	2.41E-03	0.74
5	mmu-miR-126-3p	9.98E-05	0.73
6	mmu-miR-2138	5.14E-03	0.69
7	mmu-miR-186	7.35E-05	0.68
8	mmu-miR-222	1.43E-05	0.62
9	hsa-miR-140-3p	1.01E-03	0.60
10	rno-miR-7*	1.76E-02	0.56
11	mmu-miR-19b	3.35E-04	0.55
12	hsa-miR-30e-3p	9.28E-03	0.51
13	mmu-miR-342-3p	3.96E-03	0.50
14	mmu-miR-16	1.65E-03	0.49
15	mmu-miR-24	9.96E-04	0.42
16	mmu-miR-101a	3.77E-02	0.40
17	mmu-miR-133a	5.37E-03	0.40
18	mmu-miR-126-5p	5.77E-03	0.39
19	mmu-miR-301a	1.46E-02	0.38
20	mmu-miR-29a	8.43E-03	0.36
21	hsa-miR-93*	2.49E-02	0.35
22	mmu-miR-19a	1.42E-02	0.30
23	mmu-miR-31	1.06E-02	0.30
24	mmu-miR-185	3.36E-02	-0.29
25	mmu-miR-199a-3p	1.72E-02	-0.30
26	mmu-miR-99b	5.97E-03	-0.30
27	hsa-miR-421	3.72E-02	-0.33
28	mmu-let-7b	6.91E-03	-0.37
29	mmu-miR-361	2.61E-03	-0.37
30	mmu-miR-365	1.62E-02	-0.39
31	mmu-miR-1930	2.49E-03	-0.41
32	mmu-miR-486	6.32E-03	-0.45
33	mmu-miR-210	7.34E-03	-0.46
34	mmu-miR-181a	8.82E-03	-0.46

35	hsa-miR-671-5p	5.01E-03	-0.47
36	mmu-miR-337-5p	2.69E-02	-0.49
37	mmu-miR-463*	2.01E-02	-0.49
38	hsa-miR-22*	2.97E-02	-0.51
39	mmu-miR-148a	6.17E-04	-0.52
40	mmu-miR-455	3.80E-02	-0.57
41	mmu-miR-224	2.12E-03	-0.58
42	mmu-miR-199b	4.19E-03	-0.61
43	mmu-miR-2182	1.80E-02	-0.66
44	mmu-miR-296-5p	4.77E-06	-0.72
45	mmu-miR-1928	3.83E-03	-0.91
46	hsa-miR-190b	1.25E-02	-1.65
Calibrator not detected			
47	hsa-miR-149	1.73E-02	3.82
48	hsa-miR-26b*	9.16E-03	1.87
49	hsa-miR-29a*	7.29E-04	3.52
50	hsa-miR-338-5p	3.83E-10	3.81
51	hsa-miR-425	3.73E-04	3.96
52	hsa-miR-744*	2.26E-03	2.54
53	hsa-miR-9*	0.025113978	1.84
54	mmu-let-7a*	1.35E-02	2.25
55	mmu-miR-1960	3.31E-04	3.40
56	mmu-miR-202-3p	4.23E-02	1.84
57	mmu-miR-217	6.06E-05	2.04
58	mmu-miR-23a	1.86E-03	3.73
59	mmu-miR-326	9.35E-09	4.38
60	mmu-miR-467b	1.06E-03	3.54
61	mmu-miR-700	5.43E-03	2.94
62	rno-miR-7a*	5.16E-04	4.76
Target not detected			
63	mmu-let-7e	3.36E-02	-3.04
64	mmu-miR-194	1.22E-03	-4.57

Table 37: Significantly modulated microRNAs in brain at 3 hr post injury post mild CCI injury

S.No	MicroRNA	P Value	Fold change (Log10RQ)
Valid			
1	mmu-miR-1193	4.09E-02	0.61
2	hsa-miR-136	7.88E-04	0.56
3	mmu-let-7a*	2.59E-03	0.56
4	rno-miR-350	3.56E-04	0.55
5	mmu-miR-182	1.53E-03	0.54
6	mmu-miR-2135	3.38E-04	0.53
7	hsa-let-7e*	3.14E-02	0.50
8	rno-miR-352	5.57E-03	0.48
9	hsa-miR-9*	6.24E-03	0.44
10	mmu-miR-805	2.07E-03	0.43
11	mmu-miR-337	2.97E-03	0.41
12	hsa-miR-376a*	2.81E-03	0.40
13	mmu-miR-690	1.44E-03	0.37
14	hsa-miR-29a*	1.12E-02	0.36
15	mmu-miR-300*	1.55E-02	0.35
16	mmu-miR-130b*	2.68E-02	0.34
17	mmu-miR-138	1.08E-03	0.34
18	hsa-miR-338	1.61E-03	0.34
19	mmu-miR-1949	9.36E-03	0.34
20	mmu-miR-337-5p	2.15E-03	0.33
21	mmu-miR-666-5p	6.95E-04	0.32
22	mmu-miR-592	3.40E-03	0.32
23	hsa-miR-136*	2.96E-03	0.31
24	mmu-miR-706	4.11E-03	0.31
25	mmu-miR-384-5p	6.78E-03	0.30
26	hsa-miR-27b*	7.54E-03	0.29
27	mmu-miR-340-3p	0.0051678	-0.29
28	mmu-miR-455	4.07E-02	-0.30
29	mmu-miR-380-3p	4.61E-03	-0.31
30	mmu-miR-15a	1.47E-03	-0.31
31	mmu-miR-215	1.03E-03	-0.33
32	mmu-miR-540-5p	4.35E-03	-0.33
33	mmu-miR-146b	1.18E-02	-0.34
34	rno-miR-224	0.0142256	-0.34

35	mmu-miR-199a-3p	6.53E-03	-0.34
36	mmu-miR-598	1.29E-03	-0.36
37	mmu-miR-450a-5p	2.96E-03	-0.37
38	mmu-miR-20b	1.22E-02	-0.38
39	mmu-miR-297a*	3.15E-04	-0.38
40	mmu-miR-487b	6.34E-03	-0.38
41	mmu-miR-1	1.94E-02	-0.38
42	mmu-miR-543	3.64E-03	-0.39
43	mmu-miR-495	1.59E-03	-0.41
44	mmu-miR-133b	7.06E-03	-0.41
45	hsa-miR-26b*	1.40E-02	-0.43
46	mmu-miR-216a	1.32E-02	-0.44
47	mmu-miR-770-3p	2.12E-02	-0.44
48	mmu-let-7e	8.74E-03	-0.45
49	mmu-miR-504	6.01E-03	-0.46
50	mmu-miR-202-3p	5.95E-03	-0.47
51	mmu-miR-188-5p	9.15E-03	-0.47
52	mmu-miR-214	5.45E-03	-0.48
53	mmu-miR-193	1.85E-02	-0.55
54	mmu-miR-200c	3.35E-04	-0.57
55	mmu-miR-1943	2.04E-02	-0.59
56	mmu-miR-467b	6.06E-03	-0.60
57	mmu-miR-669D	1.44E-02	-0.64
58	hsa-miR-29b-2*	2.14E-04	-0.76
59	mmu-miR-200b	4.86E-02	-0.79
60	mmu-miR-429	5.63E-06	-1.03
Calibrator not detected			
61	hsa-miR-875-5p	6.55E-03	5.11
62	mmu-miR-685	1.49E-08	5.38
Target not detected			
63	mmu-miR-466J	4.90E-02	-2.27
64	mmu-miR-496	1.43E-03	-4.66

Table 38: Significantly modulated microRNAs in brain at 24 hr post injury post mild CCI injury

S.No	MicroRNA	P Value	Fold change (Log10RQ)
Valid			
1	mmu-miR-155	1.47E-04	1.2
2	mmu-miR-331-5p	2.78E-04	1.1
3	mmu-miR-186	2.56E-04	1.0
4	mmu-miR-342-3p	8.30E-04	1.0
5	mmu-miR-489	2.08E-02	0.9
6	mmu-miR-701	4.83E-04	0.9
7	mmu-miR-383	3.66E-04	0.9
8	mmu-miR-449a	3.99E-03	0.8
9	hsa-let-7e*	1.66E-03	0.8
10	mmu-miR-872	2.28E-02	0.8
11	mmu-miR-667	1.07E-02	0.7
12	mmu-miR-125a-5p	9.35E-05	0.7
13	mmu-miR-34c*	1.76E-02	0.7
14	hsa-miR-223	1.75E-03	0.6
15	rno-miR-382*	1.24E-03	0.6
16	mmu-miR-431	1.34E-02	0.6
17	mmu-miR-1948	2.01E-02	0.6
18	mmu-miR-146b	2.56E-03	0.6
19	mmu-miR-487b	4.21E-04	0.5
20	mmu-miR-136	3.44E-04	0.5
21	mmu-miR-690	3.33E-05	0.5
22	mmu-miR-138	3.71E-04	0.5
23	mmu-miR-339-3p	0.022397876	0.5
24	mmu-miR-126-3p	1.43E-04	0.5
25	mmu-miR-805	9.86E-04	0.5
26	hsa-miR-190b	4.00E-02	0.5
27	mmu-miR-409-3p	1.52E-03	0.5
28	mmu-miR-125b*	2.68E-02	0.5
29	mmu-miR-881*	2.91E-05	0.5
30	mmu-miR-16	1.36E-02	0.5
31	hsa-miR-136*	1.79E-03	0.5
32	mmu-miR-222	1.90E-03	0.5

33	mmu-miR-592	6.62E-04	0.5
34	mmu-miR-322*	4.03E-02	0.5
35	hsa-miR-376a*	3.67E-04	0.5
36	mmu-miR-31*	5.78E-04	0.5
37	mmu-miR-547	8.39E-03	0.5
38	mmu-miR-146a	8.99E-04	0.5
39	mmu-miR-191	2.74E-03	0.5
40	mmu-miR-706	1.07E-03	0.5
41	hsa-miR-143	6.01E-03	0.5
42	mmu-miR-210	8.54E-03	0.5
43	mmu-miR-320	2.98E-04	0.5
44	mmu-miR-384-5p	8.25E-04	0.5
45	mmu-miR-376b*	5.71E-05	0.4
46	mmu-miR-193b	7.53E-04	0.4
47	hsa-miR-136	1.34E-02	0.4
48	hsa-miR-127-5p	4.14E-03	0.4
49	mmu-miR-200c	0.041042563	0.4
50	hsa-miR-421	4.25E-02	0.4
51	mmu-miR-484	2.12E-02	0.4
52	mmu-miR-186*	7.49E-04	0.4
53	hsa-miR-9*	1.74E-03	0.4
54	mmu-miR-300	6.37E-05	0.4
55	hsa-miR-338-5P	3.03E-02	0.4
56	mmu-miR-337	5.24E-04	0.4
57	mmu-miR-101b	5.41E-03	0.4
58	hsa-miR-455	6.21E-04	0.4
59	hsa-miR-338	1.11E-03	0.4
60	rno-miR-146B	3.21E-02	0.4
61	snoRNA135	6.97E-04	0.4
62	hsa-miR-27b*	1.42E-02	0.4
63	mmu-miR-376b	1.25E-03	0.4
64	mmu-miR-1949	1.35E-03	0.4
65	hsa-miR-140-3p	4.72E-02	0.4
66	hsa-miR-22*	2.99E-02	0.4
67	mmu-miR-193*	1.30E-02	0.4
68	mmu-miR-184	1.48E-02	0.3
69	mmu-miR-194	5.22E-04	0.3
70	mmu-miR-1839-5p	2.64E-03	0.3
71	hsa-miR-218-2*	7.37E-03	0.3

72	rno-miR-379*	4.02E-03	0.3
73	mmu-miR-543	3.21E-04	0.3
74	mmu-miR-1839-3p	5.40E-03	0.3
75	mmu-miR-139-5p	3.18E-03	0.3
76	mmu-miR-34b-3p	0.036751888	0.3
77	mmu-miR-30e	4.63E-03	0.3
78	mmu-miR-674*	1.19E-02	0.3
79	mmu-let-7g*	2.20E-02	0.3
80	mmu-miR-485-3p	9.49E-03	0.3
81	hsa-miR-29a*	1.45E-02	0.3
82	mmu-miR-24	3.37E-04	0.3
83	mmu-miR-133a	1.30E-02	0.3
84	mmu-miR-16*	2.49E-02	0.3
85	mmu-miR-185	7.34E-04	-0.3
86	mmu-miR-199a-3p	3.53E-02	-0.3
87	mmu-miR-490	3.21E-03	-0.3
88	mmu-miR-669C	2.71E-02	-0.3
89	mmu-miR-674	2.57E-02	-0.3
90	mmu-miR-500	6.41E-03	-0.3
91	mmu-let-7d	1.78E-03	-0.3
92	mmu-miR-29c	1.87E-03	-0.3
93	mmu-miR-330	5.19E-04	-0.3
94	mmu-miR-18a	2.81E-02	-0.3
95	mmu-miR-671-3p	4.14E-02	-0.3
96	mmu-miR-34c	6.87E-03	-0.3
97	mmu-miR-30d	1.14E-02	-0.3
98	rno-miR-758	5.24E-04	-0.3
99	mmu-miR-665	3.75E-02	-0.4
100	mmu-miR-20b	1.53E-02	-0.4
101	mmu-miR-345-5p	2.59E-03	-0.4
102	mmu-miR-495	3.30E-03	-0.4
103	mmu-miR-100	3.94E-03	-0.4
104	mmu-miR-429	2.81E-02	-0.4
105	mmu-miR-30b	1.24E-03	-0.4
106	mmu-miR-301b	6.41E-03	-0.4
107	mmu-miR-106b	6.25E-03	-0.4
108	mmu-let-7e	0.019420108	-0.4
109	mmu-miR-335-5p	6.73E-04	-0.5
110	mmu-miR-93	6.19E-03	-0.5

111	mmu-miR-32	1.10E-02	-0.5
112	mmu-miR-543	2.57E-03	-0.5
113	mmu-miR-99b	1.19E-02	-0.5
114	mmu-miR-345-3p	1.89E-03	-0.5
115	mmu-miR-350	1.07E-04	-0.5
116	mmu-miR-450a-5p	6.20E-03	-0.5
117	rno-miR-346	9.96E-04	-0.5
118	mmu-miR-652	1.25E-02	-0.5
119	mmu-miR-23b	3.55E-02	-0.5
120	mmu-miR-361	2.92E-02	-0.5
121	mmu-let-7c	4.47E-02	-0.5
122	mmu-miR-99a	4.30E-03	-0.5
123	mmu-miR-130b	3.63E-03	-0.5
124	mmu-miR-219	1.17E-03	-0.5
125	mmu-miR-25	2.76E-02	-0.5
126	mmu-miR-181c	8.52E-03	-0.5
127	mmu-miR-181a	7.17E-03	-0.6
128	mmu-miR-467e	5.36E-04	-0.6
129	mmu-miR-503	2.25E-04	-0.6
130	mmu-miR-540-5p	2.40E-03	-0.6
131	mmu-miR-501-3p	1.20E-04	-0.6
132	mmu-miR-362-3p	2.69E-04	-0.6
133	mmu-miR-1	2.09E-03	-0.6
134	mmu-miR-322	1.43E-02	-0.6
135	mmu-miR-130a	2.04E-02	-0.6
136	mmu-miR-148a	1.88E-02	-0.6
137	mmu-miR-296-3p	2.18E-02	-0.6
138	mmu-miR-423-5p	1.75E-03	-0.6
139	mmu-miR-127	1.14E-02	-0.6
140	mmu-miR-125b-5p	1.25E-02	-0.6
141	mmu-miR-467b	2.60E-03	-0.7
142	mmu-miR-7a	3.16E-02	-0.7
143	mmu-miR-215	4.21E-04	-0.7
144	mmu-let-7a	7.83E-03	-0.7
145	mmu-miR-463*	0.018963769	-0.7
146	mmu-miR-29b	3.45E-02	-0.7
147	mmu-miR-551b	2.95E-02	-0.7
148	mmu-miR-1943	1.83E-02	-0.7
149	mmu-miR-328	1.03E-03	-0.7

150	mmu-miR-497	1.00E-03	-0.7
151	mmu-miR-582-5p	5.99E-04	-0.7
152	mmu-miR-380-3p	1.40E-05	-0.8
153	mmu-miR-324-5p	2.74E-02	-0.8
154	mmu-miR-153	1.10E-04	-0.8
155	mmu-miR-598	2.73E-03	-0.8
156	mmu-miR-221	3.45E-03	-0.8
157	rno-miR-409-5p	2.41E-04	-0.8
158	hsa-miR-29b-2*	7.72E-05	-0.8
159	mmu-miR-296-5p	1.69E-04	-0.8
160	mmu-miR-381	0.026899101	-0.8
161	mmu-miR-409-5p	6.61E-03	-0.9
162	mmu-miR-455	3.61E-04	-1.2
163	mmu-miR-1941-5p	4.86E-08	-2.6
Calibrator not detected			
164	mmu-miR-1941-3p	2.62E-02	2.1
165	mmu-miR-465b-5p	1.49E-02	2.2
166	mmu-miR-685	8.47E-08	5.2
167	rno-miR-219-1-3p	3.08E-02	2.4
Target not detected			
168	mmu-miR-294	4.73E-03	-2.2
169	mmu-miR-363	2.80E-02	-2.8
170	mmu-miR-504	3.25E-02	-2.2
171	mmu-miR-546	2.58E-04	-3.8
172	mmu-miR-677	4.16E-02	-1.2
173	mmu-miR-878-3p	4.48E-02	-2.1
174	rno-miR-743a	2.77E-02	-2.1

Table 39: Significantly modulated microRNAs in brain at day 4 post injury post mild CCI injury

S.No	MicroRNA	P Value	Fold change (Log10RQ)
Valid			
1	mmu-miR-155	3.87E-05	1.29
2	rno-miR-351	1.03E-03	0.86
3	mmu-miR-331-5p	7.59E-04	0.78
4	rno-miR-532-5p	1.07E-04	0.63
5	mmu-miR-136*	8.05E-04	0.60
6	mmu-miR-449a	1.92E-02	0.60
7	mmu-miR-666-5p	1.56E-02	0.59
8	mmu-miR-182	2.22E-03	0.56
9	mmu-miR-186	3.25E-03	0.56
10	mmu-miR-138	2.68E-05	0.52
11	hsa-miR-214	1.52E-02	0.50
12	mmu-miR-142-5p	4.56E-04	0.49
13	mmu-miR-342-3p	4.29E-02	0.46
14	mmu-miR-376a*	1.66E-02	0.46
15	mmu-miR-431	9.07E-03	0.43
16	mmu-miR-146a	2.14E-03	0.42
17	hsa-miR-223	1.25E-03	0.41
18	mmu-miR-467c	2.72E-04	0.41
19	mmu-miR-16	2.07E-02	0.41
20	mmu-miR-449b	9.98E-03	0.38
21	mmu-miR-139-5p	1.95E-04	0.37
22	mmu-miR-125a-5p	2.55E-02	0.36
23	mmu-miR-322*	1.59E-03	0.36
24	mmu-miR-701	5.06E-03	0.35
25	mmu-miR-690	4.55E-04	0.35
26	mmu-miR-136	1.87E-04	0.34
27	mmu-miR-384-5p	4.32E-03	0.32
28	hsa-miR-455	4.85E-04	0.32
29	mmu-miR-337	1.06E-03	0.32
30	mmu-miR-320	3.06E-02	0.32
31	mmu-miR-223	3.11E-02	0.30
32	mmu-miR-365	3.75E-03	0.30
33	mmu-miR-193b	1.61E-02	0.29
34	rno-miR-1	3.17E-03	-0.29

35	mmu-miR-764-3p	1.30E-02	-0.30
36	mmu-let-7b	3.72E-05	-0.30
37	mmu-miR-467d	7.84E-03	-0.30
38	rno-miR-346	1.12E-04	-0.31
39	mmu-miR-217	2.78E-02	-0.32
40	mmu-miR-130a	3.55E-02	-0.32
41	rno-miR-409-5p	3.33E-02	-0.32
42	mmu-miR-540-5p	7.80E-03	-0.33
43	mmu-miR-598	3.08E-03	-0.33
44	mmu-miR-543	3.74E-03	-0.33
45	mmu-miR-125b-3p	0.0254954	-0.37
46	mmu-miR-296-5p	3.50E-03	-0.38
47	rno-miR-489	9.09E-03	-0.39
48	hsa-miR-431*	1.45E-02	-0.39
49	mmu-miR-423-5p	2.94E-03	-0.39
50	mmu-let-7c	1.29E-04	-0.39
51	mmu-miR-450a-5p	3.08E-04	-0.40
52	mmu-miR-504	3.09E-02	-0.41
53	mmu-let-7e	2.19E-02	-0.41
54	mmu-miR-490	2.10E-04	-0.41
55	mmu-miR-582-5p	2.31E-02	-0.42
56	mmu-miR-215	3.49E-05	-0.42
57	mmu-miR-1	8.01E-03	-0.43
58	rno-miR-204*	3.08E-02	-0.44
59	mmu-miR-294	1.29E-03	-0.45
60	rno-miR-450a	5.50E-03	-0.46
61	mmu-miR-377	1.91E-03	-0.46
62	mmu-miR-328	7.87E-04	-0.47
63	mmu-miR-380-3p	4.29E-04	-0.47
64	mmu-miR-878-3p	1.01E-02	-0.50
65	mmu-miR-463*	1.19E-02	-0.51
66	mmu-miR-877*	2.00E-02	-0.52
67	mmu-miR-455	4.64E-04	-0.55
68	mmu-miR-702	8.35E-07	-0.56
69	mmu-miR-501-3p	1.30E-06	-0.57
70	mmu-miR-466J	4.85E-03	-0.59
71	mmu-miR-216a	1.74E-03	-0.69
72	mmu-miR-429	1.27E-03	-0.72
73	mmu-miR-202-3p	2.30E-02	-0.79

74	mmu-miR-669D	3.33E-03	-0.80
75	hsa-miR-29b-2*	4.05E-05	-0.85
76	mmu-miR-1896	2.04E-02	-1.21
77	mmu-miR-1941-5p	1.27E-08	-2.66
Calibrator not detected			
78	mmu-miR-1947	1.24E-11	3.14
79	mmu-miR-1953	2.36E-02	0.47
80	mmu-miR-21*	3.32E-02	2.29
81	mmu-miR-465b-5p	2.33E-02	1.92
82	rno-miR-219-1-3p	3.44E-02	2.29
Target not detected			
83	mmu-miR-669C	1.21E-02	-3.45

4.1.7: Venn diagrams of significantly modulated miRNAs in brain and serum post mild CCI indicate common miRNAs between time points

Venn diagrams were constructed to show the commonality among the miRNA expression in both serum and brain samples distributed along all the three time points. In the serum miRNA profiles, around 8 miRNAs were found to common between all the three time points (Figure 33). A similar Venn diagram drawn for the dysregulated brain miRNAs post injury showed around 15 miRNAs to be common between the three time points (Figure 34).

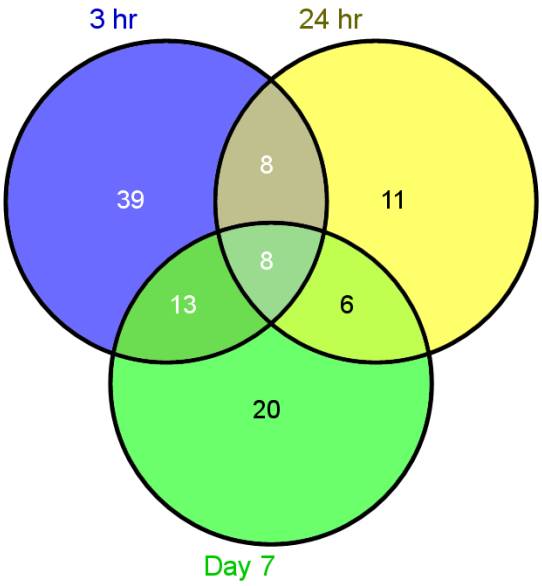

Figure 33: Venn diagram showing distribution of only valid modulated miRNAs between the three time points in serum. Eight miRNAs were found to be common between the three time points in serum post mild CCI.

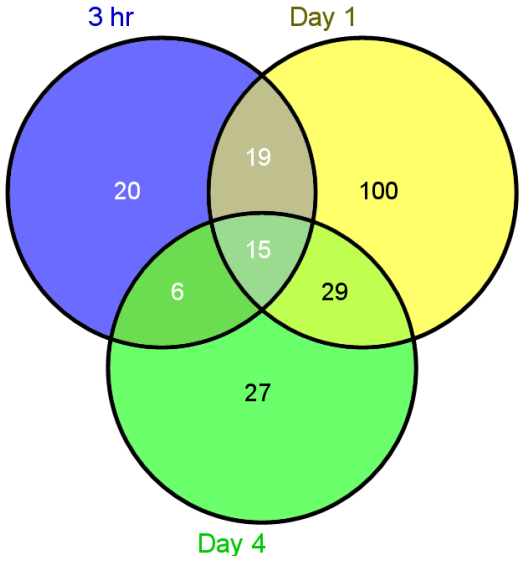

Figure 34: Venn diagram showing distribution of only valid modulated miRNAs between the three time points in brain. Fifteen miRNAs were found to be common between the three time points in brain tissue post mild CCI.

4.1.8: Pathway analysis of brain miRNAs post mild CCI indicate activation of brain-related pathways

The temporal differences in the brain miRNA expression post mild CCI was further studied by determining the pathways that could be affected which was predicted by bioinformatic analysis. Pathway analysis done using the DIANA mirPATH tool shows a difference in the nature of pathways affected among the three time points in the brain post injury. But four neurologically-relevant pathways were found to be common between all the three time points - PI3K-Akt signaling pathway, MAPK signaling pathway, regulation of actin cytoskeleton and focal adhesion (Tables 40-42). Other than the common pathways, certain pathways were found to be unique for the individual time points like the neurotrophin signaling pathway for the 3 h time point (Table 40), neuroactive ligand-receptor interaction for the 24 h time point (Table 41) and ubiquitin mediated proteolysis for the day 4 time point (Table 42). Another widely known pathway, axon guidance was absent in the 24 h time point but was associated with the 3 h and day 4 time point (Tables 40-42).

Table 40: Top ten pathways for brain miRNAs at 3 h time point post mild CCI

S.No	KEGG pathway	p-value	No. of genes	No. of miRNAs
1	PI3K-Akt signaling pathway	5.15E-60	172	50
2	Pathways in cancer	4.29E-58	178	48
3	MAPK signaling pathway	9.54E-55	136	49
4	Regulation of actin cytoskeleton	1.25E-50	121	47
5	Focal adhesion	6.24E-44	112	45
6	Axon guidance	6.96E-41	89	48
7	Wnt signaling pathway	3.20E-37	88	43
8	Ubiquitin mediated proteolysis	8.25E-31	77	40
9	Insulin signaling pathway	8.25E-31	78	46
10	Neurotrophin signaling pathway	2.88E-29	72	40

Table 41: Top ten pathways for brain miRNAs at 24 h time point post mild CCI

S.No	KEGG pathway	p-value	No. of genes	No. of miRNAs
1	Pathways in cancer	4.72E-26	229	140
2	PI3K-Akt signaling pathway	7.81E-24	220	143
3	MAPK signaling pathway	8.75E-20	172	136
4	HTLV-I infection	8.75E-20	173	136
5	Endocytosis	3.54E-18	148	133
6	Regulation of actin cytoskeleton	3.54E-18	150	140
7	Neuroactive ligand-receptor interaction	1.19E-16	143	136
8	Focal adhesion	3.49E-16	140	134
9	Transcriptional misregulation in cancer	4.11E-15	109	134
10	Protein processing in endoplasmic reticulum	4.19E-15	114	122

Table 42: Top ten pathways for brain miRNAs at day 4 time point post mild CCI

S.No	KEGG pathway	p-value	No. of genes	No. of miRNAs
1	Pathways in cancer	2.43E-66	184	64
2	PI3K-Akt signaling pathway	2.24E-63	189	67
3	MAPK signaling pathway	1.59E-47	140	63
4	Regulation of actin cytoskeleton	2.99E-44	126	65
5	Endocytosis	2.14E-42	118	63
6	Focal adhesion	1.46E-37	113	63
7	Axon guidance	2.60E-36	96	61
8	Wnt signaling pathway	2.15E-33	92	62
9	Insulin signaling pathway	8.91E-28	83	59
10	Ubiquitin mediated proteolysis	1.20E-26	78	61

4.1.9: MiRNA profile at 3 h time point and pathways at 24 h time point indicate commonality between mild CHI and mild CCI

To identify miRNAs that may be expressed in serum at an acute time point regardless of injury type (i.e., concussion or contusion), both the serum miRNA list were compared. Comparison of the 3 h serum miRNA profile in the mild CCI model with the 13 miRNA signature which is a part of the 3 h serum miRNA profile in mild CHI model (Sharma et al., 2014) indicates a list of nine common miRNAs between them (Table 43). All the nine miRNAs were up regulated in nature and a few of them like miR-124 were found to have neurological relevance (Yang et al., 2014).

Also, common biological pathways predicted to be affected by significantly modulated miRNAs in the brain tissue at 24 h post injury in three different miRNA cohorts – upregulated miRNAs post mild CHI, downregulated miRNAs post mild CHI and a cohort of both upregulated and downregulated miRNAs post mild CCI were compared with each other. Pathways common between all the four injury groups-IS1, IS2, IS3 and IS4 were seleted for the comparison study. The Venn diagram constructed between the three groups show PI3K-Akt signaling pathway as the common pathway between all the three groups. Also, between the up regulated miRNAs post mild CHI and the 24 h post mild CCI – the three pathways: MAPK signaling pathway, regulation of actin cytoskeleton along with the PI3K-Akt signaling pathway was identified. Other than the 'pathways in cancer' biological pathway which has no known significance to brain physiology only PI3K-Akt signaling pathway was found to be a common pathway between the down regulated miRNAs post mild CHI and the 24 h post mild CCI (Figure 35).

Table 43: Common miRNAs between the 13 miRNA signature in 3 h CHI serum study and 3 h CCI serum profile. Nine common miRNAs were found to be common between the earlier 13 miRNA signature specific to the acute 3 h time point post mild CHI and the serum miRNA profile at 3 h post mild CCI (CND-Calibrator not detected).

S.No	miRNA	P value	Fold change (Log10RQ)	Ct status
1	mmu-miR-376a	5.80E-04	1.93	CND
2	hsa-miR-214	3.56E-04	1.07	valid
3	mmu-miR-214	1.90E-03	0.96	valid
4	mmu-miR-337-5p	1.31E-02	1.91	CND
5	mmu-miR-574-3p	2.04E-03	0.74	valid
6	mmu-miR-434-3p	9.31E-03	0.54	valid
7	mmu-miR-218	3.37E-03	0.36	valid
8	mmu-miR-199a-3p	7.19E-04	0.53	valid
9	mmu-miR-31	3.73E-02	0.48	valid

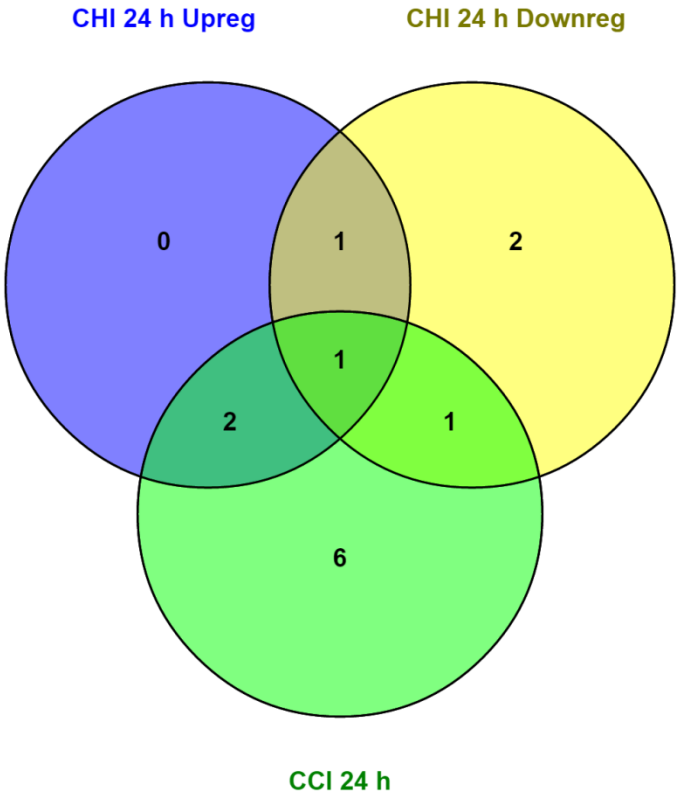

Figure 35: Venn diagram showing distribution of common pathways between the mild CHI and mild CCI model at 24h post injury in brain tissue. PI3K-Akt signaling pathway was found to be a common pathway in all the three lists of miRNA indicating its importance in the 24 h time point post TBI pathophysiology in both CHI and CCI. The pathways corresponding to the CCI 24 h group consisted of common pathways targeted by both upregulated and downregulated miRNAs.

4.2: Discussion

The heterogeneity in TBI has complicated in acquiring a better understanding of the difference in molecular level changes in the injured brain due to variable parameters like injury type, severity and post injury time frame which can help in designing personalized therapeutic strategies for TBI. Molecular level changes in brain like in protein or gene expression post TBI have been reported but the role of microRNAs in pathophysiology of mild TBI is not fully understood. The present study mainly involves studying the changes in miRNA expression in the brain tissue underlying the injury site after being subjected to mild TBI using the weight drop model which has been widely used to recreate the closed head injury or concussion seen in real-life scenario. This primarily involved creating a unilateral injury (left hemisphere in this case) by a direct impact on the skull with the mice being placed on a hard surface. MiRNA expression was determined only for the ipsilateral side of the brain that received the injury as it has already been observed in closed head injury that the changes in gene expression has been observed only in that part of the brain exposed to injury (Israelsson et al., 2009; White et al., 2013).

Histological analysis of the injury site in the brain using standard H & E staining showed no injury lesions in the brain tissue for the early as well as delayed time points – 24 hr and day 7 post injury. However, as previously reported (Sharma et al., 2014) there were clear behavioural changes post injury. Other studies have also shown the presence of behavioral deficits in the closed head injury animal models in a similar absence of overt histological or morphological changes in the injured brain (Yang et al., 2013; Khuman et al., 2011).

Brain miRNA expression in the injury site in the brain is observed to get modulated with a change in the time point post injury as well as based on injury severity. We observed a general decrease in the number of significantly modulated miRNAs at day 7 post injury compared to the 24 hr time point in all the injury groups except for the 333 g/3 cm cohort. The increase in the number of dysregulated miRNAs observed at 24 hr post injury correlate with the transient behavioral deficits and may play a role in mediating the cellular changes that lead to the behavioral deficits. Also, the general decrease in the miRNA modulation at day 7 post injury could be a defining feature of the delayed time point and a possible cause for delayed or long-term behavioral deficits that even can get chronic in certain cases of closed head injury.

The different grades of the closed head injury did not show signs of any injury lesions in the histological examination but still it might bring about molecular level changes at the injury site. The increase in injury severity by increasing the fall height does not bring about any consistent pattern of miRNA expression except that there is higher number of miRNAs expressed at day 7 post injury in the 333 g/3 cm group compared to the other three cohorts.

The presence of common injury-responsive miRNAs overlapping between the four different injury cohorts (except for day 7 downregulated miRNAs) in both the time points may indicate a role for these miRNAs in mediating common injury-responsive molecular pathways at the injury site in brain that are time point-specific. A higher number of common miRNAs (both upregulated and downregulated) at 24 hr time point compared to the day 7 time point might indicate a high activity of cellular changes in the injured brain tissue at this time point.

The temporal nature of the brain miRNA expression profile indicated by a difference in the number of miRNAs expressed in both the time points was further explored by studying the biological pathways that were predicted to be modulated by the injury-responsive miRNAs.

The difference in the pattern of the predicted biological pathways (common among all the four injury groups) at 24 hr post injury and day 7 may indicate the presence of the post injury recovery process in the brain tissue based on the time point. Also, there is difference in the pathways predicted based on whether they are affected by the set of upregulated or downregulated miRNAs suggesting a different role for the co-expressed up and downregulated miRNAs. Comparison of the common pathways at both the time points shows a clear difference between the early 24 hr timepoint where the pathways might mediate cellular changes like inflammation and cytoskeletal reconstruction and the pathways for later time points could mediate neuronal growth and other delayed recovery processes for the brain. The higher number of common biological pathways between the injury cohorts at 24 hr time period compared to the day 7 period is in line with an increase in the number of common miRNAs. These common pathways found between the injury cohorts in the same time point may indicate common post injury mechanisms in spite of difference in the injury severity. Each of the pathways predicted by the DIANA mirPATH program for both the time points can be attributed as a cause for a different facet of TBI pathophysiology.

Pathway analysis for 24 hr time point following CHI

The pathways in the **24 hr** time point are heavily skewed towards cytoskeletal and neuronal reconstruction as well as neuroprotection. The involvement of pathways like PI3K-Akt signaling neurotrophin signaling pathway and regulation of actin cytoskeleton that help in neuroprotection is found to be active in this particular post injury time point.

PI3K-Akt signaling pathway

PI3K-Akt signaling pathway is one of the most important pathways that has been identified as one of the main pathways that is activated post injury in the brain to prevent apoptosis (Chen et al., 2012). The activation of this particular pathway in both the early time points shows its importance in preventing further tissue damage and promoting cell survival.

MAPK signaling pathway

Mitogen-activated protein kinase (MAPK) signaling pathways are a family of pathways involved in signal transduction which get activated in response to various external stimuli/signals like stress and has been found to play a major role in various cellular functions like cell survival, differentiation and death and thus involved in pathology of a range of diseases from cancer to brain disorders like Alzheimer's disease (Seger and Krebs., 1995; Kim and Choi, 2009). MAPK signaling has also been found to play a strong role in neurodegeneration which occurs as a result of oxygen and nitric oxide induced stress (Schroeter et al., 2002). Long-term synaptic plasticity and memory are subjected to regulation by MAPK signaling by controlling the translation of proteins (Kelleher et al., 2004). Change in dendritic morphology has also been implicated due to activation of MAPK pathway (Wu et al., 2001). Both the above points show that MAPK signaling pathway modulation plays a major role in long-term behavioral changes.Activation of MAPK signaling pathways has been acknowledged as an important part of the post TBI secondary injury phase especially in apoptosis (Liou et al., 2003) as well as inflammation.

Regulation of actin cytoskeleton pathway

Actin cytoskeleton is a major player involved in neuroprotection (Laufs et al., 2000) and also prominently involved in synaptic modulation (Dillon and Goda, 2005). They are an essential

component in neuronal morphogenesis especially of dendrites or dendrite spines as well as axon growth (Luo, 2002)

Neurotrophin signaling pathway

Neurotrophin signaling pathway is known to play an important role in regulation of neuronal apoptosis (Miller and Kaplan et al., 2001), neuronal differentiation (Ernsberger, 2009) and also involved in synaptic modulation by mediating changes in synaptic structure and efficacy (Poo et al., 2001).

Pathway analysis for day 7 time point following CHI

The pathway pattern at **day 7** time point is primarily involved in post injury mechanisms for restoring the brain to a normative state showing that post injury repair mechanisms are still at work. Wnt signaling, axon guidance and MAPK signaling were found to be commonly expressed among all the four injury cohorts at day 7 time point and these pathways are of high importance for neuroprotection and cell survival and may be necessary for repairing the injured brain even at this delayed time point.

Wnt signaling pathway

Studies have shown that through mediating the action of several other pathways, Wnt signaling pathway has been found to play an important role in prevention of apoptosis after neuronal injury (Chong et al., 2004; Pećina-Šlaus 2010) as well as in neuronal related functions like dendritic growth and synaptic plasticity (Salinas et al., 2012; Robin et al., 2013; Onishi et al., 2014) and intracellular calcium accumulation (Niu et al., 2012).

Axon guidance pathway – common in both the time points

Axon guidance pathway was found to be commonly affected among all the injury cohorts and in both the time points showing their salient role in the post injury molecular changes in the brain and this makes them a much suitable target for therapeutic strategies without taking into interest the injury severity or even the post injury time point.

Axon guidance pathway is primarily involved in triggering axonal growth and termination based on the action of a series of guidance molecules that ultimately help in the development and maintenance of neural circuits (Curinga et al., 2008). Axon guidance is a common pathway at all

the time points and injury severity may corroborates with the fact that axonal damage is more observed than cell death in the closed head injury model compared to contusion models (Liou et al., 2003; Lu et al., 2000). Also, the increase in the number of gene targets that are a part of the two common pathways was higher in most of the injury cohorts in 24 hr compared to day 7 indicating a possible difference in the role of these pathways on the cellular changes in both the time points.

The mild CCI injury in the study induces significant modulation of miRNAs in both serum and brain at three different time points – 3 h, 24 h and day 7 time point. Clustering analysis clearly shows a difference in the miRNA expression pattern between serum and brain and also the three post injury time point groups get clustered in a different way based on the tissue sample (brain or serum). The number of miRNA expression nearly doubles at 24 h post injury but which decreases at day 4 time point indicating major injury related cellular activity in injured brain and which probably gets restored to a normative state at the day 4 time point. Venn diagrams between the significantly modulated miRNAs at three time points indicate presence of common miRNAs responsive to mild CCI in both serum and brain and these common miRNAs might be involved in common post injury pathways common in all the time points.

All the common pathways predicted to be activated in all the three time points have been implicated to play a major role in TBI related pathology. The actin cytoskeleton is very involved in synaptic modulation (Dillon and Goda, 2005). They are an essential component in neuronal morphogenesis especially of dendrites or dendrite spines as well as axon growth (Luo et al., 2002). The focal adhesion components have also been found to be involved in mechanisms post injury (Carlos et al., 2009).

PI3K-Akt signaling pathway and MAPK signaling pathway have been studied extensively to be involved in normal brain functions as well as post injury molecular changes.PI3K-Akt signaling pathway is one of the main pathways that is activated post injury in the brain to prevent apoptosis (Chen et al ., 2012). The activation of this particular pathway in both the early time points shows its importance in preventing further tissue damage and promoting cell survival. MAPK signaling pathway modulation plays a major role in long-term behavioral changes.

Activation of MAPK signaling pathways has been acknowledged as an important part of the post TBI secondary injury phase especially in apoptosis (Liou et al., 2003) as well as inflammation. Long-term synaptic plasticity and memory are subjected to regulation by MAPK signaling by controlling the translation of proteins (Kelleher et al., 2004). Change in dendritic morphology has also been implicated due to activation of MAPK pathway (Wu et al., 2001).

The presence of nine common miRNAs between the 13 miRNA signature in 3 h CHI serum study and 3 h CCI serum profile as well as the common pathways between the 24 h post CCI and the up regulated/down regulated 24 h post CHI cohorts indicate a strong commonality in the post TBI pathophysiology in both the models and these leads can be further investigated to study biomarkers for TBI irrespective of heterogeneity due to injury type.

4.3: Conclusion

A temporal pattern has been observed in the miRNA expression profiles in the injury site in the brain with a similar temporal pattern observed in the biological pathways regulated by the injury-responsive miRNAs. The pathway analysis confidently shows that miRNAs might play a critical role in most of the changes widely seen in the brain‘s secondary injury response. Thus, their role as an additional molecular level of control shows that they can be exploited for therapeutic strategies for treating the wide range of post TBI cellular changes in the injury site which might lead to behavioral deficits.

Axon guidance pathway may be an important part of the molecular changes seen in the post injury brain across both the early and delayed time points and can aid in studying injury progression exclusively post mTBI especially the closed head injury type and also in the discovery of novel drug targets that can be acted upon irrespective of the post injury time frame or injury severity. Majority of the pathways that were predicted to be regulated by the injury-responsive miRNAs in the injury site in the different post-injury time points have been known to have multifarious but essential roles in TBI pathophysiology.

MicroRNA expression profiling studies in mild CCI model also indicates a temporal pattern in the miRNA expression and the possible pathways getting activated in the three different time points of 3 h, 24 h and day 4 post injury. Also, identification of common miRNAs as well as

pathways between the mild CHI and mild CCI model indicates a commonality in molecular level changes in spite of difference in injury type.

Chapter 5: Blast Overpressure Injury induces Upregulation of MiRNA let-7i in Serum and Cerebrospinal Fluid

5.1: Results

5.1.1: MiRNA expression profile in serum samples of rats exposed to repeated BOP

The objective of this study was to identify serum miRNAs as biomarkers of BOP-induced TBI in war fighters. On the battlefield, military personnel may be exposed to multiple blasts which can happen in a short interval of time or over a span of days. An ideal biomarker should be one that has the sensitivity and specificity to predict injury in these scenarios. To address this, in our study we included two injury groups, SII and LII, as shown in Figure 36. Serum samples from these groups were analyzed for their miRNA modulation in response to BOP injury. In the case of SII, a total of 123 and 75 miRNAs were dysregulated in samples collected at 3 h and 24 h post-BOP injury, respectively. Among these, 33 miRNAs were common in both of the subgroups. In the case of LII, 17 and 19 miRNAs were dysregulated in serum samples collected 3 h and 24 h after the last BOP injury, respectively (Table 44 and Table 45). This difference in the number of miRNAs modulated between SII and LII may be attributable to the fact that in the case of LII, the time between the two injuries was 24 h, and this may help in recovery from the trauma. Previously, in a rat model it was shown that increasing the time from 15 min to 4 h between three successive 26-psi blast exposures decreased the mortality rate from 87% to 27%. If the time interval was increased to 1 day, the mortality dropped further to 7% (Stuhmiller et al., 1991). This suggests that increasing the time interval between repetitive blasts allows a physiological recovery time, which may explain the reduced miRNA modulation in the LII group observed in this study. Moreover, many miRNAs were found to be modulated in specific injury groups, whereas others were modulated in multiple injury groups. From a biomarker perspective, miRNAs that show modulation in various injury groups will be the best candidates for aBOP injury biomarker.

In our study, we found a total of 47 miRNAs modulated in two or more groups with a significance level of $p < 0.05$. Further, five candidate miRNAs were selected for analysis, which includes miR-let-7i, miR-122, miR-340-5p, miR-200b*, and miR-874, since these miRNAs were modulated in the serum of three injury groups. No miRNA was found to be significantly

modulated in all the injury groups (Table 46). MiR-let-7i, miR-122, and miR-340-5p were upregulated in both SII and LII, whereas miR-874 was downregulated. Mir- 200b was upregulated in SII, but was found to be downregulated in one injury subgroup of LII. An important role of these miRNAs in various pathologic conditions of the central nervous system (CNS) has been reported (Table 47). Among these miRNAs, miR-122 and let-7i were shown to be upregulated in liver and brain, respectively, upon exposure to an RDX blast, whereas miR-200b was downregulated in brain tissue (Zhang and Pan, 2009). Further, members of the let-7 miRNA family are highly enriched in brain, specifically in the hippocampus and frontal cortex (Bak et al., 2008; Lagos-Quintana et al., 2002). These studies suggest that among all the modulated miRNAs in serum post-BOP injury, miR-let-7i may be a good candidate biomarker for BOP injury, since it is upregulated in three injury groups and is highly enriched in brain.

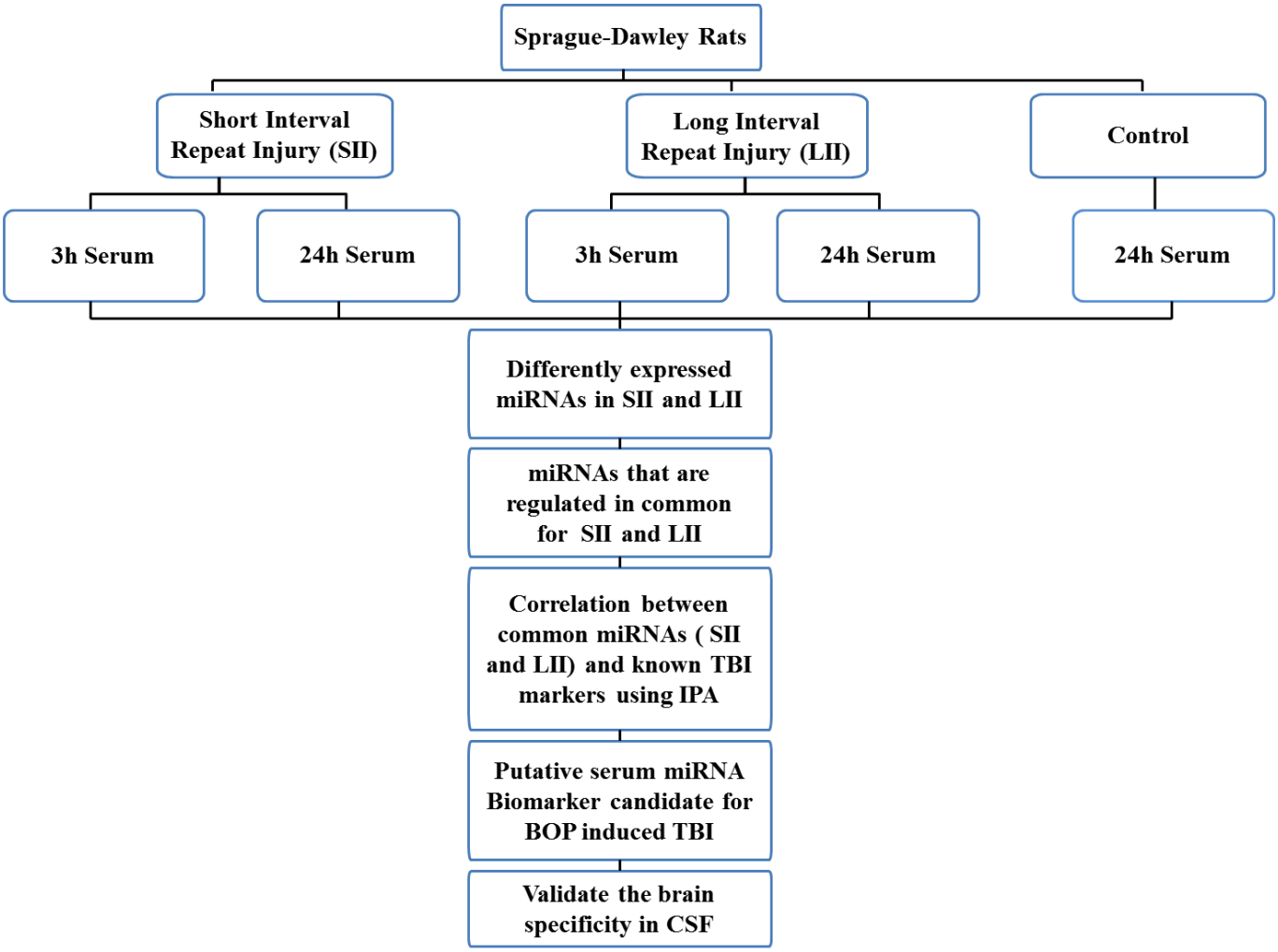

Figure 36: Flow chart showing the experimental design of blast overpressure experiments in the rat. Four different injury groups based on injury interval/time point and control group were used for studying serum/ CSF miRNA expression post blast exposure (CSF, cerebrospinal fluid; IPA, Ingenuity Pathway Analysis).

Table 44: List of BOP exposure modulated miRNAs expressed in short interval injury group

S.No	3 BOP at 2h interval			
	3h serum		24h serum	
	miRNA	Log10RQ	miRNA	Log10RQ
1	mmu-miR-9*	1.665	mmu-miR-16	1.180
2	mmu-miR-18a	1.270	mmu-miR-106a	1.340
3	mmu-miR-126-5p	1.010	mmu-miR-125a-3p	1.168
4	rno-miR-336	1.202	mmu-miR-130b	1.364
5	rno-miR-352	2.437	mmu-miR-132	1.977
6	mmu-miR-690	1.126	mmu-miR-148b	1.688
7	mmu-miR-706	0.802	mmu-miR-155	1.507
8	mmu-miR-10b	2.575	mmu-miR-15a	1.702
9	rno-miR-125b*	1.371	mmu-miR-182	1.449
10	mmu-miR-125b*	1.258	mmu-miR-186	1.179
11	rno-miR-136*	1.417	mmu-miR-193b	1.152
12	mmu-miR-136	1.245	mmu-miR-195	1.184
13	mmu-miR-141	1.385	mmu-miR-20a	1.408
14	mmu-miR-146b*	1.208	mmu-miR-221	1.538
15	rno-miR-148b-5p	2.288	mmu-miR-222	1.212
16	mmu-miR-152	0.891	mmu-miR-223	1.435
17	mmu-miR-15b*	3.092	mmu-miR-224	1.285
18	mmu-miR-181a-1*	1.635	mmu-miR-23b	1.363
19	mmu-miR-183*	1.497	mmu-miR-25	1.231
20	mmu-miR-188-5p	-1.220	mmu-miR-320	1.333
21	mmu-miR-191*	1.555	mmu-miR-335-3p	1.483
22	mmu-miR-199b*	1.177	rno-miR-339-3p	1.238
23	mmu-miR-200a*	2.055	mmu-miR-339-5p	1.707
24	rno-miR-204*	1.544	mmu-miR-345-5p	1.231
25	mmu-miR-205	1.431	mmu-miR-34a	2.041
26	rno-miR-20a*	1.987	mmu-miR-362-3p	1.612
27	rno-miR-20b-5p	1.783	mmu-miR-363	1.979
28	mmu-miR-214*	1.602	mmu-miR-376c	1.663
29	mmu-miR-218-1*	1.659	mmu-miR-451	1.261
30	mmu-miR-218-2*	1.381	mmu-miR-484	1.217
31	mmu-miR-22	2.361	mmu-miR-582-3p	1.277
32	rno-miR-23a*	2.332	mmu-miR-671-3p	1.543
33	mmu-miR-24-2*	1.434	mmu-miR-99b*	-1.178

34	rno-miR-25*	1.856	34	mmu-miR-92a	1.380
35	mmu-miR-27a*	1.133	35	mmu-miR-26a	1.232
36	mmu-miR-27b*	2.508	36	mmu-miR-30c	1.408
37	rno-miR-28*	2.690	37	mmu-let-7c-1*	-1.669
38	mmu-miR-28*	1.809	38	mmu-let-7i	1.881
39	mmu-miR-297c	1.643	39	mmu-miR-1	-1.696
40	mmu-miR-298	1.517	40	mmu-miR-101a*	1.128
41	mmu-miR-29a*	2.930	41	mmu-miR-106b*	1.487
42	rno-miR-29b-2*	1.469	42	mmu-miR-122	1.449
43	mmu-miR-30a*	1.489	43	mmu-miR-130a	1.698
44	mmu-miR-30b*	2.454	44	mmu-miR-140	1.132
45	rno-miR-30d*	2.885	45	mmu-miR-142-5p	1.311
46	mmu-miR-30e*	1.335	46	mmu-miR-148a	1.700
47	mmu-miR-322	1.635	47	mmu-miR-17*	1.307
48	mmu-miR-326	1.312	48	mmu-miR-185	1.259
49	mmu-miR-33*	2.138	49	mmu-miR-192	1.607
50	mmu-miR-330*	1.558	50	mmu-miR-193*	1.606
51	mmu-miR-331-5p	1.074	51	mmu-miR-194	1.794
52	rno-miR-345-3p	1.291	52	mmu-miR-19a	1.352
53	mmu-miR-370	1.297	53	mmu-miR-200b*	1.235
54	mmu-miR-374	3.255	54	mmu-miR-20b*	1.225
55	mmu-miR-376a	1.431	55	mmu-miR-21*	1.097
56	mmu-miR-376b*	1.372	56	mmu-miR-26b*	1.233
57	mmu-miR-378	1.673	57	mmu-miR-29c*	-1.337
58	mmu-miR-379	1.515	58	mmu-miR-301a	1.243
59	mmu-miR-431	1.890	59	mmu-miR-301b	1.325
60	rno-miR-450a	0.898	60	mmu-miR-31*	1.485
61	mmu-miR-467b*	1.395	61	mmu-miR-340-5p	1.273
62	mmu-miR-499	2.288	62	mmu-miR-350	1.623
63	mmu-miR-501-3p	2.076	63	mmu-miR-429	1.317
64	mmu-miR-532-5p	3.523	64	mmu-miR-449a	1.821
65	mmu-miR-592	1.395	65	mmu-miR-450a-5p	2.241
66	mmu-miR-665	1.477	66	mmu-miR-455*	1.446
67	mmu-miR-674*	1.295	67	mmu-miR-652	2.317
68	mmu-miR-7a*	1.324	68	mmu-miR-744*	1.536
69	mmu-miR-720	1.048	69	mmu-miR-802	1.493
70	mmu-miR-764-5p	2.112	70	mmu-miR-872	3.198
71	rno-miR-7a*	2.128	71	mmu-miR-93*	1.274
72	mmu-miR-7b	1.373	72	rno-miR-219-1-3p	1.571

73	mmu-miR-875-5p	2.294	73	rno-miR-505	1.840
74	mmu-miR-879*	2.221	74	rno-miR-532-5p	1.101
75	rno-miR-99a*	2.051	75	mmu-miR-874	-1.676
76	mmu-let-7a*	2.214			
77	mmu-let-7g*	2.016			
78	mmu-miR-365	0.786			
79	mmu-miR-199a-5p	1.451			
80	mmu-miR-19b	1.052			
81	mmu-miR-219	1.465			
82	mmu-miR-29b	1.361			
83	mmu-let-7c-1*	0.978			
84	mmu-let-7i	1.325			
85	mmu-miR-101a*	1.861			
86	mmu-miR-101b	3.077			
87	mmu-miR-106b*	3.178			
88	mmu-miR-122	1.602			
89	mmu-miR-130a	1.677			
90	mmu-miR-140	1.161			
91	mmu-miR-148a	1.453			
92	mmu-miR-17*	1.603			
93	mmu-miR-185	1.021			
94	mmu-miR-192	1.243			
95	mmu-miR-193*	1.700			
96	mmu-miR-19a	1.024			
97	mmu-miR-200b*	1.411			
98	mmu-miR-20b*	1.965			
99	mmu-miR-21*	1.649			
100	mmu-miR-26b*	1.124			
101	mmu-miR-29c*	1.327			
102	mmu-miR-301a	1.015			
103	mmu-miR-301b	1.048			
104	mmu-miR-31*	1.797			
105	mmu-miR-340-5p	1.543			
106	mmu-miR-34c*	1.069			
107	mmu-miR-350	1.525			
108	mmu-miR-425*	2.347			
109	mmu-miR-449c	2.333			
110	mmu-miR-450a-5p	2.592			
111	mmu-miR-455*	2.304			

112	mmu-miR-542-3p	1.283
113	mmu-miR-652	1.148
114	mmu-miR-744*	1.638
115	mmu-miR-7a	1.554
116	mmu-miR-802	2.113
117	mmu-miR-872	1.620
118	mmu-miR-93*	0.882
119	rno-let-7e*	1.989
120	rno-miR-219-1-3p	1.077
121	rno-miR-363*	1.802
122	rno-miR-505	1.671
123	rno-miR-532-5p	0.927

Table 45: List of BOP exposure modulated miRNAs expressed in long interval injury group

	3 BOP at 24h interval				
	3h serum		S.No	24h serum	
S.No	miRNA	Log10RQ		miRNA	Log10RQ
1	mmu-miR-107	1.583	1	mmu-miR-103	-1.849
2	mmu-miR-134	0.961	2	mmu-miR-10a	-1.340
3	mmu-miR-323-3p	1.469	3	mmu-miR-181a	-1.250
4	mmu-miR-490	1.853	4	mmu-miR-187	-1.957
5	mmu-let-7i	1.574	5	mmu-miR-200c	-1.516
6	mmu-miR-1	-1.236	6	mmu-miR-203	-1.732
7	mmu-miR-122	1.054	7	rno-miR-207	-1.478
8	mmu-miR-142-5p	-1.030	8	mmu-miR-210	-1.462
9	mmu-miR-340-5p	1.069	9	rno-miR-351	-1.386
10	mmu-miR-425*	1.588	10	mmu-miR-351	-1.464
11	mmu-miR-449a	1.055	11	rno-miR-489	1.233
12	mmu-miR-449c	1.096	12	mmu-miR-672	-1.249
13	mmu-miR-542-3p	1.126	13	mmu-miR-877*	1.286
14	mmu-miR-7a	1.366	14	mmu-miR-101b	1.492
15	rno-let-7e*	1.245	15	mmu-miR-194	-1.253
16	rno-miR-363*	1.967	16	mmu-miR-200b*	-1.810
17	mmu-miR-874	-1.423	17	mmu-miR-34c*	1.397
			18	mmu-miR-429	-1.822
			19	mmu-miR-874	-1.914

Table 46: MiRNAs Modulated in Serum of Animals Exposed to BOP Injury in Both SII and LII Groups. The data were normalized using endogeneous control by using Statminer software ($p < 0.05$). Highlighted MiRNAs are modulated in three injury groups.

S No.	miRNA	Fold change (Log10)			
		Blast 2h interval X 3 times		Blast 24h interval X 3 times	
		3h serum	24h serum	3h serum	24h serum
1	mmu-let-7c-1*	0.978	-1.669		
2	**mmu-let-7i**	**1.560**	**1.881**	**1.574**	
3	mmu-miR-1		-1.696	-1.236	
4	mmu-miR-101a*	1.861	1.128		
5	mmu-miR-101b	3.077			1.492
6	mmu-miR-106b*	3.178	1.487		
7	**mmu-miR-122**	**1.602**	**1.449**	**1.054**	
8	mmu-miR-130a	1.677	1.698		
9	mmu-miR-140	1.161	1.132		
10	mmu-miR-142-5p		1.311	-1.030	
11	mmu-miR-148a	1.453	1.700		
12	mmu-miR-17*	1.603	1.307		
13	mmu-miR-185	1.021	1.259		
14	mmu-miR-192	1.243	1.607		
15	mmu-miR-193*	1.700	1.606		
16	mmu-miR-194		1.794		-1.253
17	mmu-miR-19a	1.024	1.352		
18	**mmu-miR-200b***	**1.411**	**1.235**		**-1.810**
19	mmu-miR-20b*	1.965	1.225		
20	mmu-miR-21*	1.649	1.097		
21	mmu-miR-26b*	1.124	1.233		
22	mmu-miR-29c*	1.327	-1.337		
23	mmu-miR-301a	1.015	1.243		
24	mmu-miR-301b	1.048	1.325		
25	mmu-miR-31*	1.797	1.485		
26	**mmu-miR-340-5p**	**1.543**	**1.273**	**1.069**	
27	mmu-miR-34c*	1.069			1.397
28	mmu-miR-350	1.525	1.623		
29	mmu-miR-425*	2.347		1.588	
30	mmu-miR-429		1.317		-1.822
31	mmu-miR-449a		1.821	1.055	

32	mmu-miR-449c	2.333		1.096	
33	mmu-miR-450a-5p	2.592	2.241		
34	mmu-miR-455*	2.304	1.446		
35	mmu-miR-542-3p	1.283		1.126	
36	mmu-miR-652	1.148	2.317		
37	mmu-miR-744*	1.638	1.536		
38	mmu-miR-7a	1.554		1.366	
39	mmu-miR-802	2.113	1.493		
40	mmu-miR-872	1.620	3.198		
41	mmu-miR-93*	0.882	1.274		
42	rno-let-7e*	1.989		1.245	
43	rno-miR-219-1-3p	1.077	1.571		
44	rno-miR-363*	1.802		1.967	
45	rno-miR-505	1.671	1.840		
46	rno-miR-532-5p	0.927	1.101		
47	**mmu-miR-874**		**-1.676**	**-1.423**	**-1.914**

Table 47: Modulated miRNAs and their association with neuropathology

S.No	miRNA	Neuropathological Function	References
1	miR-122	Liver specific, altered in hepatic ischemia-reperfusion (I=R) injury, hypoxic human neuroblastoma cells Significantly upregulated in mouse brain tissue after RDX exposure	Takada et al., 2006; Xu et al., 2009; Yamagata et al., 2010; Zhang and Pan 2009
2	miR-340	Induced during ischemic preconditioning	Lusardi et al., 2010
3	Let-7i	Brain-enriched miRNA 1) induced during ischemic preconditioning 2) induced during the Triggering receptor expressed on myeloid cells involved in inflammatory diseases and septic shock, expressed on myeloid cells, mainly macrophages and neutrophils. Significantly upregulated in mouse brain tissue after RDX exposure	Podolska et al 2011; Syed et al., 2010; Zhang and Pan 2009
4	miR-200b*	Upregulated early after ischemic preconditioning and the miR-200 family was neuroprotective, mainly by downregulating prolyl hydroxylase-2 levels Significantly downregulated in mouse brain tissue after RDX exposure	Bak et al., 2008; Lee et al., 2010; Zhang and Pan 2009
5	miR-874	MicroRNAs induced during ischemic preconditioning	Lee et al., 2010

5.1.2: Validation of miR-let-7i expression

Most of the published studies to quantify miRNAs for the purpose of biomarker development involve the use of realtime PCR. However, due to low serum concentrations of miRNAs, a pre-amplification step is performed, which can potentially introduce bias in later quantitation (Scholer et al., 2010). Hence, we validated miR-let-7i in the serum of animals exposed to short-interval BOP with independent Taqman miRNA assays in which pre-amplification of the cDNA is not required. We found that miR-let-7i expression was significantly up regulated in SII in both the subgroups. In the case of LII, no significant change in the expression of miR-let-7i was observed. Overall these results correlate with the miRNA profiling done using TLDA cards (Figure 37).

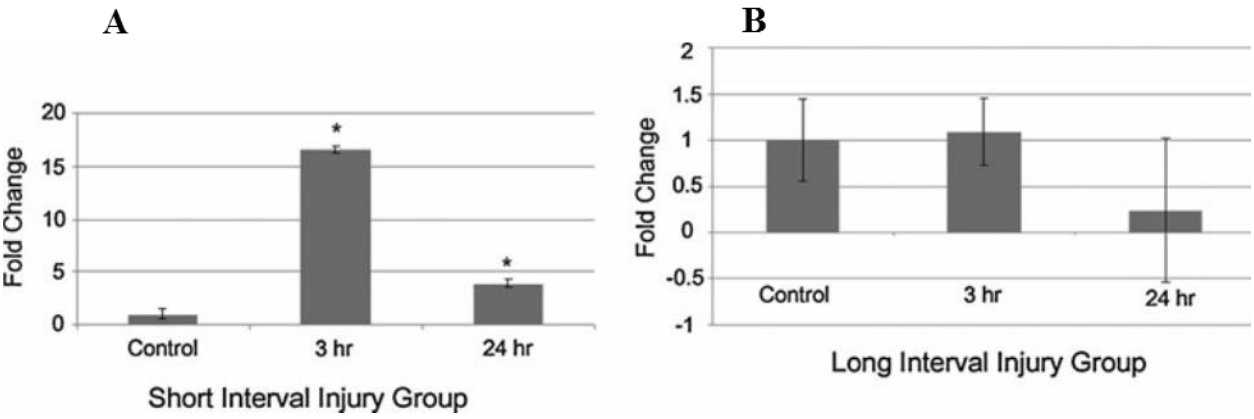

Figure 37: Validation of miR-let 7i miRNA in the short interval (SII) and long interval (LII) groups. The levels of miRNA were normalized by the level of MammU6 endogenous control RNA and all reactions were performed in triplicate. (A) Expression levels of let-7i were significantly upregulated in SII. (B) No significant upregulation of let-7i was observed in both subgroups of LII (*$p < 0.05$).

5.1.3: MiR-Let-7i expression in CSF

Earlier, it was suggested that a biomarker of brain injury is often detected in serum as well as CSF (Papa et al., 2010), therefore we also analyzed the expression of miR-let-7i in the CSF of the animals exposed to BOP injury. CSF samples from the same group of animals were analyzed using a specific real-time miRNA assay for miR-let-7i. Overexpression of miR-let-7i in CSF samples in the SII group of more than eightfold and fivefold were observed for the 3 h and 24 h groups, respectively ($p < 0.05$), whereas in the LII group only the 3 h injury group showed a twofold increase, which failed to reach significance. The group with injuries 24 h apart, and samples collected 24 h after the last BOP exposure, also did not show any significant increase over the control samples (Figure 38). This pattern correlates with the expression of miR-let-7i in the serum of these animals, which further supports our hypothesis that increased expression of miR-let-7i in serum and CSF is of CNS origin.

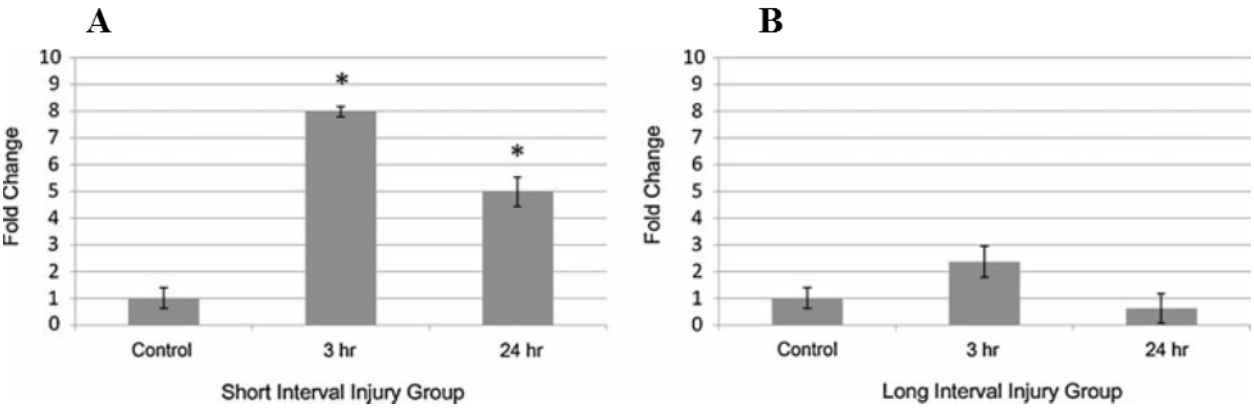

Figure 38: Expression of miR-let-7i in both the short interval (SII) and long interval (LII) groups in cerebrospinal fluid (CSF) of rats exposed to blast overpressure (BOP). (A) A significant increase in the expression of miR-let-7i was observed in the SII groups post-BOP exposure. (B) No significant difference was observed in the expression of miR-let-7i in the LII groups (*$p < 0.05$).

5.1.4: Functional pathway analysis of modulated miRNAs

Even though only five miRNAs were found to be modulated in both the SII and LII groups out of a total of 47, the remaining miRNAs may also play important roles in the pathology of BOP injury. Biological pathways are the major functional units that are involved in physiological and pathological processes. A single miRNA can act on several targets, and a single mRNA can be targeted by multiple miRNAs. To determine the role of the modulated miRNAs after BOP injury in different biological pathways, we performed DIANA mirPath analysis of 47 modulated miRNAs (Table 46). Three miRNA prediction web tools; DIANA microT 4.0, TargetScan 5, and PicTar, were used by this program, which identified axon guidance and Wnt signaling as the two topmost KEGG pathways (Table 48). Previously, neuropathology of the brains of the animals exposed to 120-kPa blasts was studied. Diffuse axonal injury was observed, including mechanical shearing of axons, damage to the cytoskeleton, interruption of axoplasmic flow, and calcium influx into axons, resulting in interruption of traffic along these interconnecting pathways. Further, BBB breakdown and oxidative stress were also observed (Readnower et al., 2010; Young, 2010). Wnt signaling has been implicated in the regulation of the BBB, along with the regulation of b-catenin, claudin, occludins, and other tight junction proteins in the endothelial cells that form the blood vessels (Polakis, 2008). Together, the results of the pathway analysis and the evidence from the previous studies indicate that miRNAs modulated in the serum after BOP injury may play an important role in neurological pathways.

Table 48: Functional Pathway Analysis Using DIANA mirPath Software

S No.	KEGG Pathway – Target Scan	No. of Genes (Union)	-In (p-value) Union	KEGG Pathway – microT4	No. of Genes (Union)	-In (p-value) Union	KEGG Pathway -PicTar	No. of Genes (Union)	-In (p-value) Union	KEGG Pathway – Target Scan-PicTar-microT4	No. of Genes (Union)	-In (p-value) Union
1	Axon guidance	63	29.59	Axon guidance	67	38.55	Axon guidance	44	24.8	Axon guidance	85	44.34
2	Focal adhesion	84	28.9	MAPK signaling pathway	106	33.93	MAPK signaling pathway	69	21.6	Wnt signaling pathway	86	33.73
3	MAPK signaling pathway	99	23.59	Focal adhesion	84	30.96	Wnt signaling pathway	45	20.33	Focal adhesion	105	31.86
4	Glioma	35	21.89	Renal cell carcinoma	40	27.8	Colo-rectal cancer	27	12.72	MAPK signaling pathway	128	29.14
5	TGF-beta signaling pathway	43	19.15	Glioma	36	25.13	Melano-genesis	29	11.46	Colorectal cancer	54	24.96
6	Chronic myeloid leukemia	38	18.55	Regulation of actin cyto-skeleton	84	24.11	TGF-beta signaling pathway	27	11.4	Regulation of actin cytoskeleton	106	24.86
7	Renal cell carcinoma	35	17.16	Wnt signaling pathway	63	23.1	mTOR signaling pathway	18	10.1	Melano-genesis	58	22.01
8	Wnt signaling pathway	59	16.67	Chronic myeloid leukemia	39	21.39	Insulin signaling pathway	35	9.14	ErbB signaling pathway	52	21.07
9	Adherens junction	36	16.47	Colorectal cancer	42	20.99	Renal cell carci-noma	21	8.77	Glioma	41	20.46
10	Regulation of actin cyto-skeleton	78	16.13	ErbB signaling pathway	41	18.79	ErbB signaling pathway	24	8.26	Chronic myeloid leukemia	47	20.37

For all the significantly modulated microRNAs (miRNAs), the DNA intelligent analysis (DIANA) mirPath algorithm combined with miRNA target prediction web tools of DIANA microT 4.0, TargetScan 5, and PicTar was performed. The results represented here indicate the top predicted biological functions of modulated miRNAs (KEGG-Kyoto Encyclopedia of Genes and Genomes).

5.1.5: MiR-Let-7i may regulate important TBI-related proteins

The DIANA mirPath analysis for all the modulated miRNAs indicated their role in neurological processes. We further specifically studied miR-let-7i for its role in the regulation of neurological pathways. We used IPA network analysis software and made a network using all of the important TBI-related proteins and correlated it with miR-let-7i gene targets. The analysis showed that miR-let-7i may regulate many proteins and inflammatory cytokines, including S100b and UCH-L1, which are currently proposed as candidate protein biomarkers for TBI through intermediate downstream molecules (Figure 39).

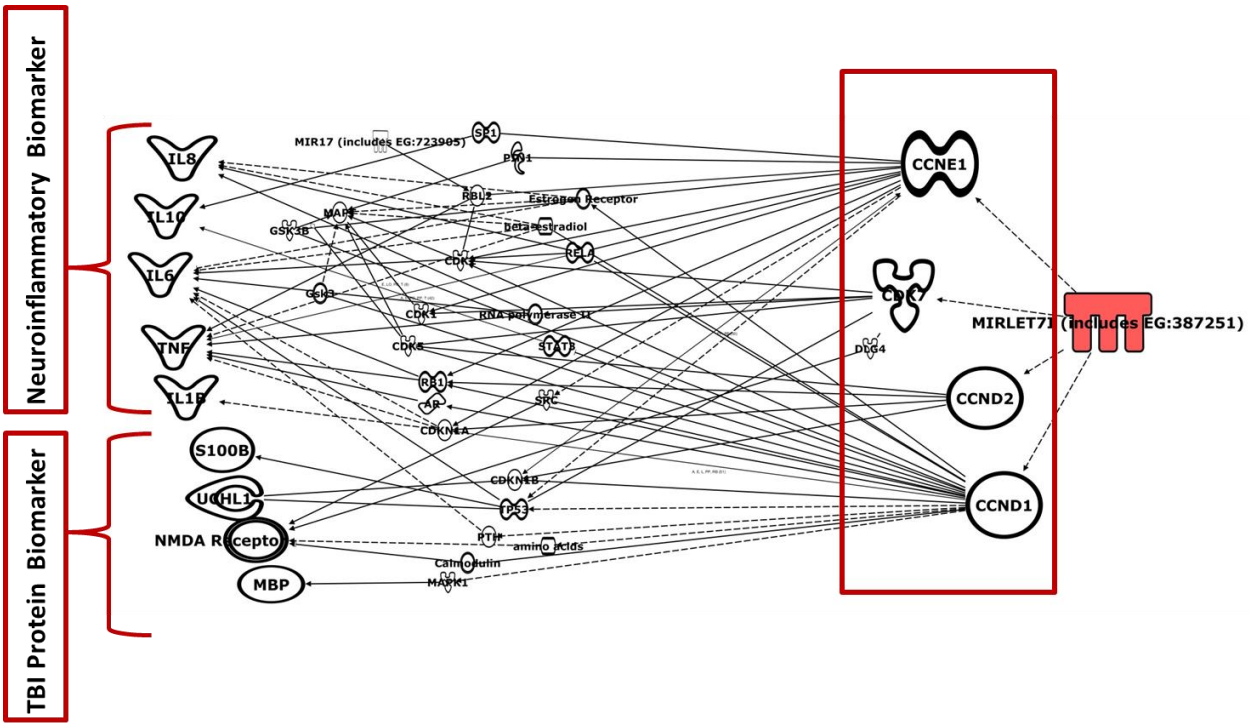

Figure 39: Functional interaction networks of known TBI-related protein biomarkers and inflammatory molecules predicted to be regulated targets of miRNA let-7i as predicted by the Ingenuity Pathway Analysis program (NMDA, N-methyl-Daspartate; UCHL-1, ubiquitin C-terminal hydrolase-1; TNF-a, tumor necrosis factor-a; IL-1b, interleukin-1b; IL-6, interleukin-6; IL-8, interleukin-8).

5.2: Discussion

Biomarker development often uses two approaches: ‘‘topdown’’ and ‘‘bottom-up’’ (Dash et al., 2010). Most commonly a top-down approach has been used for biomarker discovery. In this method, a biomarker, commonly a protein will be identified in the injured brain, and then its presence in peripheral body fluids is evaluated. Depending on its level of expression, its utility as a diagnostic marker is determined; this might be lessened if its expression is too low. In this study, we have followed a bottom-up approach, in which a biomarker in the peripheral fluid is first determined, and then is correlated with the pathology of brain injury. A collective signature of these biomarkers may help in diagnosing a condition such as brain injury.

Serum-based biomarkers are valuable for the early diagnosis of TBI due to the non-invasive method of sample collection. Many serum proteins have been studied for developing a diagnostic biomarker for TBI. S100b is a small dimeric calcium-binding protein that has been extensively studied as a TBI biomarker. It is abundantly expressed in glial cells of the CNS, and is detected in serum following TBI (Geyer et al., 2009). Another promising candidate is GFAP, a filamentous protein found only in the astroglial cytoskeleton. Although GFAP has shown to be a good biomarker for severe TBI, no significant changes have been detected in GFAP expression in cases of mild TBI (Pelinka et al., 2004). Recently, UCH-L1, a neuronal marker, has been identified as a biomarker for severe TBI. Elevated levels of UCH-L1 were detected in serum and CSF samples in patients with severe TBI (Papa et al., 2010). High levels of UCH-L1 were detected in serum and CSF of rats exposed to severe BOP injury (Svetlov et al., 2010). Despite the numerous studies on the development of protein-based biomarkers for TBI, none of the candidates have been successfully used in the clinic to diagnose patients. Recently, miRNAs have emerged as novel diagnostic biomarkers for various diseases, including neurodegenerative disorders like Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and schizophrenia (Barbato et al., 2009; Lukiw et al., 2008). Further, miRNAs have also been implicated as circulating biomarkers in tissue injury, including liver, muscle, and brain (Laterza et al., 2009).

The systemic effects of the level of blast wave (~120 kPa) used in the present study were previously characterized and were shown to produce acute reflex suppression in rats, which is consistent with concussion and mild TBI, and closely resembles the clinical manifestations of

blast-induced TBI (Bruns and Jagoda, 2009; Jones et al., 2007). After exposure to 120 kPa BOP, a substantial reactive astrocytosis was observed in the cerebellum and hippocampus as early as 3 h post blast (Cullen et al., 2011). Widespread increased expression of GFAP and inflammation, including microglial activation and neutrophil infiltration, was observed in the cerebrum, hippocampus, and cortex region (Cullen et al., 2011; Readnower et al., 2010). In our experiments, we used two different groups for comparing the short-term and long-term effects of multiple BOP injury. We found more miRNAs modulated in the SII groups than in the LII groups. This can be correlated with the recent study by Readnower and associates (2010), who demonstrated that the maximum BOP injury severity is visible immediately after injury, and that the animals show signs of recovery by the end of third day. However, we did not observe any increase in the number and extent of miRNA modulation between 3 and 24 h post-injury.

Another important factor to keep in mind when studying about biomarkers for TBI is whether the specific biomolecule is modulated directly in response to the injury or is a secondary effect of the injury. A promising biomarker candidate will be one that is not only detected for an extended period of time after injury, but also appears immediately after the injury. Keeping these criteria in mind, we analyzed the data and selected five miRNAs to study (miR-let-7i, miR-122, miR-200b*, miR-340- 5p, and miR-874). We searched the literature for the expression and role of these miRNAs and found that only miR-let-7i is highly enriched in brain and is overexpressed upon blast exposure. Mir-122 is also up regulated following blast, but has been shown to be liver-specific. Based on these observations it appeared that increased expression of miR-let-7i was a direct effect of injury to the brain, and therefore it may be a good candidate for further studies. The next step was to see if its expression was increased in CSF of the animals, since most of the neuronal markers that are detected in serum are also suggested to be present in CSF (Papa et al., 2010). The increased expression of miR-let-7i in the CSF samples correlated with that of serum, which further indicated that expression of miR-let-7i was caused from brain injury. To further validate this hypothesis, we selected the existing TBI biomarker candidates, such as protein and neuroinflammatory cytokine biomarkers (Kövesdi et al., 2010; Svetlov et al., 2009), and the five aberrant miRNAs in rat serum that are expressed in both SII and LII BOP exposures. These were added to ‘‘my pathway workflow’’ in Ingenuity Pathway Analysis to build customized pathways. Among the five aberrant miRNAs, only miR-let-7i showed

involvement in the regulatory pathways of several proteins and neuroinflammatory cytokine biomarkers. Recently, Redell and colleagues (2010) have shown miRNA modulation in a patient with TBI. Five miRNAs, miR-16, miR- 26a, miR-92a, miR-638, and miR-765, were modulated in severe TBI patient samples. In our study, we did not observe significant modulation of these miRNAs in any of the injury groups. This may be because the type of injury was different in our study in comparison to the patient study, in which there was no BOP exposure. This suggests that more studies are required to understand whether a single miRNA signature can be used as a biomarker for different types of TBI.

5.3: Conclusion

Some molecular biomarkers are released very early after brain injury, while others may not appear until 24–48 h after the injury. The biomarkers that are released very early after TBI will be the most relevant to the early diagnosis, particularly for the field diagnosis of mild and moderate TBI. In this study, we have for the first time shown modulation of miRNAs in serum and CSF in response to BOP injury. Specifically, miR-let-7i appeared at elevated levels in the serum as early as 3 h post-injury, and was upregulated in the SII groups. Moreover, we found elevated levels of miR-let-7i in the CSF samples in the SII groups, whereas a modest up regulation was also observed in the LII groups. Together, these indicate that miR-let-7i can be used as a biomarker for the early detection of blast-induced TBI. Validation of its role may lead to new therapeutic interventions for TBI involving miR-let-7i. To our knowledge this is the first study to report miR-let-7i as a biomarker for blast-induced TBI.

Chapter 6: Traumatic Stress induced Serum and Amygdala MicroRNA Signatures as a Potential Biomarker for Post-traumatic Stress Disorder

6.1: Results

6.1.1: Analysis of miRNA signatures in serum and correlation with amygdala miRNAs following exposure to traumatic stress

RNA quantity and quality analysis done using Small RNA kit in the Bioanalyzer instrument showed sufficient quantity of miRNA of good quality that can be used for the purpose of real-time PCR (Figure 40). The miRNA expression profiling identified 82 miRNAs, which were differentially expressed at day 14 after traumatic stress, whereas only 18 miRNAs were modulated in serum at day 0 after the cessation of stress (Table 49). Our primary objective of this study was to identify miRNA candidates in serum to diagnose PTSD; therefore, we also evaluated the miRNAs expression in amygdala due to its critical role in fear conditioning (Morey et al., 2012). A comparison of miRNAs expression profile in amygdala at day 0 and day 14 with serum miRNAs indicated a similar miRNA modulation pattern (Table 50). Fourteen miRNAs were modulated at day 0 whereas 60 miRNAs were modulated at day 14 after the cessation of stress. We also observed that most of the modulated miRNAs at day 0 were significantly down regulated in both serum (27 out of 31) and amygdala (8 out of 14). However, this trend of miRNA down regulation at day 0 was reversed at day 14 post stress where 78 out of 82 miRNAs were up regulated in serum and all 60 significantly modulated miRNAs were up regulated in amygdala. No common miRNAs were found between all the four groups. However, comparison of serum and amygdala profiles showed 9 common miRNAs at day 14. No similar miRNAs between serum and amygdala were observed at day 0. Comparison of miRNAs in serum samples at day 0 and 14 showed 18 common miRNAs whereas only 4 miRNAs were common in amygdala profiling data at day 0 and day 14 (Figure 41). The symptoms and pathophysiology of PTSD in our model has been previously reported to develop at day 14 after stress exposure, which also correlates with the changes in the miRNA expression profile. Moreover, PTSD in humans has been shown to develop over a period of time after the traumatic stress (Jia et al., 2012). Therefore, we compared miRNA profiles of day 14 serum and amygdala to diagnose PTSD in our stress animal model and identified 9 upregulated miRNAs as common viz., miR-

142-5p, miR-19b, miR-1928, miR-223-3p, miR-322*, miR-324, miR-421-3p and miR-463* and miR-674* (Table 51). However, this panel of miRNAs represents a small subset of miRNAs, and it is possible that the other serum miRNAs may also be good biomarker of the traumatic stress. Therefore, in this study, we have only focused on miRNAs which possibly have a source from amygdala and may be involved in exaggerated fear.

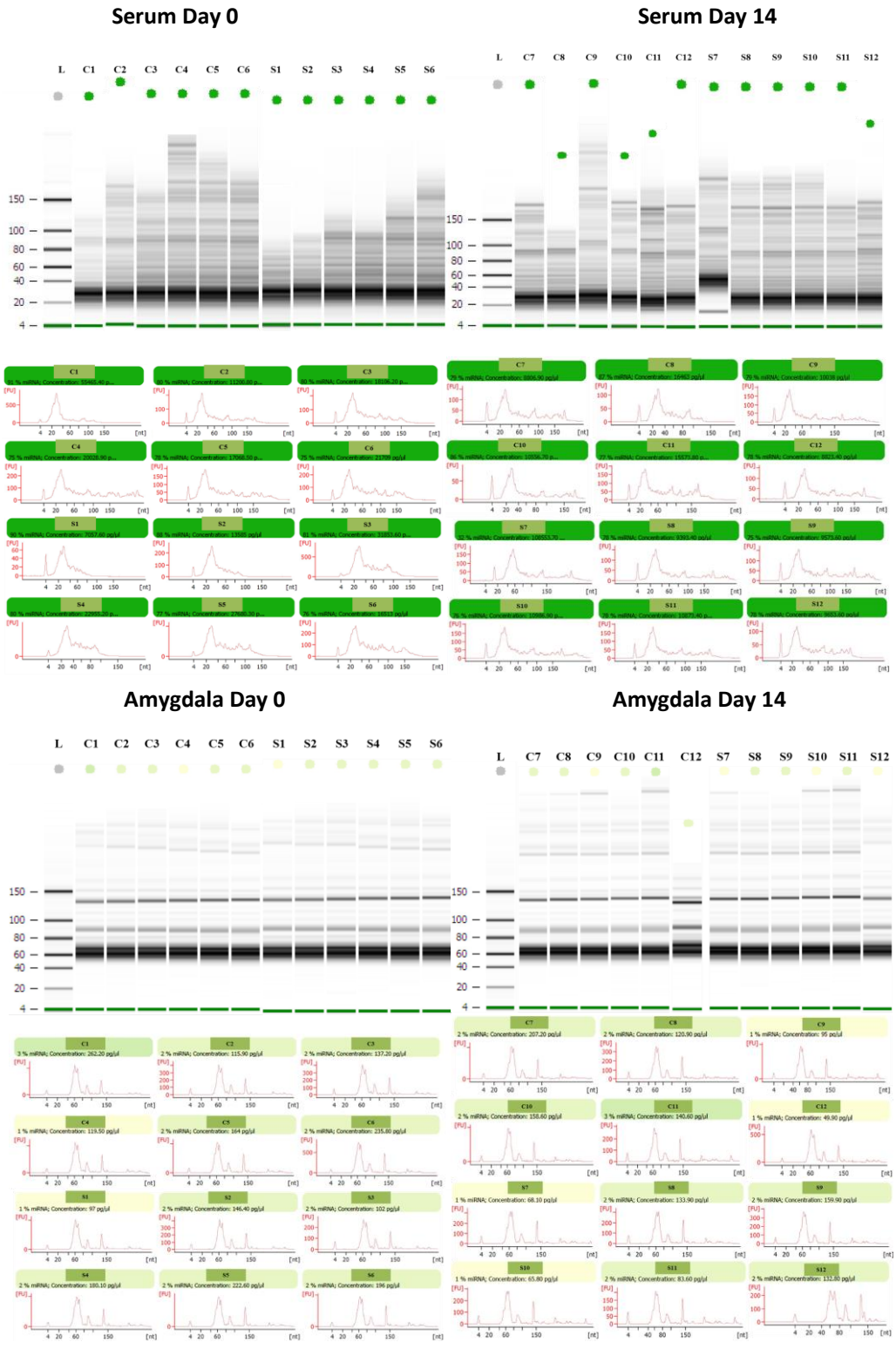

Figure 40: Electronic gel and electropherogram images of small RNA assay from the serum and amygdala RNA samples of both control and stress animals. Bioanalyzer data shows good quality and sufficient quantity of miRNA in the available total RNA samples.

Table 49: Posttraumatic stress exposure altered miRNAs in serum

S No.	Serum Day 0			Serum Day 14		
	miRNA	**Fold change (RQ)**	**P value**	**miRNA**	**Fold change (RQ)**	**P value**
1	mmu-miR-188-5p	7.90	0.04	mmu-miR-673	12.60	0.00
2	mmu-miR-511	3.61	0.01	mmu-miR-1928	11.23	0.00
3	hsa-miR-183*	2.64	0.02	mmu-miR-463*	9.97	0.01
4	mmu-miR-224	2.56	0.02	mmu-miR-326	9.94	0.00
5	mmu-miR-130a	-6.34	0.03	rno-miR-148b-5p	8.38	0.00
6	**hsa-miR-27a***	-5.83	0.00	hsa-miR-106b*	7.36	0.00
7	**mmu-miR-328**	-5.00	0.01	rno-miR-7a*	6.74	0.00
8	**mmu-miR-128a**	-4.70	0.02	**hsa-miR-27a***	6.35	0.00
9	**mmu-miR-148a**	-4.65	0.00	mmu-miR-101b	6.12	0.00
10	mmu-miR-29c	-4.58	0.01	**hsa-miR-26b***	6.05	0.00
11	mmu-miR-28	-4.42	0.00	mmu-miR-17*	5.59	0.01
12	mmu-miR-26b	-3.82	0.03	**mmu-miR-328**	5.41	0.00
13	mmu-miR-31	-3.78	0.00	hsa-miR-338-5p	5.36	0.00
14	**mmu-miR-151-3p**	-3.71	0.00	**mmu-miR-128a**	5.36	0.00
15	**mmu-miR-223**	-3.62	0.01	mmu-miR-24-2*	5.11	0.00
16	**hsa-miR-425**	-3.47	0.02	mmu-miR-877*	4.94	0.00
17	**hsa-miR-26b***	-3.42	0.00	mmu-miR-16*	4.84	0.01
18	**mmu-miR-652**	-3.37	0.05	mmu-miR-345	4.80	0.00
19	mmu-miR-31*	-3.17	0.01	mmu-miR-18a*	4.73	0.00
20	**rno-miR-7***	-3.11	0.00	**mmu-miR-652**	4.66	0.00
21	**hsa-miR-223**	-3.03	0.01	mmu-miR-181c	4.62	0.00
22	**mmu-miR-210**	-3.02	0.04	**hsa-miR-425**	4.55	0.01
23	**mmu-miR-339-5p**	-3.01	0.02	hsa-miR-9*	4.53	0.04
24	**mmu-miR-26a**	-2.81	0.04	mmu-miR-301b	4.43	0.02
25	**mmu-miR-191**	-2.73	0.01	mmu-miR-92a	4.35	0.00
26	**mmu-miR-872***	-2.71	0.03	rno-miR-339-3p	4.34	0.00
27	mmu-miR-200a	-2.67	0.02	mmu-miR-301a	4.27	0.01
28	mmu-miR-30d	-2.46	0.04	**mmu-miR-223**	4.25	0.00
29	mmu-miR-138	-2.33	0.04	mmu-miR-494	4.20	0.02
30	**mmu-miR-331-3p**	-2.30	0.03	mmu-miR-19a	4.16	0.00
31	**mmu-miR-191***	-2.21	0.05	hsa-miR-421	3.96	0.00

32				mmu-miR-350	3.90	0.01
33				mmu-miR-130b	3.90	0.01
34				**mmu-miR-191***	3.88	0.00
35				mmu-miR-342-3p	3.86	0.00
36				mmu-miR-18a	3.84	0.01
37				mmu-miR-20b	3.82	0.01
38				mmu-miR-106a	3.55	0.01
39				mmu-miR-872	3.51	0.04
40				hsa-miR-423-3p	3.51	0.00
41				**mmu-miR-339-5p**	3.42	0.00
42				rno-miR-17-3p	3.37	0.03
43				mmu-miR-20a	3.31	0.01
44				**mmu-miR-191**	3.27	0.00
45				mmu-miR-17	3.27	0.01
46				mmu-miR-324-5p	3.26	0.01
47				mmu-miR-29a	3.17	0.01
48				mmu-miR-19b	3.13	0.02
49				**hsa-miR-223**	3.11	0.02
50				mmu-miR-24	3.04	0.00
51				mmu-miR-142-5p	2.95	0.03
52				mmu-miR-138*	2.92	0.01
53				**mmu-miR-331-3p**	2.90	0.00
54				**mmu-miR-872***	2.88	0.01
55				mmu-miR-186	2.80	0.03
56				mmu-miR-27a	2.74	0.03
57				hsa-miR-23a*	2.71	0.04
58				**mmu-miR-148a**	2.71	0.02
59				mmu-miR-340-3p	2.70	0.05
60				mmu-miR-142-3p	2.70	0.05
61				rno-miR-664	2.69	0.00
62				hsa-miR-22*	2.68	0.02
63				mmu-let-7a*	2.67	0.04
64				mmu-miR-744	2.60	0.00
65				**mmu-miR-26a**	2.53	0.04

66				**mmu-miR-210**	2.51	0.02
67				**mmu-miR-151-3p**	2.49	0.01
68				mmu-miR-320	2.37	0.00
69				mmu-miR-93	2.37	0.02
70				mmu-miR-674*	2.30	0.04
71				mmu-miR-193b	2.26	0.01
72				mmu-miR-322*	2.25	0.05
73				hsa-miR-671-5p	2.25	0.00
74				**rno-miR-7***	2.23	0.05
75				mmu-miR-484	2.12	0.02
76				hsa-miR-324-3p	2.11	0.03
77				mmu-miR-324-3p	2.06	0.01
78				mmu-miR-720	2.01	0.02
79				rno-miR-190b	-27.95	0.02
80				mmu-let-7i	-4.41	0.03
81				rno-miR-224	-2.29	0.02
82				mmu-miR-125b-5p	-2.11	0.01

miRNAs marked in bold letters are common between day 0 and day 14.

Table 50: Posttraumatic stress exposure altered miRNAs in amygdala

S No.	Amygdala Day 0			Amygdala Day 14		
	miRNA	Fold change (RQ)	P value	miRNA	Fold change (RQ)	P value
1	mmu-miR-429	2.02	0.01	rno-miR-632	742.43	0.01
2	mmu-miR-29b	2.58	0.05	hsa-miR-190b	14.49	0.00
3	mmu-miR-205	2.31	0.01	mmu-miR-1928	7.85	0.00
4	mmu-miR-130b*	2.25	0.04	hsa-miR-124*	4.93	0.00
5	mmu-miR-690	2.16	0.05	mmu-miR-141	4.47	0.00
6	mmu-miR-186	-3.02	0.00	mmu-miR-706	3.73	0.00
7	mmu-miR-449a	-2.84	0.01	mmu-miR-291a-3p	3.67	0.00
8	mmu-miR-331-5p	-2.44	0.01	mmu-miR-1982.2	3.49	0.00
9	rno-miR-632	-25.01	0.03	rno-miR-673	3.43	0.01
10	mmu-miR-342-3p	-2.24	0.02	mmu-miR-1896	3.32	0.00
11	mmu-miR-376a*	-2.16	0.02	hsa-miR-653	3.29	0.03
12	mmu-miR-467b	-2.07	0.01	mmu-miR-362-5p	3.28	0.01
13	mmu-miR-16	-2.00	0.02	mmu-miR-463*	3.16	0.01
14	hsa-miR-27b*	-2.00	0.01	rno-miR-547	3.05	0.01
15				rno-miR-219-1-3p	3.01	0.02
16				mmu-miR-146b	2.93	0.00
17				mmu-miR-204	2.85	0.03
18				mmu-miR-300*	2.84	0.00
19				mmu-miR-1188	2.83	0.01
20				mmu-miR-433-5p	2.80	0.00
21				mmu-miR-200c	2.79	0.00
22				mmu-miR-487b	2.77	0.00
23				rno-miR-345-3p	2.59	0.00
24				mmu-miR-130b*	2.58	0.00
25				mmu-miR-363	2.51	0.00
26				rno-miR-409-3p	2.49	0.00
27				mmu-miR-10a	2.45	0.00
28				mmu-miR-342-	2.42	0.01

				3p		
29				mmu-miR-199b	2.41	0.00
30				mmu-miR-28*	2.38	0.01
31				mmu-miR-19b	2.37	0.00
32				hsa-miR-28-3p	2.36	0.02
33				hsa-miR-136*	2.35	0.05
34				mmu-miR-124	2.35	0.00
35				mmu-miR-125b*	2.32	0.03
36				mmu-miR-217	2.25	0.02
37				hsa-miR-412	2.23	0.01
38				hsa-miR-875-5p	2.23	0.01
39				mmu-miR-674*	2.22	0.02
40				mmu-miR-103	2.21	0.00
41				mmu-miR-671-3p	2.19	0.00
42				hsa-miR-30e-3p	2.18	0.00
43				mmu-miR-134	2.17	0.02
44				mmu-miR-223	2.16	0.03
45				rno-miR-146B	2.14	0.01
46				mmu-miR-467b	2.12	0.00
47				hsa-miR-421	2.10	0.01
48				mmu-miR-142-5p	2.10	0.00
49				hsa-miR-151-5p	2.09	0.02
50				hsa-miR-455	2.07	0.00
51				mmu-miR-9	2.06	0.00
52				mmu-miR-216b	2.05	0.00
53				mmu-miR-99a	2.05	0.03
54				rno-miR-344-3p	2.04	0.00
55				hsa-miR-340	2.04	0.00
56				mmu-miR-383	2.03	0.01
57				mmu-miR-140	2.02	0.00
58				mmu-miR-188-5p	2.01	0.02
59				hsa-miR-189	2.00	0.02
60				mmu-miR-322*	2.00	0.01

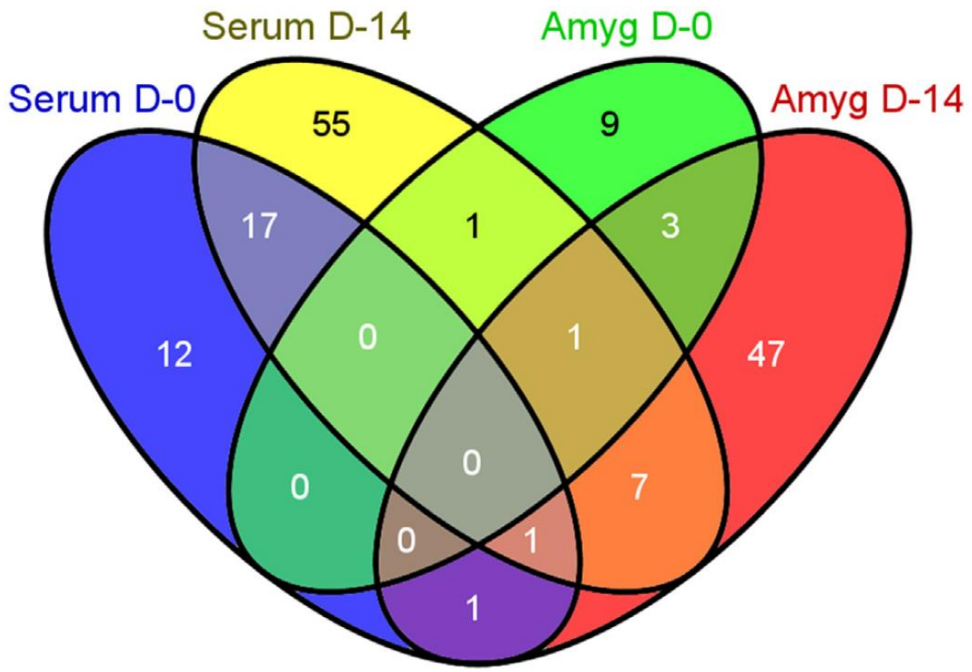

Figure 41: Overlapping miRNAs data analysis for the significantly modulated miRNAs among the four traumatic stress groups. Comparison of miRNAs in serum and amygdala at day 0 and day 14 in both serum and amygdala showed 18 common miRNAs whereas only 4 miRNAs were common in amygdala expression data at both the days. Venn diagram was done using the online Venn diagram generation tool (Oliveros, 2007).

Table 51: Posttraumatic stress altered day 14 common miRNAs in serum and amygdala

S No.	miRNA (TLDA ID)	MicroRNA Symbol	MirBase ID	Mature Sequence	Serum Day 14		Amygdala Day 14	
					Fold change	P value	Fold change	P value
1	mmu-miR-142-5p-002248	rno-miR-142-5p	MIMAT0000847	cauaaaguagaaagcacuacu	2.95	0.029	2.10	0.001
2	mmu-miR-19b-000396	rno-miR-19b-3p	MIMAT0000788	ugugcaaauccaugcaaaacuga	3.13	0.018	2.37	0.000
3	mmu-miR-1928-121164_mat	rno-miR-221-3p	MIMAT0000890	agcuacauugucugcuggguuuc	11.23	0.001	7.85	0.000
4	mmu-miR-223-002295	rno-miR-223-3p	MIMAT0000892	ugucaguuugucaaaauacccc	4.25	0.001	2.16	0.033
5	mmu-miR-322#-002506	rno-miR-322-3p	MIMAT0000547	aaacaugaagcgcugcaaca	2.25	0.048	2.00	0.013
6	mmu-miR-324-3p-002509	rno-miR-324-3p	MIMAT0000554	ccacugcccaggugcugcugg	2.06	0.015	2.42	0.007
7	hsa-miR-421-002700	rno-miR-421-3p	MIMAT0017175	aucaacagacauuaauugg	3.96	0.001	2.10	0.009
8	mmu-miR-463#-002582	rno-miR-463-5p	MIMAT0017309	uaccuaauuuguugucca uca	9.97	0.006	3.16	0.010
9	mmu-miR-674#-001956	rno-miR-674-3p	MIMAT0005330	cacagcuccccaucucagaacaa	2.30	0.037	2.22	0.016

6.1.2: Validation of differential expression in TaqMan miRNA assay

Global miRNA screening platforms can introduce bias in the miRNA profiling which can occur because of the reproducibility of the platform used, pre-amplification step due to low serum concentration and stable endogenous controls. All these factors may contribute and lead to an identification of false positives (Balakathiresan et al., 2012). Therefore, we validated the miRNA profiling data obtained from low-density array platform by performing individual miRNA assay. We selected miR-223 for validation as it was expressed in both serum and amygdala. MiRNA-223 was found to be up regulated by two fold both in TLDA and individual miRNA assay. MiR-223 is reported to be enriched in hippocampus, midbrain, and cortex (Harraz et al., 2012). MiR-223 is also implicated in studies related to brain injury and stroke. MiR-223 was reported to be prevalent in the relatively large vessel like structures scattered throughout the brain after TBI (Redell et al., 2009). In stroke animal model, miR-223 overexpression in hippocampus showed the neuroprotective effect by regulating the expression of glutamate receptor subunits, GluR2 and NR2B (Harraz et al., 2012). In our validation assays, we used U6snRNA as an endogenous normalization control. Since, our criteria for selection for miRNAs was based on p value <0.05 and fold change >1.5, we randomly selected one additional miRNA, miR-128a to confirm the TLDA data. The singleplex PCR assay for miR-128a and miR-223 confirmed and validated its expression for the same set of animals from the multiplex platform strengthening the validity of the candidate identified from the multiplex platform (Figure 42 and Figure 43).

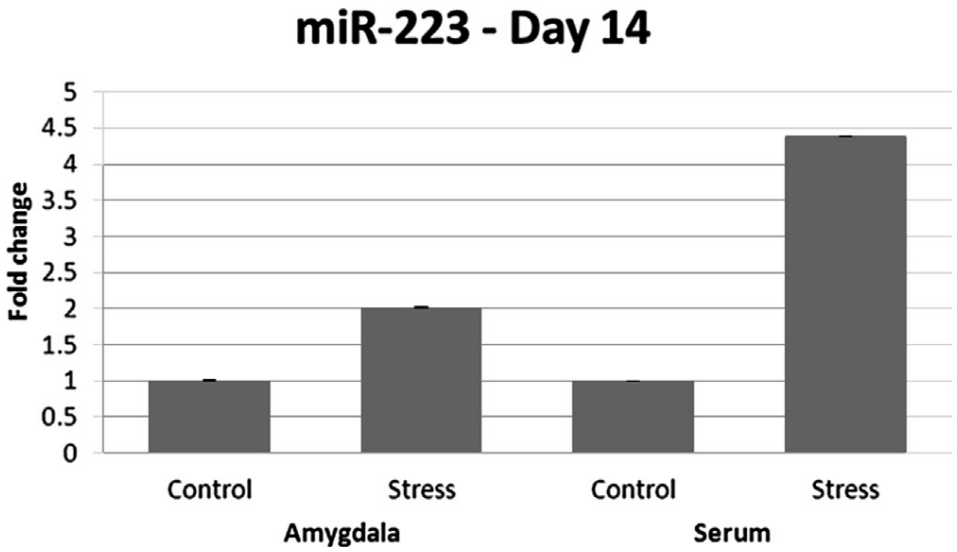

Figure 42: Validation of miR-223 expression in amygdala and serum samples of day 14. Expression of miR-223 was observed to be up regulated in a similar trend as observed in the TLDA data. The levels of miRNA were normalized by the level of U6 snRNA endogenous control and all reactions were performed in triplicate.

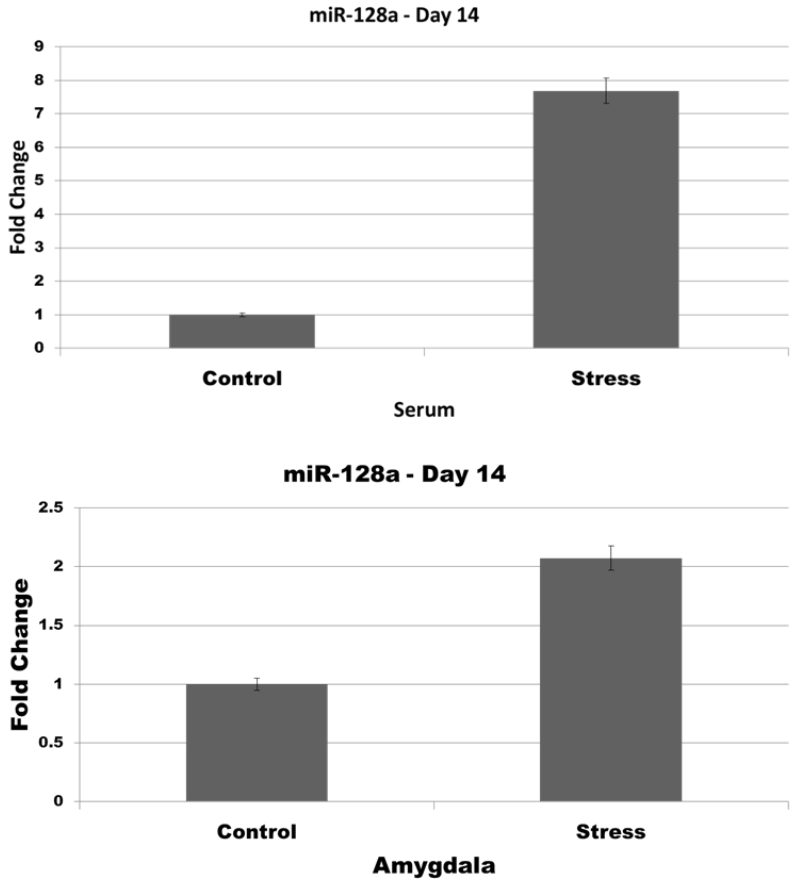

Figure 43: Validation of miR-128a expression in amygdala and serum samples of day 14. Expression of miR-128a was observed to be up regulated in a similar trend as observed in the TLDA data. The levels of miRNA were normalized by the level of U6 snRNA endogenous control and all reactions were performed in triplicate.

6.1.3: Prediction of traumatic stress altered miRNA targets and their pathway analysis

In an effort to understand the role of 9 miRNAs which are common to both serum and amygdala in PTSD pathophysiology, we performed bioinformatics analysis to identify their gene targets. Analysis in MiRWalk database showed 331 experimentally validated gene targets (Table 52). Among these genes, we found that genes involved in anxiety regulation or developments are among the targets of the modulated miRNAs. Two genes stathmin (STMN1) and aquaporin 4 (AQP4) were identified and the role of these two genes is well studied in anxiety disorder. Moreover, they were identified as direct target of miR-223. Pathway analysis of validated gene targets by IPA program suggested cell death and survival as one of the top most biofunctions in the molecular and cellular functional category (Figure 44). In canonical pathways, glucocorticoid receptor signaling pathway was among the top five pathways which is regulated by miRNAs (Figure 45). Molecular functional network was constructed using fear related genes and molecules suggest that miR-223, miR-1928 (miR-221) may have direct role in STMN1 regulation (Figure 46). Taken together, these data suggest that the selected nine miRNAs may have a potential role in PTSD development as their modulation is observed in both serum and amygdala and makes them an ideal biomarker candidate(s).

Table 52: Posttraumatic stress potential biomarker miRNA candidates experimentally validated targets from miRWalk database

S No.	MicroRNA Name	StemLoop Name	miR_ Chr.	Gene Name	Entrez ID	Pubmed ID
1	rno-miR-322*	rno-mir-322	X	Egfr	24329	17889671
2	rno-miR-322*	rno-mir-322	X	MBP_RAT	24547	20215419
3	rno-miR-223	rno-mir-223	X	Stx1a	116470	18258830
4	rno-miR-223	rno-mir-223	X	Akt1	24185	19074548
5	rno-miR-223	rno-mir-223	X	Igf1r	25718	22425712
6	rno-miR-223	rno-mir-223	X	Blr1	29363	22984081
7	rno-miR-223	rno-mir-223	X	Fgf16	60464	18258830
8	rno-miR-223	rno-mir-223	X	Mmp9	81687	18258830
9	rno-miR-223	rno-mir-223	X	NOTC1_RAT	25496	20826802
10	rno-miR-223	rno-mir-223	X	Adora1	29290	18258830
11	rno-miR-223	rno-mir-223	X	Scn3a	497770	18258830
12	rno-miR-223	rno-mir-223	X	Itch	311567	19074548
13	rno-miR-223	rno-mir-223	X	Frap1	56718	22425712
14	rno-miR-223	rno-mir-223	X	Cd4	24932	23153510
15	rno-miR-223	rno-mir-223	X	Capn8	170808	18258830
16	rno-miR-223	rno-mir-223	X	Kcnj16	29719	18258830
17	rno-miR-223	rno-mir-223	X	Kitl	60427	20826802
18	rno-miR-223	rno-mir-223	X	CPG2	499010	18258830
19	rno-miR-223	rno-mir-223	X	Gad1	24379	18258830
20	rno-miR-223	rno-mir-223	X	Zap70	301348	19144983

21	rno-miR-223	rno-mir-223	X	Cd4	24932	22527633
22	rno-miR-223	rno-mir-223	X	Bcl2	24224	23208072
23	rno-miR-223	rno-mir-223	X	Dhcr24	298298	18258830
24	rno-miR-223	rno-mir-223	X	Runx1	50662	18416028
25	rno-miR-223	rno-mir-223	X	Frap1	56718	20826802
26	rno-miR-223	rno-mir-223	X	Ptges	59103	18258830
27	rno-miR-223	rno-mir-223	X	Fgfr1	79114	18258830
28	rno-miR-223	rno-mir-223	X	Lmo2	362176	19278969
29	rno-miR-223	rno-mir-223	X	Tnf	24835	22562984
30	rno-miR-223	rno-mir-223	X	Tra1_predicted	362862	23208072
31	rno-miR-223	rno-mir-223	X	Madd	94193	18258830
32	rno-miR-223	rno-mir-223	X	Stmn1	29332	18555017
33	rno-miR-223	rno-mir-223	X	Zap70	301348	20862275
34	rno-miR-223	rno-mir-223	X	Vsnl1	24877	18258830
35	rno-miR-223	rno-mir-223	X	Il6	24498	22959936
36	rno-miR-223	rno-mir-223	X	Dclk1	83825	18258830
37	rno-miR-223	rno-mir-223	X	Cd4	24932	19297609
38	rno-miR-223	rno-mir-223	X	Akt1	24185	23208072
39	rno-miR-223	rno-mir-223	X	Klf15	85497	18258830
40	rno-miR-223	rno-mir-223	X	Ifng	25712	18791161
41	rno-miR-223	rno-mir-223	X	NP_001102651.1	499593	21109969
42	rno-miR-223	rno-mir-223	X	Golph3	78961	18258830
43	rno-miR-223	rno-mir-223	X	Casp4	114555	22959936
44	rno-miR-223	rno-mir-223	X	Neurod1	29458	18258830

45	rno-miR-223	rno-mir-223	X	Frap1	56718	23208072
46	rno-miR-223	rno-mir-223	X	Slc17a7	116638	18258830
47	rno-miR-223	rno-mir-223	X	Rhob	64373	19850724
48	rno-miR-223	rno-mir-223	X	Bcl2	24224	17260024
49	rno-miR-223	rno-mir-223	X	Nos2	24599	18791161
50	rno-miR-223	rno-mir-223	X	NP_001099865.1	294515	21926415
51	rno-miR-223	rno-mir-223	X	Nol3	85383	18258830
52	rno-miR-223	rno-mir-223	X	LOC685953	29184	22959936
53	rno-miR-223	rno-mir-223	X	Itgb1	24511	18258830
54	rno-miR-223	rno-mir-223	X	Mgst1	171341	18258830
55	rno-miR-223	rno-mir-223	X	Sars1	266975	19915717
56	rno-miR-223	rno-mir-223	X	Runx1	50662	17996649
57	rno-miR-223	rno-mir-223	X	Hyou1	192235	18258830
58	rno-miR-223	rno-mir-223	X	Cd4	24932	19014482
59	rno-miR-223	rno-mir-223	X	Smad7	81516	21940491
60	rno-miR-223	rno-mir-223	X	Il10	25325	22959936
61	rno-miR-223	rno-mir-223	X	Vim	81818	18258830
62	rno-miR-223	rno-mir-223	X	Gpd1	60666	18258830
63	rno-miR-223	rno-mir-223	X	Cd4	24932	19931339
64	rno-miR-223	rno-mir-223	X	Aqp4	25293	18258830
65	rno-miR-223	rno-mir-223	X	Akap6	64553	18258830
66	rno-miR-223	rno-mir-223	X	Lmo2	362176	19017354
67	rno-miR-223	rno-mir-223	X	Clec4d	362432	22145958
68	rno-miR-223	rno-mir-223	X	Tnf	24835	22959936

69	rno-miR-223	rno-mir-223	X	Ogt	26295	18258830
70	rno-miR-223	rno-mir-223	X	Gnb1	24400	18258830
71	rno-miR-223	rno-mir-223	X	Slc2a4	25139	20080987
72	rno-miR-223	rno-mir-223	X	Syt4	64440	18258830
73	rno-miR-223	rno-mir-223	X	Tagln	25123	18258830
74	rno-miR-223	rno-mir-223	X	Lmo2	362176	19047678
75	rno-miR-223	rno-mir-223	X	NOTC1_RAT	25496	22424712
76	rno-miR-223	rno-mir-223	X	Fos	314322	22959936
77	rno-miR-223	rno-mir-223	X	Tpm1_v7	24851	18258830
78	rno-miR-223	rno-mir-223	X	Mapre1	114764	18258830
79	rno-miR-223	rno-mir-223	X	Cd4	24932	20448109
80	rno-miR-223	rno-mir-223	X	Hmox1	24451	18258830
81	rno-miR-223	rno-mir-223	X	Acvr1	79558	18258830
82	rno-miR-223	rno-mir-223	X	Itgam	25021	19059913
83	rno-miR-223	rno-mir-223	X	Igf1r	25718	22424712
84	rno-miR-223	rno-mir-223	X	Scd2	83792	22959936
85	rno-miR-223	rno-mir-223	X	Mapk1	116590	18258830
86	rno-miR-223	rno-mir-223	X	Nr4a1	79240	18258830
87	rno-miR-223	rno-mir-223	X	NP_001102651.1	499593	20676373
88	rno-miR-223	rno-mir-223	X	Gmfb	81661	18258830
89	rno-miR-221	rno-mir-221	X	Zbtb16	353227	18417445
90	rno-miR-221	rno-mir-221	X	Zfhx1b	311071	20516212
91	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19767219
92	rno-miR-221	rno-mir-221	X	Met	24553	21537871

93	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19150885
94	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	20975375
95	rno-miR-221	rno-mir-221	X	Pten	50557	20021821
96	rno-miR-221	rno-mir-221	X	Icam1	25464	22535415
97	rno-miR-221	rno-mir-221	X	BIM_RAT	64547	19438724
98	rno-miR-221	rno-mir-221	X	Agt	24179	21310411
99	rno-miR-221	rno-mir-221	X	Mycn	298894	17943719
100	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	20428775
101	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	18417445
102	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	20547861
103	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19859555
104	rno-miR-221	rno-mir-221	X	Cd4	24932	21788445
105	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	19150885
106	rno-miR-221	rno-mir-221	X	NP_001102171.1	362686	21076613
107	rno-miR-221	rno-mir-221	X	Kras	24525	20093556
108	rno-miR-221	rno-mir-221	X	Tnf	24835	22562984
109	rno-miR-221	rno-mir-221	X	Mapk3	50689	19438724
110	rno-miR-221	rno-mir-221	X	Vcam1	25361	21310411
111	rno-miR-221	rno-mir-221	X	Myc	24577	17943719
112	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	20428775
113	**rno-miR-221**	**rno-mir-221**	**X**	**Stmn1**	**29332**	**18555017**
114	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	20618998
115	rno-miR-221	rno-mir-221	X	Dbi	25045	19953484

116	rno-miR-221	rno-mir-221	X	Adam17	57027	22009755
117	rno-miR-221	rno-mir-221	X	Cdkn1a	114851	19153141
118	rno-miR-221	rno-mir-221	X	Runx1	50662	21076613
119	rno-miR-221	rno-mir-221	X	Map2k1	170851	20299489
120	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	22992757
121	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19615744
122	rno-miR-221	rno-mir-221	X	Socs1	252971	21355095
123	rno-miR-221	rno-mir-221	X	Dnd1	307492	18155131
124	rno-miR-221	rno-mir-221	X	Ttpa	25571	20435889
125	rno-miR-221	rno-mir-221	X	Cxcr4	60628	18647411
126	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	20618998
127	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19953484
128	rno-miR-221	rno-mir-221	X	Akt1	24185	22009755
129	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19153141
130	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	21109963
131	rno-miR-221	rno-mir-221	X	Fos	314322	20299489
132	rno-miR-221	rno-mir-221	X	Met	24553	23380809
133	rno-miR-221	rno-mir-221	X	Bmf	246142	19671867
134	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	21355095
135	rno-miR-221	rno-mir-221	X	Tnf	24835	18246122
136	rno-miR-221	rno-mir-221	X	Hnrpd	79256	20435889
137	rno-miR-221	rno-mir-221	X	Ephb1	24338	18704095
138	rno-miR-221	rno-mir-221	X	Pten	50557	20618998
139	rno-miR-221	rno-mir-221	X	Amacr	25284	20014922

140	rno-miR-221	rno-mir-221	X	Nos3	24600	22037549
141	rno-miR-221	rno-mir-221	X	Kras	24525	19153141
142	rno-miR-221	rno-mir-221	X	Adm	25026	21122348
143	rno-miR-221	rno-mir-221	X	Ephb1	24338	20299489
144	rno-miR-221	rno-mir-221	X	Axin2	29134	23380809
145	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19671867
146	rno-miR-221	rno-mir-221	X	Bcl2	24224	21400558
147	rno-miR-221	rno-mir-221	X	Tnfsf10	246775	18246122
148	rno-miR-221	rno-mir-221	X	Tnf	24835	20435889
149	rno-miR-221	rno-mir-221	X	Pten	50557	18704095
150	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	20818387
151	rno-miR-221	rno-mir-221	X	Ddit4	140942	20018759
152	rno-miR-221	rno-mir-221	X	Cdkn2a_v1	25163	22037549
153	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	19153141
154	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	21226887
155	rno-miR-221	rno-mir-221	X	Tpm1_v7	24851	20417062
156	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	19671867
157	rno-miR-221	rno-mir-221	X	Tp53	24842	21400558
158	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	18246122
159	rno-miR-221	rno-mir-221	X	Bcl2	24224	20460378
160	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	18708351
161	rno-miR-221	rno-mir-221	X	Pdc	25343	20822813
162	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	20018759
163	rno-miR-221	rno-mir-221	X	Mmp14	81707	22213426

164	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19264608
165	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	21226887
166	rno-miR-221	rno-mir-221	X	LOC685953	29184	17379065
167	rno-miR-221	rno-mir-221	X	Rtn4	83765	20417062
168	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19730150
169	rno-miR-221	rno-mir-221	X	Fas	246097	21400558
170	rno-miR-221	rno-mir-221	X	Bcl2	24224	18382364
171	rno-miR-221	rno-mir-221	X	Stat5a	24918	20489169
172	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19088079
173	rno-miR-221	rno-mir-221	X	Cd4	24932	20822813
174	rno-miR-221	rno-mir-221	X	Frap1	56718	20018759
175	rno-miR-221	rno-mir-221	X	Cdkn2a_v1	25163	22213426
176	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19351832
177	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	21273047
178	rno-miR-221	rno-mir-221	X	NP_001099207.1	89804	17379831
179	rno-miR-221	rno-mir-221	X	Inppl1	65038	20417062
180	rno-miR-221	rno-mir-221	X	NP_001099886.1	294790	20492666
181	rno-miR-221	rno-mir-221	X	Pten	50557	19730150
182	rno-miR-221	rno-mir-221	X	Akt1	24185	21481725
183	rno-miR-221	rno-mir-221	X	Tp53	24842	18382364
184	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19107213
185	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	20822813
186	rno-miR-221	rno-mir-221	X	Bcl2	24224	20021821
187	rno-miR-221	rno-mir-221	X	Egfr	24329	22213426

188	rno-miR-221	rno-mir-221	X	Npepps	50558	19351832
189	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	21278784
190	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	17569667
191	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	20417062
192	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	20492666
193	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19749093
194	rno-miR-221	rno-mir-221	X	Pten	50557	21481725
195	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	18413744
196	rno-miR-221	rno-mir-221	X	Fabp4	79451	19126397
197	rno-miR-221	rno-mir-221	X	Cxcl12	24772	20975375
198	rno-miR-221	rno-mir-221	X	Tp53	24842	20021821
199	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	22473819
200	rno-miR-221	rno-mir-221	X	Akt1	24185	19401561
201	rno-miR-221	rno-mir-221	X	Kcnh8	246325	21310411
202	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	17627278
203	rno-miR-221	rno-mir-221	X	Pdcd4	64031	20417062
204	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	18413744
205	rno-miR-221	rno-mir-221	X	Pi3	408230	20505758
206	rno-miR-221	rno-mir-221	X	Tnfsf10	246775	19767219
207	rno-miR-221	rno-mir-221	X	Eno2	24334	21487968
208	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	19126397
209	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	20975375
210	rno-miR-221	rno-mir-221	X	Cdkn1b	83571	20021821
211	rno-miR-221	rno-mir-221	X	NP_001028929.1	246060	22473819

212	rno-miR-221	rno-mir-221	X	Ephb1	24338	19438724
213	rno-miR-221	rno-mir-221	X	Agtr1a	24180	21310411
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215	rno-miR-221	rno-mir-221	X	NP_001102171.1	362686	20425795
216	rno-miR-19b	rno-mir-19b-1	15	Scpep1	114861	21527938
217	rno-miR-19b	rno-mir-19b-2	X	Hoxa7	500126	22362744
218	rno-miR-19b	rno-mir-19b-2	X	Bace1	29392	18434550
219	rno-miR-19b	rno-mir-19b-2	X	Pten	50557	20851997
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221	rno-miR-19b	rno-mir-19b-1	15	Myc	24577	21664042
222	rno-miR-19b	rno-mir-19b-2	X	BIM_RAT	64547	22362744
223	rno-miR-19b	rno-mir-19b-2	X	Socs1	252971	18728182
224	rno-miR-19b	rno-mir-19b-2	X	Ctgf	64032	21501375
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226	rno-miR-19b	rno-mir-19b-1	15	BIM_RAT	64547	21664042
227	rno-miR-19b	rno-mir-19b-2	X	Tp53	24842	18728182
228	rno-miR-19b	rno-mir-19b-2	X	Rhob	64373	21527938
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230	rno-miR-19b	rno-mir-19b-1	15	Aps	114203	21794077
231	rno-miR-19b	rno-mir-19b-2	X	Stat3	25125	19713220
232	rno-miR-19b	rno-mir-19b-2	X	Scpep1	114861	21527938
233	rno-miR-19b	rno-mir-19b-1	15	Kras	24525	20089119
234	rno-miR-19b	rno-mir-19b-1	15	Bcl2	24224	21883694
235	rno-miR-19b	rno-mir-19b-2	X	Nr3c2	25672	19944075

236	rno-miR-19b	rno-mir-19b-2	X	Myc	24577	21664042
237	rno-miR-19b	rno-mir-19b-1	15	Fmr1	24948	20435064
238	rno-miR-19b	rno-mir-19b-1	15	BIM_RAT	64547	21883694
239	rno-miR-19b	rno-mir-19b-2	X	Myc	24577	20008931
240	rno-miR-19b	rno-mir-19b-1	15	NP_001100814.1	306825	17575136
241	rno-miR-19b	rno-mir-19b-2	X	BIM_RAT	64547	21664042
242	rno-miR-19b	rno-mir-19b-1	15	Myc	24577	20851997
243	rno-miR-19b	rno-mir-19b-1	15	Runx1	50662	22362744
244	rno-miR-19b	rno-mir-19b-2	X	Cdkn1a	114851	20089119
245	rno-miR-19b	rno-mir-19b-1	15	Hipk3	83617	17575136
246	rno-miR-19b	rno-mir-19b-2	X	Aps	114203	21794077
247	rno-miR-19b	rno-mir-19b-1	15	Kras	24525	20851997
248	rno-miR-19b	rno-mir-19b-1	15	Hoxa7	500126	22362744
249	rno-miR-19b	rno-mir-19b-2	X	Kras	24525	20089119
250	rno-miR-19b	rno-mir-19b-1	15	Bace1	29392	18434550
251	rno-miR-19b	rno-mir-19b-2	X	Bcl2	24224	21883694
252	rno-miR-19b	rno-mir-19b-1	15	Pten	50557	20851997
253	rno-miR-19b	rno-mir-19b-1	15	BIM_RAT	64547	22362744
254	rno-miR-19b	rno-mir-19b-2	X	Fmr1	24948	20435064
255	rno-miR-19b	rno-mir-19b-1	15	Socs1	252971	18728182
256	rno-miR-19b	rno-mir-19b-2	X	BIM_RAT	64547	21883694
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260	rno-miR-19b	rno-mir-19b-1	15	Tp53	24842	18728182
261	rno-miR-19b	rno-mir-19b-2	X	Runx1	50662	22362744
262	rno-miR-19b	rno-mir-19b-1	15	Rhob	64373	21527938
263	rno-miR-19b	rno-mir-19b-2	X	Hipk3	83617	17575136
264	rno-miR-19b	rno-mir-19b-2	X	Kras	24525	20851997
265	rno-miR-19b	rno-mir-19b-1	15	Stat3	25125	19713220
266	rno-miR-142-5p	rno-mir-142	10	Ifng	25712	21085987
267	rno-miR-142-5p	rno-mir-142	10	Nos2	24599	21085987
268	rno-miR-142-5p	rno-mir-142	10	Adarb1	25367	16369484
269	rno-miR-142-5p	rno-mir-142	10	Phb2	114766	21569818
270	rno-miR-142-5p	rno-mir-142	10	Scpep1	114861	16369484
271	rno-miR-142-5p	rno-mir-142	10	Ifit3	309526	22367717
272	rno-miR-142-5p	rno-mir-142	10	Apcs	29339	19794140
273	rno-miR-142-5p	rno-mir-142	10	Apcs	29339	22549634
274	rno-miR-142-5p	rno-mir-142	10	Cxcl9	246759	20178649
275	rno-miR-142-5p	rno-mir-142	10	Cd4	24932	22549634
276	rno-miR-142-5p	rno-mir-142	10	Twist2	59327	20178649

277	rno-miR-142-5p	rno-mir-142	10	Elovl6	171402	20178649
278	rno-miR-142-5p	rno-mir-142	10	Ddit4l	140582	20178649
279	rno-miR-142-5p	rno-mir-142	10	Fmr1	24948	20435064
280	rno-miR-142-5p	rno-mir-142	10	Tnf	24835	21085987
281	rno-miR-421	rno-mir-421	X	Pten	50557	19175831
282	rno-miR-421	rno-mir-421	X	Mycn	298894	20080624
283	rno-miR-421	rno-mir-421	X	Smad4	50554	21352803
284	rno-miR-421	rno-mir-421	X	Nr1h4	60351	22146319
285	mmu-miR-674	mmu-mir-674	2	Mbp	17196	20215419
286	mmu-miR-674	mmu-mir-674	2	Lin28	83557	20413612
287	mmu-miR-463	mmu-mir-463	X	Tnp2	21959	15901636
288	mmu-miR-463	mmu-mir-463	X	Mat1a	11720	19507003
289	mmu-miR-463	mmu-mir-463	X	Mbp	17196	20215419
290	mmu-miR-463	mmu-mir-463	X	Lin28	83557	20413612
291	mmu-miR-324-3p	mmu-mir-324	11	Ctdspl	69274	17369397
292	mmu-miR-324-3p	mmu-mir-324	11	Hprt1	15452	17369397
293	mmu-miR-	mmu-mir-324	11	Oog4	242737	17369397

	324-3p					
294	mmu-miR-324-3p	mmu-mir-324	11	Dnmt3b	13436	17369397
295	mmu-miR-324-3p	mmu-mir-324	11	H2afx	15270	17369397
296	mmu-miR-324-3p	mmu-mir-324	11	Fgf21	56636	17369397
297	mmu-miR-324-3p	mmu-mir-324	11	Mos	17451	17369397
298	mmu-miR-324-3p	mmu-mir-324	11	Mtpn	14489	15538371
299	mmu-miR-324-3p	mmu-mir-324	11	Mt1	17748	17369397
300	mmu-miR-324-3p	mmu-mir-324	11	Cdh1	12550	19559694
301	mmu-miR-324-3p	mmu-mir-324	11	Ccne1	12447	17369397
302	mmu-miR-324-3p	mmu-mir-324	11	Ccnb2	12442	17369397
303	mmu-miR-324-3p	mmu-mir-324	11	Stat3	20848	19559694
304	mmu-miR-324-3p	mmu-mir-324	11	Zp3	22788	17369397
305	mmu-miR-324-3p	mmu-mir-324	11	Rfpl4	192658	17369397
306	mmu-miR-324-3p	mmu-mir-324	11	Fgf10	14165	19559694
307	mmu-miR-324-3p	mmu-mir-324	11	Sycp3	20962	17369397
308	mmu-miR-	mmu-mir-324	11	H2afz	51788	17369397

	324-3p					
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310	mmu-miR-324-3p	mmu-mir-324	11	Camk2g	12325	17369397
311	mmu-miR-324-3p	mmu-mir-324	11	Dicer1	192119	17369397
312	mmu-miR-324-3p	mmu-mir-324	11	Mapk14	26416	19559694
313	mmu-miR-324-3p	mmu-mir-324	11	Pou5f1	18999	17369397
314	mmu-miR-324-3p	mmu-mir-324	11	H1foo	171506	17369397
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317	mmu-miR-324-3p	mmu-mir-324	11	Dppa3	73708	17369397
318	mmu-miR-324-3p	mmu-mir-324	11	Lin28	83557	20413612
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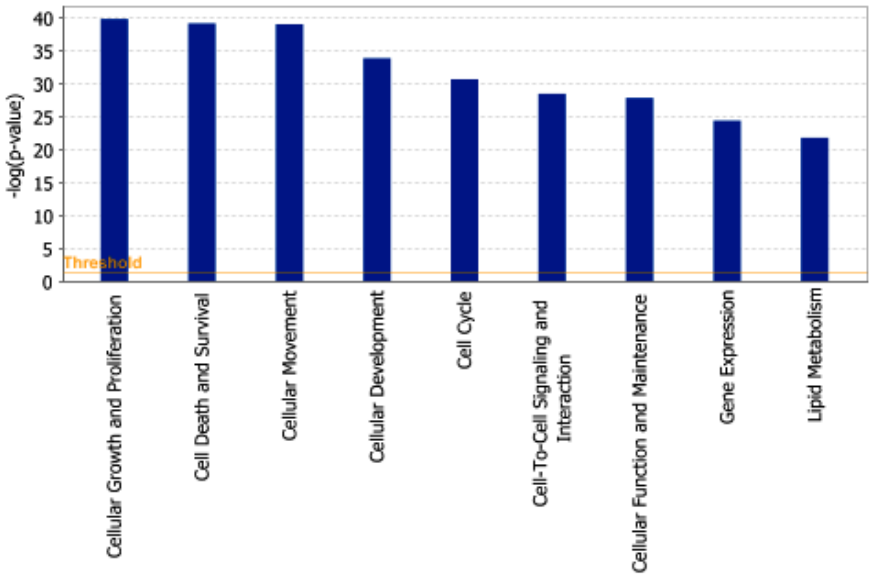

Figure 44: Top 10 functional pathways of posttraumatic stress altered day 14 serum and amygdala common miRNAs and their validated targets from miRWalk database using Ingenuity pathway analysis program.

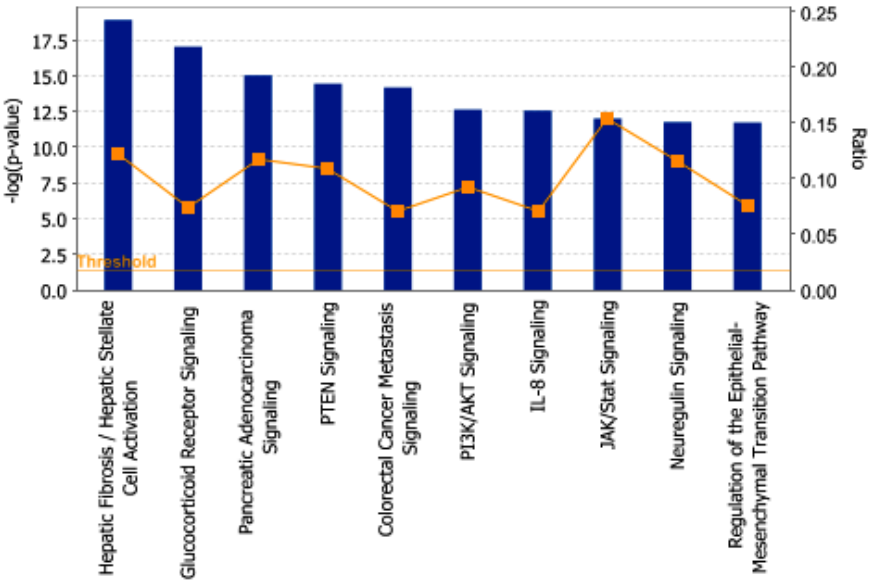

Figure 45: Top 10 canonical pathways of posttraumatic stress altered day 14 serum and amygdala common miRNAs and their validated targets from miRWalk database using Ingenuity pathway analysis program.

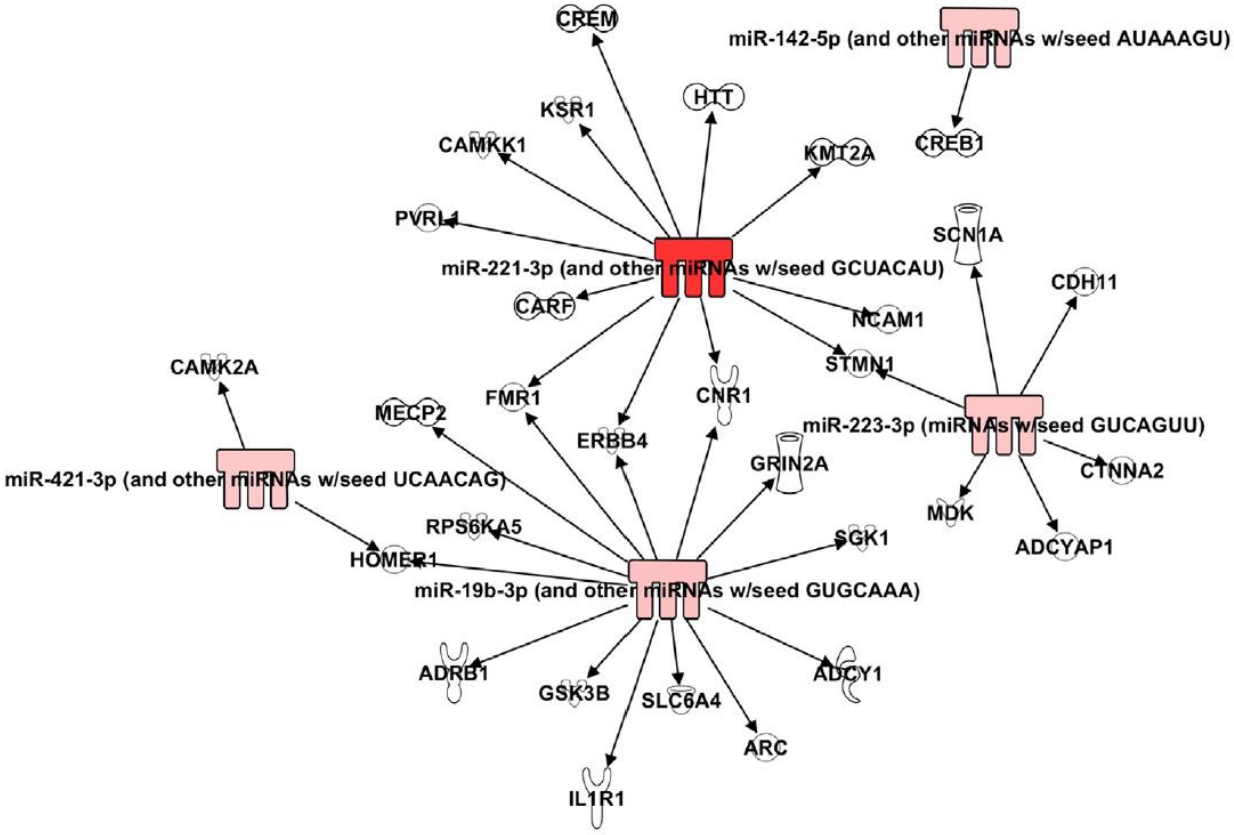

Figure 46: Network analysis of posttraumatic stress altered day 14 serum and amygdala common miRNAs and their fear related gene targets based on published literatures and available in Ingenuity Pathway Analysis (IPA) software. The network correlation between miRNAs and their targets relevant to fear response were custom-built using "my pathway" tool in IPA. Molecular functional network suggests that miR-223, miR-1928 (miR-221) may have direct role in STMN1 regulation. Red color indicates the up regulated expression of miRNAs.

6.2: Discussion

In this study, we used an animal model of PTSD which involves a restraint and tail shock for three continuous days. We performed a global miRNA expression profiling at day 0 and day 14 of serum and amygdala samples after the traumatic stress exposure to identify potential biomarker candidates in serum as well as the involvement of modulated miRNAs in fear memory formation and consolidation in the amygdala. The miRNA expression at day 0 immediately after the cessation of stress showed that most of the miRNAs were found to be down regulated in amygdala and this may be due to the "de novo protein synthesis" that supports long-lasting functional and structural plasticity which is a molecular requirement for new memory formation (Griggs et al., 2013). The down regulated miRNAs were also shown to regulate memory formation in amygdala by repressing actin-regulating proteins that are involved in plasticity and memory (Griggs et al., 2013). Furthermore, the global reduction of several miRNAs expression in rodents forebrain such as amygdala, hippocampus and cortex were shown to regulate learning and memory (Gao et al., 2010; Konopka et al., 2010; Lin et al., 2011; Griggs et al., 2013).

Much evidence indicates that the newly formed fear memories are being consolidated into stable long-term memories in the amygdala which are believed to be the site of fear memory storage (Fanselow and LeDoux, 1999; Nader et al., 2000). In order to identify miRNAs that are involved in consolidation and long-term stability of fear memories, we performed miRNA profiling in amygdala at day 14 after the cessation of traumatic stress. Analysis of day 14 miRNAs in amygdala revealed a substantial alteration of the posttranscriptional machinery characterized by a global increase in miRNA expression. This change could indicate the development and ongoing pathophysiology of the PTSD, as each miRNA is able to regulate the expression of several target genes (Beveridge et al., 2010). For example, we observed two fold upregulation of miR- 124, which has been shown to directly target mineralocorticoid receptor (MR) which regulates CORT secretion (Mannironi et al., 2013). Interestingly, Jia et al. (2012) demonstrated the downregulation of MR in amygdala enhanced the secretion of CORT for several days and the development of anxiety. Due to the alteration of large number of miRNAs (60 miRNAs; >2 fold) in day 14 amygdala, we selected only those miRNAs which were common (9 miRNAs) between serum and amygdala of day 14 for further analysis such as correlation with fear related genes.

Network analysis of these 9 miRNAs with their fear related gene targets that are available in IPA showed only 5 of them were correlated with fear related genes (Fig. 3). For instance, cAMP responsive element binding protein 1 (Creb1) was identified as a direct target of miR-142-3p. Creb1 was recently reported to be down regulated in rat brain exposed to repeated inescapable shock (Smalheiser et al., 2011). This suggests that miR-142-3p may regulate the expression of Creb1 and may play an important role in stress related response (Figure 55). Further, miR-221 and miR-223 were also found to regulate the expression of stathmin1 (STMN1), an important amygdala molecule involved in fear conditioning (Shumyatsky et al., 2005). IPA analysis suggested an involvement of five miRNAs viz., miR- 142-5p, miR-19b, miR-1928, miR-223 and miR-421-3p in the regulation of genes associated with delayed and exaggerated fear.

We further explored these five miRNAs for their brain specificity and/or their functions related to any neurological conditions. MiR- 142-5p was found to be enriched in microglia and has been shown to be upregulated after brain injury (Lei et al., 2009; Wu et al., 2011; Lau et al., 2013). Further, auditory fear training in Rattus norvegicus, down regulated the expression of miR-142-5p in lateral amygdala of naïve animals suggesting its involvement in memory formation dysfunction (Griggs et al., 2013). MiR-19b-3p that copurify with polyribosomes in mammalian neurons showed significantly higher expression in 6-hydroxydopamine-injured MN9D cells, indicating its role in neurodegenerative diseases by contributing to dopaminergic neuronal apoptosis (Li et al., 2013). MiR-221-3p expression was also upregulated in distal axons of superior cervical ganglia (SCG) after spinal cord injury (Liu et al., 2009, Wu et al., 2011). MiR-223 and miR-19 are also enriched in glial cells and were shown to inhibit aberrant glial expression of neuronal proteins and phenotypes (Jovicic et al., 2013). The miR-421 was first identified in neocortex and hippocampus from developing rat brain and predicted to play a role in neurodegenerative disorders (Miska et al., 2004; Taguchi 2013). Recent studies also suggest participation of miR-421 in the regulation of plasminogen activator Inhibitor-1 (PAI-1) which is known to induce neuronal apoptosis, disrupt the blood brain barrier (BBB) and contribute to neurotoxicity in ischemic brain damage after stroke (Abu Fanne et al., 2010; Marchand et al., 2012).

For biomarker identification, we selected only day 14 serum miRNA profiles for the analysis since the day 14 animals showed delayed and exaggerated startle response, enhanced plasma CORT and retarded body weight gain after several days (10-21 days) of posttraumatic stress in rats (Jia et al., 2012). Modulation of miRNAs in serum can occur either because of the change in the miRNAs expression in the regions of the brain which controls the stress response. These miRNAs can leach out in the serum by different ways as previously described (Andrews and Neises 2012). However, there is a possibility that serum miRNA modulation may occur due to a bystander effect of the stress on other organs which can potentially alter the serum miRNA expression profile. Such miRNAs can be a marker for organ stress but cannot be used as marker for psychological stress. To identify the true candidate biomarkers, we performed miRNA profiling of amygdala which is believed to play a critical role in regulation of fear conditioning in this animal model (Andero et al., 2013). Therefore, 9 miRNAs which are found to be up regulated in both amygdala and in serum were selected and analyzed for their correlation with PTSD pathophysiology by computational analysis to validate their potential as diagnostic biomarkers of PTSD. Since miRNA regulates the cell physiology by targeting the mRNA and altering the corresponding protein expression, the validated gene targets of 9 candidate miRNAs were identified using miRWalk program. These gene targets were used to identify the pathways involved using IPA. Interestingly, stress related glucocorticoid receptor signaling pathway appeared as one of the major canonical pathway which was predicted to be regulated by the 9 miRNAs. These computational analyses suggest that the candidate biomarkers of PTSD may have an important role in stress response and hence are good candidates for further biomarker validation studies.

6.3: Conclusion

In conclusion, our data suggest that traumatic stress associated with a global decrease in day 0 and global increase in day 14 in miRNA expression in amygdala could have profound psychopathological implications in the context of PTSD development by influencing genes involved in fear memory formation and consolidation. A panel of dysregulated miRNAs present in both serum and amygdala after exposure to traumatic stress and their correlation with PTSD pathophysiology suggested them as promising candidates for biomarkers. To the best of our knowledge, this is the first study, where we have identified the serum miRNAs which may have

potential for the diagnosis of PTSD. Further studies with human clinical samples will be required to identify the best biomarker candidate for their use as both diagnostic biomarkers and therapeutic targets for PTSD.

Chapter 7: Summary, Conclusion and Future scope of work

Traumatic brain injury (TBI) has now currently emerged as one of the most widely prevalent public disorder in the world. Mild traumatic brain injury which is of lesser severity compared to moderate or severe grade of TBI is normally of a lesser health hazard with just transient behavioral changes but still a percentage of patient population can suffer from persistent symptoms due to the TBI. A prompt and better diagnosis of an incident of mild TBI can effectively aid in treating the patient population with TBI-related symptoms and this has led to a search for novel biomarkers for mild TBI. Post-traumatic stress disorder, an anxiety disorder that develops after exposure to traumatic stress has a high overlap of symptoms with mild TBI leading to a clear difficulty in diagnosis between both the disorders.

MicroRNAs are a species of small noncoding RNA that have emerged as ideal candidates as biomarkers and as therapeutic targets for a range of diseases. The current study has explored to study the role of miRNAs in both mild traumatic brain injury and post-traumatic stress disorder.

TBI is a very highly heterogeneous disorder with the injury depending upon multiple factors like the brain area subjected to injury, type of force (physical or blast), damage to skull (closed head or penetrating) and also whether the injury has primarily affected the neurons or is axonal in nature. This heterogeneity makes sure that research information gleaned from a single animal model of TBI will be insufficient to provide useful scientific information for treatment of TBI.

Weight drop injury model was the main animal model of TBI used in this project. This model is characterized by presence of transient behavioral deficits along with absence of histological damage. The recovery of the animals at later time points after the presence of deficits around 24 hr time point measured by behavioral tests such as neurological severity scale test, open field locomotion and acoustic startle response indicate a mild grade of TBI. Serum miRNA expression of four different grades of mild injury induced using the weight drop device were studied and a thirteen miRNA common serum miRNA signature was identifed with potential as acute biomarker for exposure to mild TBI.

Another study was done using the same weight drop device to study temporal changes in miRNA expression at the site of brain injury and a clear discrimination in miRNA expression between an

early (24 hr) and delayed (day 7) time point was observed which in turn leads to activation of unique set of biological pathways which can be predicted using a bioinformatics approach.

Another TBI model, the controlled cortical impact (CCI) model was also used to study post TBI changes in serum and brain miRNA expression. The time-dependent miRNA profiling data shows a difference from the miRNA expression patterns observed in the CHI mouse model.

TBI caused due to exposure to blast wave is entirely different from a TBI due to physical impact. We have also studied the serum miRNA expression in a rat model of blast TBI exposed to three serial blast exposures. Levels of microRNA let 7i were found to be elevated in both serum and cerebrospinal fluid making it an ideal biomarker candidate unique to this particular injury model.

A tail shock model based on learned helplessness paradigm was used to study miRNA expression changes in serum and amygdala post exposure to a stress protocol. A panel of nine stress-responsive miRNAs viz., miR-142-5p, miR-19b, miR-1928, miR-223-3p, miR-322*, miR-324, miR-421-3p and miR-463* and miR-674* were identified in this study and may have potential as biomarker(s) for PTSD. Five of the nine miRNA (miR-142-5p, miR-19b, miR-1928, miR-223 and miR-421-3p) were found to be associated with regulation of genes associated with delayed and exaggerated fear.

Hence to summarise the results, heterogeneity was generally observed at the molecular level among the TBI models based on the difference seen in their respective miRNA signatures inspite of few common miRNAs like the overlapping miRNAs observed at 3 h timepoint in the CHI and CCI serum. Also, there was an absence of overlap between the miRNA signatures of PTSD model and the TBI models suggesting that they have potential as biomarker candidates for differentiating between both the disorders and can help circumvent the difficulty arising due to overlap of symptoms between mild TBI and PTSD.

The major future implication for this work is the potential for using the differentially expressed microRNA candidates as biomarker candidates or therapeutic targets in human studies. One of the biggest research gaps in the field of TBI involved a lack of knowledge on molecular changes especially that of microRNA expression between the different models. The differential expression if observed between the three TBI animal models can help in giving a molecular

explanation for the heterogeneity seen in human populations who have been exposed to different types or subtypes of TBI. Specific miRNAs identified through serum miRNA expression profiling and validation can be used in further studies on human cases of TBI. The knowledge on the pattern of pathways being predicted to get differentially modulated based on differences in TBI types, and also within the model but based on a temporal difference has immense potential to study the pathophysiology of TBI at the molecular level which in turn can help in designing better drug candidates for treating TBI related complications. The difference in the serum miRNA expression signature between the PTSD and TBI animal models has a bright potential to be used for human studies on differential diagnosis of both the disorders which have been proved to be often difficult based on the high overlap of their symptoms. A robust testing of the miRNA candidates identified in this study in human samples based on different injury types of TBI as well as with onset of PTSD can be much desired for rapid diagnosis of both the disorders.

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http://www.intechopen.com/books/anxiety-disorders/ptsd-and-currenttranslational-research.

LIST OF PUBLICATIONS

1. N.S. Balakathiresan, M. Bhomia, **R. Chandran**, M. Chavko, R.M. McCarron and R.K. Maheshwari. (2012). MicroRNA Let-7i is a Promising Serum Biomarker for Blast Induced Traumatic Brain Injury. Journal of Neurotrauma. 29 (7):1379-1387.
2. N.S. Balakathiresan*, **R. Chandran***, M. Bhomia, M. Jia, H. Li and R.K. Maheshwari. (2014). Serum and Amygdala MicroRNA Signatures of Posttraumatic Stress: Fear Correlation and Biomarker Potential. Journal of Psychiatric Research. 57: 65–73.
3. A. Sharma*, **R. Chandran***, E.S. Barry, M. Bhomia, M.A. Hutchison, N.S. Balakathiresan, N.E. Grunberg and R.K. Maheshwari. (2014). Identification of serum microRNA signatures for diagnosis of mild traumatic brain injury in a closed head injury model. PLoS One. 9(11): e112019.
4. **R. Chandran***, A. Sharma*, M. Bhomia, N.S. Balakathiresan and R.K. Maheshwari. Temporal Differences in Brain MicroRNA Expression Pattern in injury site post mild closed head injury (Manuscript under preparation).
5. P. Gupta*, **R. Chandran***, N.S. Balakathiresan, M. Bhomia and R.K. Maheshwari. Temporal Differences in Serum and Brain MicroRNA Expression Signature post mild CCI (Manuscript under preparation).

Brief Biography of Candidate

Education:

Degree	Institute	Year	GPA
Ph.D	Birla Institute of Technology & Science, Pilani - Pilani Campus, Rajasthan, India (Joint program with Uniformed Services University of the Health Sciences, Bethesda, MD)	2010-2015	-
M.E (Biotechnology)	Birla Institute of Technology & Science, Pilani - K K Birla Goa Campus, Goa, India	2008-2010	(9.09/10)
B.Tech (Biotechnology)	SRM University, Kattankulathur, Tamil Nadu, India	2004-2008	8.4/10.0 (First class with distinction)

Professional Experience:

- **Graduate Research Assistant**: **(2010-Present).** Uniformed Services University of the Health Sciences, Bethesda, Maryland, India.
- **Teaching Assistant**: **(2008-2010).** Birla Institute of Technology and Science, Pilani, Goa Campus, India.

Honors and Awards:

- **Student Travel grant** awarded for for poster presentation at National Neurotrauma Society Symposium 2014 (June 29 - July 02, 2014), San Francisco, CA
- Teaching Assistantship, Birla Institute of Technology & Science, Pilani - K K Birla Goa Campus, 2008-2010

Research Experience:

- **Uniformed Services University of the Health Sciences**, Bethesda, MD
  Graduate Research Assistant; Advisor: Dr. Radha K. Maheshwari; 2010-Present
  MicroRNA expression profiling studies in animal models of Mild Traumatic Brain Injury (TBI) and Post traumatic Stress Disorder (PTSD)
    - Examined global microRNA expression changes in serum and brain post brain injury or stress in rodent models of TBI and PTSD using microarray analysis to identify possible biomarker candidates

- Conducted various behavioral tests in mice like open field locomotion, acoustic startle response, Morris water maze, rotarod and neurological severity scale test
- Analyzed microRNA-target relationships and resultant biological pathway changes using bioinformatic approaches
- Examined brain sections post brain injury using different histological staining techniques
- Managed laboratory functions including organization, purchasing and biohazard disposal
- Mentored summer interns

- **SN ONGC Department of Genetics and Molecular Biology, Sankara Nethralaya Research Foundation,** Chennai, Tamil Nadu, India
  Summer Intern; Advisor: Dr. Sripriya Sarangapani; May 2009 – July 2009
  Optimization of PCR conditions for amplification of CHX10 gene for studies on micropthalmia in South Indian population
  - Optimized touch-down PCR protocols for amplifying exons of micropthalmia-associated CHX10 gene
  - Analyzed genetic polymorphisms in micropthalmia patients using automated sequencing technique

- **KJ Research Foundation**, Chennai, Tamil Nadu, India
  Research Student; Advisor: Dr. R. Narayani; January 2008-April 2008
  Biopolymeric Controlled Drug Delivery system for Oral Delivery of therapeutic agents
  - Used *in vitro* release kinetics studies to analyze alginate beads as a viable oral drug delivery system

- **AU-KBC Research Centre, Department of Bioinformatics**, Chennai, Tamil Nadu, India
  Summer Intern; Advisor: Dr. Gopal Ramesh Kumar; June 2007 – July 2007
  Analysis of microarray data of human breast tumors using BRB Array tools
  - Analyzed microarray data of human breast tumors obtained from online databases using BRB Array tools platform

TECHNICAL SKILLS

- RNA isolation and quantitative/qualitative analysis using Bioanalyzer instrument
- Global microRNA profiling of serum and brain samples using TaqMan microarray platform
- Quantitative PCR (96 well) for validation of miRNA candidates
- Statistical analysis of qPCR and microarray data using Statminer software
- MicroRNA target prediction analysis using *in silico* tools like DIANA mirPATH online software
- Biological pathway analysis using Ingenuity Pathway analysis software and other related computational tools
- General rodent (mouse) handling procedures including blood collection (tail vein/cardiac puncture routes), cardiac perfusion and brain harvesting for histology

- Behavioral testing on mice like open field locomotion, acoustic startle response, Morris water maze, rotarod and neurological severity scale test
- Histology: Preparation of brain sections, analysis using staining techniques like H & E, silver staining, fluorojade-C and β-APP staining and digital archiving of brain section slides using Nanozoomer instrument
- Mammalian cell culture and maintenance

TALKS/PRESENTATIONS

- 'MicroRNAs as Biomarkers for Traumatic Brain Injury and PTSD' presented at Uniformed Services University of the Health Sciences, Bethesda, MD as part of Pathology department seminar series.

- 'MicroRNAs as biomarkers' and 'Lab techniques-Animal handling and molecular biology techniques' presented at Birla Institute of Technology & Science, Dubai campus, Dubai, UAE for senior, junior biology undergraduates and a group of clinical physicians

PROFESSIONAL ASSOCIATIONS

National Neurotrauma Society (May 2013-Present)

TRAINING AND CERTIFICATION

- **Rodent handling techniques** (Uniformed Services University, Bethesda, MD, August 2010)
- **Bioinformatics and Computational Biology techniques** (BITS Pilani Goa Campus in association with Indian Institute of Technology, Delhi, April 2010)
- **Drug Discovery and Development through Bioinformatics** (AU-KBC Research Centre, Chennai, India, July 2006)
- **Microarray Data Analysis** (AU-KBC Research Centre, Chennai, India, June 2006)

BOOK CHAPTERS

- N.S. Balakathiresan, A. Sharma, **R. Chandran**, M. Bhomia, Z. Zhang, K.K.W. Wang and R.K. Maheshwari. (2014). Molecular Mechanisms and Biomarker Perspective of MicroRNAs in Traumatic Brain Injury. In: K.K.W. Wang, Z. Zhang and F.H. Kobeissy (Eds.), Biomarkers of Brain Injury and Neurological Disorders (pp. 76–115). CRC Press (Book chapter).

- Balakathiresan NS, Bhomia M, Gupta P, **Chandran R**, Sharma A and Maheshwari RK. MicroRNAs as Brain injury Biomarker. In: Preedy VR, Patel VB (Eds.), Biomarkers in Disease: Methods, Discoveries and Applications - General Methods and their Applications. (In press). Springer (Book chapter).

CONFERENCE PRESENTATIONS

Oral Presentation

- **R. Chandran**, N.S. Balakathiresan, M. Bhomia, A. Sharma, M. Jia, H. Li and R.K. Maheshwari. MicroRNA expression altered in Serum and Amygdala of rats exposed to repeated inescapable stress and their pathophysiology with Posttraumatic Stress disorder. CNRM Annual Meeting, May 2012.

Poster Presentations

- **R. Chandran** , A. Sharma , N.S. Balakathiresan, M. Bhomia , E.S. Barry, M.A. Hutchison, N.E. Grunberg and R.K. Maheshwari. (2014). Serum MicroRNA Signatures of Closed Head Injury in Mouse: A Potential Biomarker for Mild Traumatic Brain Injury. National Neurotrauma Society Symposium 2014 (June 29 - July 02, 2014), San Francisco, CA.

- **R. Chandran**, N.S. Balakathiresan, M. Bhomia, A. Sharma, P. Gupta and R.K. Maheshwari. Serum MicroRNA Signatures as Potential Biomarkers of Traumatic Brain Injury: A Comparative Study of Three Different Rodent Models of TBI. National Neurotrauma Society Symposium 2013 (August 4-7, 2013), Nashville, TN.

- **R. Chandran**, A. Sharma, E.S. Barry, N.S. Balakathiresan, M.A. Hutchison, M. Bhomia, J.T. McCabe, N.E. Grunberg and R.K. Maheshwari. Mild Closed Head Injury leads to Neurobehavioral Deficits and an Altered Serum microRNA Profile in Mice. USU Research Days, May 2013.

- **R. Chandran**, A. Sharma, E.S. Barry , N.S. Balakathiresan , M.A. Hutchison, M. Bhomia, J.T. McCabe , N.E. Grunberg and R.K. Maheshwari. Altered Serum MicroRNA Expression and Neurobehavioral deficits following Mild Closed Head Injury in Mice. National Capital Area TBI Research Symposium (CNRM), April 2013.

- **R. Chandran**, N.S. Balakathiresan, M. Bhomia, A. Sharma, M. Jia, H. Li and R.K. Maheshwari. Altered microRNA expression in Serum and Amygdala of Rats exposed to repeated inescapable stress and their pathophysiology with Posttraumatic Stress disorder. USUHS Research Week, May 2012.

- **R. Chandran**, N.S. Balakathiresan, M. Bhomia, A. Sharma, M. Jia, H. Li and R.K. Maheshwari. Aberrant expression of serum and amygdala miRNAs in traumatic stress induced rats. Seventh Annual Amygdala, Stress and PTSD conference at USUHS, April 24, 2012.

Brief Biography of Supervisor

NAME	POSITION TITLE		
RADHA K. MAHESHWARI	**Professor of Pathology**		
EDUCATION/TRAINING			
INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Lucknow University, India	B.S.	1968	Biological Sciences
Birla Institute of Technology & Sciences, India	M.S.	1970	Biological Sciences
Kanpur university, Kanpur, India (Ph.D Dissertations work was carried out at the Central Drug Research Institute, Lucknow)	Ph.D.	1974	Virology, Interferon and Immunology

A. Personal Statement

I have been extensively involved in studies of viral pathogenesis and host response (mRNA as well as microRNA) studies over the past several years. My lab has been studying the Vennezuelan equine encephalitis virus (VEEV) pathogenesis and identification of potential therapeutic and targets for treatment of VEEV disease. Other than this, my lab has also been engaged in identification of biomarkers of blast overpressure injury and post-traumatic stress syndrome studying the molecular mechanisms of viral pathogenesis and neuro-degeneration specifically looking at identification of serum biomarkers, role of inflammation and innate immune response in alphavirus pathogenesis the host responses and during neurodegenerative viral infections. in rat model and the weight drop model in mice for identification of serum biomarkers. In past, I have also studied the protective efficacy of phytochemicals in prevention of hemorrhage, ischemia and reperfusion-induced injury; chemoprevention of cancer and enhancement of wound healing as evident through the peer reviewed publications. Over the years, my laboratory has been involved successfully and productively in interdisciplinary

collaborative approach and based on the experience I am well suited for my role to serve as PI for the proposed study.

B. Positions and Employment

1977-1980: Post-Doctoral Fellow, National Institute of Arthritis, Metabolism and Digestive Diseases, NIH, Bethesda.

1977-1981: Senior Research Associate, Department of Pathology, USUHS, Bethesda, Maryland.

1977-1982: Adjunct Faculty, Dept. of Microbiology, School of Medicine, Georgetown University, Washington, D.C.

1984-1989: Assistant Professor, Department of Pathology, USUHS, Bethesda, Maryland.

1990-1994: Associate Professor, Department of Pathology, USUHS, Bethesda, Maryland.

1990-present: Adjunct Professor, Birla Institute of Technology and Science, Pilani, India.

1995-Present Professor, Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda

Other Experience and Honors

- Organized scientific meetings, symposia, and served as Chairman in scientific sessions;
- Invited speaker at many national and international meetings; and invited to participate in discussion groups
- Served on the editorial board and reviewed papers for journals
- Invited to write articles/chapters in books
- Appointed as the Coordinator of Indo-US Programs at USUHS
- Co-edited two books, "Interferon in Biomedical Research", and "Cell-Mediated Immunity in Tropical Diseases".
- Adjunct Faculty, Birla Institute of Technology and Science, Pilani, India.
- Served as a review member for Fogarty Center, NIH and for the United Nations.

C. *Selected Peer-reviewed Publications* *(Selected from 125 peer-reviewed publications)*

1. Anuj Sharma, Raghavendar Chandran, Erin S Barry, Mary Anne Hutchison, Balakathiresan Nagaraja, Neil E Grunberg, **Radha K Maheshwari**. Identification of serum microRNA signatures for diagnosis of mild traumatic brain injury in a closed head injury model. PLoS One. 2014 Nov 7;9(11):e112019. doi: 10.1371/journal.pone.0112019. eCollection 2014.
2. Shelley Honnold, Russell Bakken, Diana Fisher, Cathleen Lind, Jeffrey Cohen, Lori Eccleston, Kevin Spurgers, Anuj Sharma, **Radha Maheshwari**, Pamela Glass. Second generation inactivated eastern equine encephalitis virus vaccine candidates protect mice against a lethal aerosol challenge. PLoS One. 2014 Aug 12;9(8):e104708. doi: 10.1371/journal.pone.0104708. eCollection 2014.
3. Balakathiresan NS, Chandran R, Bhomia M, Jia M, Li H, **Maheshwari RK**. Serum and amygdala microRNA signatures of posttraumatic stress: fear correlation and biomarker potential. J Psychiatr Res. 2014 Oct;57:65-73. doi: 10.1016/j.jpsychires.2014.05.020. Epub 2014 Jun 21.
4. Manish Bhomia, Anuj Sharma, Manoshi Gayen, Paridhi Gupta and **Radha K Maheshwari**. Artificial MicroRNAs can Effectively Inhibit Replication of Venezuelan Equine Encephalitis Virus**.** Antiviral Res. 2013 Nov; 100(2):429-34. doi: 10.1016/j.antiviral.2013.08.010. Epub 2013 Aug 27.
5. Thangapazham RL, Sharad S, Maheshwari RK. Skin regenerative potentials of curcumin. Biofactors. 2013 Jan-Feb; 39(1):141-9. doi: 10.1002/biof.1078. Epub 2013 Jan 11.
6. 2. Sharma A, Gupta P, Maheshwari RK. Inactivation of Chikungunya virus by 1,5 iodonapthyl azide. Virol J. 2012 Dec 4; 9:301.
7. 3. Gaidamakova EK, Myles IA, McDaniel DP, Fowler CJ, Valdez PA, Naik S, Gayen M, Gupta P, Sharma A, Glass PJ, Maheshwari RK, Datta SK, Daly MJ. Preserving immunogenicity of lethally irradiated viral and bacterial vaccine epitopes using a radio-protective Mn2+-Peptide complex from Deinococcus. Cell Host Microbe. 2012 Jul 19;12(1):117-24.
8. Balakathiresan N, Bhomia M, Chandran R, Chavko M, McCarron RM and **Maheshwari RK**. MicroRNA Let-7i is a promising serum biomarker for blast induced traumatic brain injury. J Neurotrauma (2012).
9. Sharma A, Bhomia M, Honnold SP, **Maheshwari RK**. Role of adhesion molecules and inflammation in Venezuelan equine encephalitis virus infected mouse brain. Virol J. 2011 Apr 29;8(1):197.
10. Bhomia M, Balakathiresan N, Sharma A, Gupta P, Biswas R, **Maheshwari RK**. Analysis of MicroRNAs induced by Venezuelan Equine Encephalitis virus infection in mouse brain. Biochem Biophys Res Commun. 2010 Mar 18.
11. Sharma A, **Maheshwari RK (2009)** . Oligonucleotide Array Analysis of Toll Like Receptors and Associated Signaling Genes in Venezuelan Equine Encephalitis Virus Infected Mouse Brain. *J Gen Virol.*

12. Sharma A, Bhattacharya B, Puri RK, **Maheshwari, RK (**2008). Venezuelan equine encephalitis virus infection causes modulation of inflammatory and immune response genes in mouse brain. *BMC Genomics.16;9:289.*

13. Thangapazham RL, Shaheduzzaman S, Kim KH, Passi N, Tadese A, Vahey M, Dobi A, Srivastava S, **Maheshwari R.K** (2008)Androgen responsive and refractory prostate cancer cells exhibit distinct curcumin regulated transcriptome.*Cancer Biol Ther.7(9):1436-40.*

14. Thangapazham, R.L, Sharma, A, and **Maheshwari, R.K** (2008). Protectve Effect of phytochemicals in cancer chemoprevention, wound healing and ischemia/reperfusion injury. p.135-144. Ethnopharmacology- Recent Advances (edited by Puspangadan, George and Janardhan), Daya Publishing House, Delhi.

15. Singh, A.K., Warren, J., Madhavan, S., Kumar, R., Steele, K., Sharma, A., Sharma, S., Kulshreshtha, D., and **Maheshwari, R.K.** (2007) Picroliv accelerates epithelialization and angiogenesis in rat wounds. *Planta Medica* 73,251-256.

16. Rollwagen, F., S. Madhavan, A. Singh, Y. Li, K. Wolcott, and **R. Maheshwari**. (2006) IL-6 protects enterocytes from hypoxia-induced apoptosis by bcl-2 and fas mRNA regulation. *Biophysical Biochemical Research Communications* 347:1094-1098.

17. Steele, K, Seth, P., Catlin, K., Sconeboom, B., Husain, M., Grieder, F., and **Maheshwari, R.K.** (2006) Tunicamycin Enhances Neuroinvasion and neurodegeneration in mice with Venezuelan Equine Encephalitis virus. *Veterinerary Pathology* 43:904-913.

18. Thangapazham R. L, Sharma A, **Maheshwari R. K.** (2006). Biomarkers of Angiogenesis in Wound Healing and Cancer: Role of Botanicals and Phytochemicals. *Journal of Horticulture* 720,129-136 .

19. Thangapazham R. L, Sharma A, **Maheshwari R. K.** (2006). Multiple Molecular Targets in Cancer Chemoprevention by Curcumin. *Journal of the American Association of Pharmaceutical Sciences* 8(3):E 443-449.

20. Sharma A., Singh, A.K., Warren J., Thangapazham, R., and **Maheshwari, R.K.** (2006) Differential Expression of Angiogeneic Genes in diabetic Wound Healing. *Journal of Investigative Dermatology* 126:2321-2331.

21. Sundar, Shirin V., Li, Ying-Yue, Rollwagen, Florence M., and **Maheshwari, Radha K.** (2005). Hemorrhagic shock induces differential gene expression in mouse liver. *Biochem. Biophy. Res. Comm.* 332,688-696.