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Assessment of alterations in neurometabolites and brain microstructure after repetitive mild traumatic brain injury and its association with the behavioral outcome

1.Review of literature

Traumatic brain injury (TBI) is a major public health concern, responsible for one-third of all injury-related fatalities (Roozenbeek et al., 2013). Worldwide, TBI affects over 27 million individuals, with motor vehicle accidents, falls, and assaults being the leading causes of hospitalization (James et al., 2019). It is a primary contributor to both mortality and long-term disability, affecting civilian and military populations alike. Those who survive often face persistent cognitive, motor, and personality changes, highlighting the urgent need for further research in this area. TBI is defined as a disruption in brain function or pathology caused by an external force (Menon et al., 2015). This broad definition encompasses a complex range of injury types, which often overlap, and recent efforts have focused on distinguishing these subtypes to develop more precise treatments based on specific pathobiological processes (Strianese et al., 2020). These approaches must also account for variations in host responses, which may be influenced by concurrent trauma or pre-existing conditions. TBI involves both primary and secondary injury mechanisms. Primary injury occurs immediately upon impact, resulting in mechanical damage to brain tissue such as contusions, hemorrhage, and axonal shearing. Secondary injury, which develops in the minutes to months following the initial trauma, involves a cascade of cellular and molecular events that cause further brain cell death, tissue damage, and atrophy. Unlike primary injury, secondary injury provides a window for therapeutic intervention, offering opportunities to prevent additional damage and improve long-term recovery. TBI severity is typically classified as mild, moderate, or severe. The Glasgow Coma Scale (GCS) is commonly used to assess TBI severity. However, the evolving nature of secondary injury and its associated pathophysiological responses highlight the complexity of TBI as a disease, which complicates treatment and patient care (Masel & DeWitt, 2010). Mild, moderate, and severe TBI are classified according to GCS scores, with mild TBI ranging from 13 to 15, moderate TBI from 9 to 13, and severe TBI from 3 to 8. Even mild to moderate injuries can result in cognitive and behavioral impairments. The rising incidence of Alzheimer's disease, chronic traumatic encephalopathy (CTE), and Parkinson's disease in individuals with severe or repeated mild TBI has drawn increasing concern. Mild TBI, the most common form, can cause neurological, cognitive, and behavioral changes. Neurological symptoms may include headaches, nausea, dizziness, sleep disturbances, and vision issues, while cognitive symptoms can involve attention, memory, language, communication, and executive function impairments. Behavioral changes often include irritability, aggression, impulsiveness, and poor social judgment.

Animal models are essential for understanding the mechanistic changes associated with TBI, as they help simulate clinical scenarios and produce reproducible results. Clinical TBI cases exhibit varying pathophysiological conditions due to factors such as injury location, severity, and pre-existing conditions, including genetics, age, health, gender, and substance abuse. In India, limited data indicates that TBI occurs frequently, with a notable percentage of cases (69%) occurring in individuals aged 15-35 years (Verma & Tewari, 2004). Road traffic accidents (60%), falls (20-25%), and violence (10%) are the leading causes of TBI (Gururaj, 2002). Developing countries like India suffer significant socioeconomic losses due to TBI, and the country has the highest global fatality rate from road traffic accidents (Samabasivan, 1991; Ramamurthi, 1991). Approximately 70% of TBI cases are classified as mild (miTBI) (Belanger, 1996), with 20-23% falling into the moderate or severe categories (moTBI). Alarmingly, about 40% of individuals with miTBI do not seek medical care (Sosin, 1991). Mild TBI can lead to post-concussion symptoms, which include neurological, cognitive, and behavioral manifestations. Although most of these symptoms resolve within 10 days to two weeks post-injury (d'Hemecourt, 2011), more than 25% of individuals continue to experience symptoms long after the injury (Dikmen, 2010; Lannsjo, 2009; Sigurdardottir, 2009).

Primary Injury

Primary injury in traumatic brain injury (TBI) refers to the initial damage due to direct impact to the head, rapid acceleration or deceleration, penetrating injuries, or blast events. This damage often results in membrane disruption, axonal injury, and blood-brain barrier (BBB) breakdown. Diffuse axonal injury (DAI) is one of the most prevalent pathologies in closed head injuries, often leading to coma (Smith et al., 2000; Povlishock & Katz, 2005). Primary axotomy occurs when axons are directly severed during TBI, leading to cytoskeletal distortion (Povlishock & Christman, 1995). Additionally, micropores may form in cell membranes, allowing sodium and calcium influx, which causes depolarization of the membrane potential (Pettus et al., 1994; Farkas & Povlishock, 2007; Krishnamurthy & Laskowitz, 2016). The blood-brain barrier (BBB), is a structure which separates brain parenchyma from the blood, consists of endothelial cells connected by tight junctions. This barrier regulates the influx of nutrients and ions while restricting harmful molecules, ensuring homeostasis and maintaining an immunologically privileged environment. TBI disrupts the BBB by damaging endothelial cells, disrupting tight junctions, and permitting atypical molecular transport (Campbell et al., 2012). This disruption contributes to neuronal dysfunction and initiates secondary injury cascades, compounding brain damage.

Secondary Injury Cascades

Secondary injury encompasses the pathological processes triggered by the primary injury. The central mechanism of secondary injury is excitotoxicity (Krishnamurthy & Laskowitz, 2016), characterized by an excessive release of excitatory neurotransmitters, particularly glutamate, leading to calcium overload in post-synaptic neurons. Under normal conditions, glutamate released from pre-synaptic neurons into the synaptic cleft binds to ligand-gated ion receptors such as N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) on post-synaptic membranes, facilitating sodium and calcium influx. However, after TBI, excessive glutamate release occurs in proportion to injury severity, with extracellular glutamate levels rising up to 50-fold compared to normal levels (Faden et al., 1989; Palmer et al., 1994; Bullock, 1995). This abnormal glutamate surge overwhelms the system, impairing the function of astrocytic glutamate transporters and exacerbating calcium influx into neurons. This cascade disrupts normal neuronal signaling, contributes to cellular damage, and perpetuates the cycle of neurotoxicity, ultimately leading to widespread neuronal death and further brain dysfunction. Increased intracellular calcium can lead to further neuronal damage and even death by several means, one of which is mitochondrial dysfunction (Boya, Gonzalez-Polo et al. 2003). Mitochondria have been described as “calcium sinks” as they will sequester large amounts of calcium from the cytosol in an effort to maintain calcium homeostasis (Ichas and Mazat 1998). Under injury conditions where calcium is overloaded in the cell, the large quantities of calcium taken up by the mitochondria lead to increases in mitochondrial production of reactive oxygen species (ROS) (Sullivan, Thompson et al. 1999; Chen, Yang et al. 2007), loss of ATP production (Jacobus, Tiozzo et al. 1975; Vercesi, Reynafarje et al. 1978), and opening of the mitochondrial permeability transition pore (Bernardi 1996). The consequence of these events is the release of cytochrome c and other pro-apoptotic factors which trigger the formation of the caspase-3 cleaving apoptosome. Cleavage of caspase-3 induces apoptosis of the cell, resulting in cell death. Another feature of calcium overload after TBI is activation of cytoskeletal-cleaving calpains (Araujo, Carreira et al. 2010). Breakdown of filaments and microtubules can cause damage to the structural integrity of the neuron (Liu, Liu et al. 2008) and dysfunction of the transported networks within the neuron (Saatman, Abai et al. 2003). Immunohistochemical labeling of transport proteins after TBI, such as amyloid precursor protein (APP), have been used to identify damaged axons within hours (Roberts, Gentleman et al. 1991; Roberts, Gentleman et al. 1994). However, not all axons within a region appear damaged by the primary injury, likely due to the heterogeneity among axons in their size and myelination. In contrast, some dysfunctional axons can also appear normal depending on the marker used to detect injury (Tomei, Spagnoli et al. 1990). Calpains are also responsible for cleaving the mGluR1 receptor blocking the neuroprotective PI3K-AKT

pathway (Liu, Liu et al. 2008). These events are spread via positive feedback, as the initial glutamate release in injured neurons induces further neurotransmitter release from the post-synaptic cell (Werner and Engelhard 2007). Disruption of the BBB initiates additional secondary injury cascades by allowing an influx of thrombin, fibrinogen and albumin into the parenchyma initiating a coagulation cascade (Price, Wilson et al. 2016). Decreased cerebral blood flow causes ischemia at the site of impact. A lack of oxygen and nutrients in the impact area limits the production of ATP needed for cell function (del Zoppo and Mabuchi 2003; Schwarzmaier, Kim et al. 2010). Alongside coagulation factors, neutrophils and peripheral immune cells, such as macrophages, also infiltrate brain tissue (Chodobski, Zink et al. 2011). Because the brain is considered immunologically privileged, the infiltrating immune cells are believed to become active, secreting cytokines (Kumar and Loane 2012). Secretion of these cytokines then initiates the activation of microglia and astrocytes. Microglia and astrocytes express pattern recognition receptors on their cell surface called Toll-like receptors (TLR) that allow them to recognize foreign or damaged cells (Gorina, Font-Nieves et al. 2011). Recognition by TLRs of trigger molecules induces the production and release of inflammatory chemokine and cytokines initiating the inflammatory cascade. Under normal conditions, astrocytes play an important role in maintaining homeostasis of neurons by the uptake of glutamate from synapses (Chen and Swanson 2003). Under injury conditions in which excessive amounts of neurotransmitters are released into the synaptic cleft, astrocytes downregulate production of glutamate transporters (Beschorner, Simon et al. 2007). This down-regulation is believed to perpetuate the excessive amounts of glutamate in the synaptic cleft resulting in cell death. Astrocytes are also responsible for maintaining the integrity of the BBB (Risau and 10 Wolburg 1990). Following TBI, astrocytes secrete inflammatory molecules that modulate reactivity and suppression of astrocyte function (Gorina, Font-Nieves et al. 2011). Secretion of the cytokine IL-6 by astrocytes has been reported to increase the permeability of the BBB (Schwaninger, Sallmann et al. 1999), further damaging the already delicate BBB allowing for greater infiltration of peripheral cells (Jin, Liu et al. 2012). In addition, astrocytes increase expression of glial fibrillary acidic protein (GFAP) and vimentin, two intermediate filament proteins (Pekny, Eliasson et al. 1999) and increase self-proliferation in areas of injury (Bardehle, Kruger et al. 2013). Within a week following injury in rodent models, reactive astrocytes have been shown to form a glial scar which effectively surrounds the injured tissue separating it from nearby healthier tissue (Villapol, Byrnes et al. 2014). The glial scar is beneficial in stopping the influx of peripheral immune cells that contribute to a continued inflammatory response (Dardiotis, Hadjigeorgiou et al. 2008), but creates a barrier for the regeneration of damaged axons. Microglia are the brain's resident immune cells. In their resting state, they constantly monitor the brain's environment, looking for signs of damage or infection. When injury occurs, microglia become activated, releasing pro-inflammatory cytokines to enhance their reactivity and assist in clearing debris through phagocytosis. While the removal of debris is beneficial there is growing literature that microglia can remain activated long after injury and may contribute to continued neurodegeneration (Holmin and Mathiesen 1999; Block and Hong 2005; Chen, Johnson et al. 2009). Similar to astrocytes, microglia exhibit heterogeneity. Activated microglia are thought to exist in three distinct states: classically activated, alternatively activated, and acquired deactivation. Classically activated microglia (also referred to as M1 stage) are believed to release pro-inflammatory cytokines and reactive oxygen species, which can lead to white matter damage and cell death (Karve, Taylor et al., 2016). In contrast, alternatively activated microglia (M2b stage) are thought to play a role in tissue repair and healing by producing scavenger receptors. Microglia in acquired deactivation states (also known as stage M2c) promote inflammation resolution (Cao, Thomas et al. 2012; Karve, Taylor et al. 2016). Several studies have reported a transient increase in M2 microglial responses following injury that is followed by a delayed increase in M1 microglia (Hu, Li et al. 2012; Jin, Liu et al. 2012; Wang, Zhang et al. 2013) suggesting the activated microglia observed chronically after injury are deleterious.

Mechanisms of Blast and Blunt-Induced Traumatic Brain Injury

The mechanisms of blast-induced and blunt-induced traumatic brain injuries involve complex and distinct processes. Blast-induced TBI is primarily caused by rapid pressure changes from blast waves, leading to diffuse axonal injury, neuroinflammation, and oxidative stress. In contrast, blunt-induced TBI results from direct mechanical forces, causing coup-contrecoup injuries, shear forces, and skull fractures. Research into these mechanisms continues to provide valuable insights into the pathophysiology of TBIs and informs the development of targeted treatments to improve outcomes for affected individuals.

Blast-Induced Traumatic Brain Injury (bTBI)

Blast-induced TBI primarily affects military personnel and individuals exposed to explosive devices. The unique nature of blast waves results in complex injury mechanisms that differ from other forms of TBI.

Mechanisms of Blast-Induced TBI:

1. **Primary Blast Injury**: This is caused by the direct impact of the blast wave on the brain. The rapid overpressure followed by under-pressure can cause significant brain injury without physical penetration. The mechanisms include:
   - **Rapid Pressure Changes**: The blast wave induces rapid compression and decompression of brain tissues, leading to shear forces and diffuse axonal injury (DAI) (Bauman, et al., 2009).
   - **Cavitation**: The negative phase of the blast wave can create cavities within brain tissues, leading to microvascular damage and hemorrhage (Garman, et al., 2011).
2. **Secondary Blast Injury**: This results from shrapnel and debris propelled by the blast. The impact can cause penetrating brain injuries and additional blunt trauma.
3. **Tertiary Blast Injury**: The blast wave can displace the body, causing it to collide with objects. This can lead to blunt trauma, similar to other forms of TBI.
4. **Quaternary Blast Injury**: This includes all other injuries from the blast, such as burns and inhalation of toxic gases, which can exacerbate brain injury.

Blunt-Induced Traumatic Brain Injury

Blunt-induced TBI typically results from impacts to the head, such as falls, motor vehicle accidents, or sports injuries. The mechanisms of injury involve direct mechanical forces applied to the skull and brain.

Mechanisms of Blunt-Induced TBI:

1. **Coup-Contrecoup Injury**: When the head strikes an object, the brain impacts the inside of the skull at the site of impact (coup) and the opposite side (contrecoup). This can cause bruising, bleeding, and axonal damage.
   - **Coup Injury**: The brain is damaged directly under the site of impact.
   - **Contrecoup Injury**: The brain is damaged on the opposite side due to the rebound effect (Gennarelli, et al., 1982).
2. **Shear Forces**: Rotational forces during the impact cause shearing of brain tissues, leading to diffuse axonal injury (DAI) (Adams, et al., 1982).
3. **Skull Fractures**: Blunt force can cause fractures of the skull, leading to additional brain damage from bone fragments and increased intracranial pressure.

1.1 Animal models of TBI

Animal models of traumatic brain injury (TBI) play a crucial role in advancing our understanding of TBI pathophysiology, evaluating potential treatments, and exploring therapeutic mechanisms. Animal models allow us to recreate TBI conditions in a controlled and reproducible manner. By inducing controlled injuries that mimic the mechanical forces and impact experienced in human TBI, these models helps in understanding pathophysiology, assessing neurobehavioral outcomes, exploring therapeutic interventions, investigating long-term effects and translational research.

Fluid percussion injury models

Fluid Percussion Injury (FPI) is a widely utilized and well-established model for traumatic brain injury (TBI). It has been effectively applied in several animal species, including rabbits, cats, rats, mice, and pigs. The injury can be delivered either centrally (midline FPI), along the sagittal suture between bregma and lambda, or laterally (lateral FPI), over the parietal cortex. This model produces graded neurological, histological, and cognitive outcomes that closely resemble the effects of human TBI (Dixon, 1987). The injury is induced by delivering a brief fluid pulse (~20 msec) to the dura mater through a craniotomy, causing a temporary deformation of the brain tissue. By varying the intensity of the fluid pulse and the location of the impact, the FPI model can mimic both diffuse and focal injury types, resulting in corresponding neurological impairments and neuropathological alterations. In the FPI model, injury is generated by a pendulum striking a piston connected to a fluid reservoir, which creates a pressure pulse transmitted to the dura mater through the craniotomy. This can be applied centrally along the midline or laterally over the parietal bone, between bregma and lambda. The impact causes a brief displacement and deformation of the brain tissue. The severity of the injury depends on the intensity of the pressure pulse (McIntosh et al., 1989)

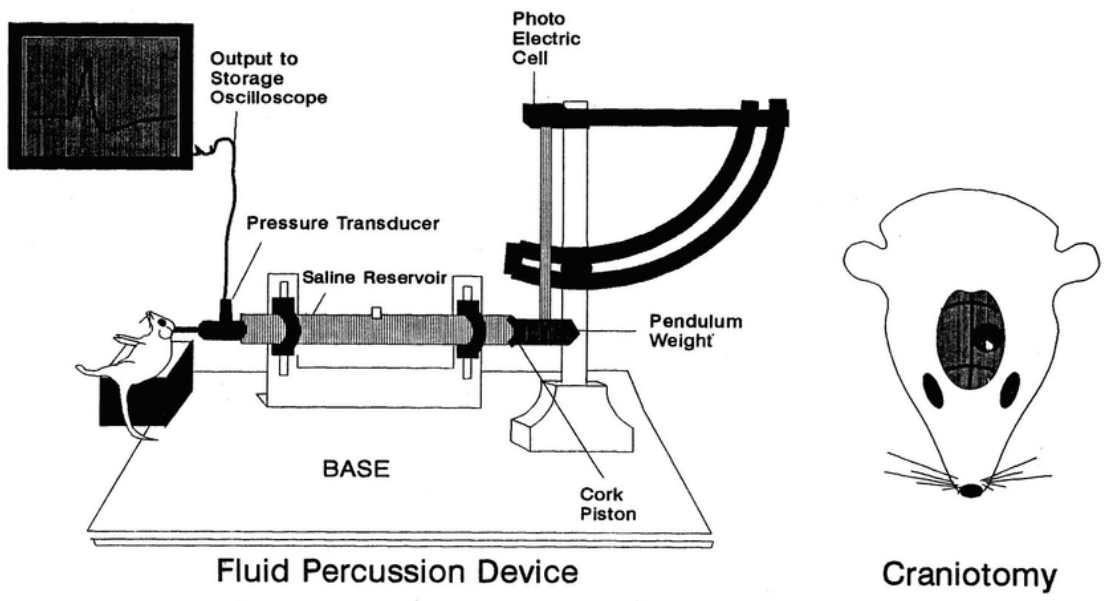


Figure: Fluid Percussion Device

Controlled cortical impact injury model

The CCI model uses a pneumatic or electromagnetic impact device to drive a rigid impactor onto the exposed intact dura, and mimics cortical tissue loss, acute subdural hematoma, axonal injury, concussion, blood–brain barrier (BBB) dysfunction and even coma (Morales DM, et al, 2005). The advantage of this injury model over other TBI models is the ease at which mechanical factors, such as time, velocity and depth of impact, can be controlled; thus, it may be more useful than the FPI model for biomechanical studies of TBI (Wang HC, Ma YB., 2010).

Weight drop TBI model

In weight drop models, the skull is exposed (with or without a craniotomy) to a free falling, guided weight (Morales DM, et al, 2005). Injury severity in these models can be altered by adjusting the mass of the weight and the height from which it falls. One such example is Marmarou weight drop apparatus.

Marmarou Weight Drop Apparatus

One widely used model is Marmarou's weight drop model, which simulates diffuse brain injury in rodents. The Marmarou's weight drop model was developed in the 1990s by Anthony Marmarou to create a reproducible and clinically relevant method for studying diffuse brain injury in animals. This model involves dropping a weight from a predetermined height onto a rodent's head, causing rapid acceleration and deceleration forces similar to those experienced in human TBIs. Marmarou's weight drop model of traumatic brain injury has been a cornerstone in TBI research, providing valuable insights into the pathophysiology and potential treatments for diffuse brain injury. Its ability to replicate key features of human TBIs, such as diffuse axonal injury and neuroinflammation, makes it an indispensable tool for researchers. The animal, typically a rat or mouse, is anesthetized and placed on a foam bed to absorb the impact. A weight, of 450 grams was dropped dropped from a specified height onto a small metal disc placed on the skull. The impact results in a diffuse brain injury, characterized by widespread axonal damage, edema, and hemorrhage. This model effectively mimics the biomechanical forces of human TBIs, making it a valuable tool for studying injury mechanisms and potential treatments.

Marmarou's weight drop model has significantly contributed to our understanding of TBI's pathophysiology. Its ability to induce diffuse axonal injury (DAI), a common feature in human TBIs, has made it particularly valuable for exploring the cellular and molecular responses to brain injury. Studies have shown that the model induces axonal swelling and disconnection, mirroring DAI observed in humans, neuroinflammation, oxidative stress, blood brain barrier disruption and cognitive and motor deficits (Smith, et al., 1997; Kumar, et al., 2015; Fang, et al., 2013; Hamm, et al., 1994) .

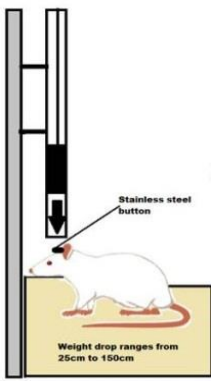


Figure: Marmarou Weight Device

Blast induced TBI model

Shock wave-generated traumatic brain injury (TBI) models have become a cornerstone in the study of blast-related brain injuries, particularly those experienced by military personnel and victims of explosions. These models aim to replicate the primary blast wave's effects on the brain, providing valuable insights into injury mechanisms and potential therapeutic interventions. Shock wave-generated TBI models are designed to simulate the primary blast wave, characterized by a rapid rise in pressure followed by a negative phase. This sudden pressure change can cause significant brain injury without physical penetration, making it a crucial area of study for understanding blast-induced TBI. The primary blast wave in shock wave-generated TBI models is typically produced using a shock tube, a device that generates controlled shock waves. The basic setup involves a high-pressure chamber separated from a low-pressure chamber by a diaphragm. When the diaphragm is ruptured, it creates a shock wave that travels through the low-pressure chamber, exposing animal to the blast.

Shock wave-generated TBI models have been instrumental in elucidating the pathophysiological mechanisms underlying blast-induced brain injuries. Previous studies showed these models replicate key features of human bTBI, such as diffuse axonal injury, neuroinflammation, and blood-brain barrier disruption, oxidative stress and cognitive decline (Garman, et al., 2011; Goldstein, et al., 2012; Kuriakose & Younger, 2013; Hoffer, et al., 2013; Elder, et al., 2010). Another study reported that the intensity and duration of the shock wave significantly influence the severity of brain injury (Taylor & Ford, 2009).

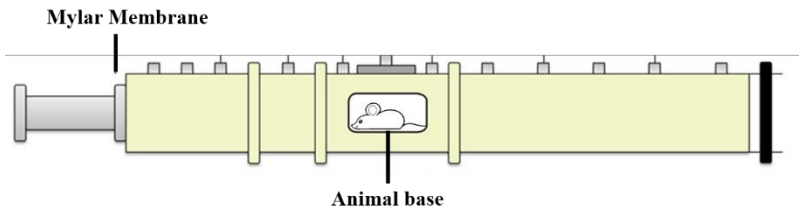


Figure: compression driven shock wave tube.

Metabolomics in TBI

TBI pathology and progression appears to have a metabolic basis. Studies have tried to establish evidence of a significant metabolic abnormalities such as oxidative stress (Bayir 2006, Viant 2005), mitochondrial dysfunction and excitotoxicity (Prasad and Bondy 2015, Viant 2005) in TBI. These changes are resultant of alterations in the cellular levels of different metabolites viz glucose, glutamate, lactate, pyruvate, NAA, NAAG, creatines etc (Timofeev 2011). Inflammation has shown to play a key role in TBI pathology (Yatsiv 2002), where metabolites like ATP and lactate have been reported as sensitive markers of inflammation. These findings suggest that metabolomics could play a pivotal role in investigating TBI (Fitzpatrick and Young 2013). However, there are few studies on TBI metabolomics reported in literature. Earlier study by Viant et al. (2005) employed 1H NMR to study the brain and plasma metabolome from rats following a TBI model of lateral fluid percussion. They report that the metabolic fingerprint following TBI comprises information on oxidative stress, excitotoxic injury, membrane disruption and neuronal injury (Viant 2005). Pascual (2007) report the use of 1H MRS to study Sprague–Dawley rats that were subjected to a closed head impact and examined over a period of 72 h. They found that using a stepwise multivariate model they were capable of discriminating amongst the various time groups (1, 9, 24, 48 and 72 h) based on a combination of taurine and myoinositol (Pascual 2007). Casey et al. (2008) reported alterations in cerebral metabolism over a 7 days period of immature rats following TBI. Brain tissue samples were analyzed using 1H NMR, revealing changes in metabolic processes between 4 and 24 hours after traumatic brain injury (TBI). Specifically, there was an increase in glycolysis and/or oxidative metabolism, as indicated by the elevated ratio of lactate to creatine. Additionally, at 24 hours and 7 days post-TBI, signs of neuronal damage were detected, as reflected by the reduced ratio of N-acetyl aspartate to creatine. These findings suggest that metabolic disturbances begin as early as 24 hours after TBI and persist for up to one week (Casey, 2008). Similarly, Lemaire (2011) used 1H NMR to study TBI in rats at 3, 24, and 48 hours after injury, noting that lactate, valine, and ascorbate were the first metabolites significantly altered following TBI. Robertson (2013), building on Casey's study, utilized both 1H and 13C NMR to track the utilization of labeled glucose in the brains of immature rats 24 hours post-TBI. They found that oxidative glucose metabolism in the immature rat brain begins to recover within 24 hours after injury, though they suggested further research to explore the pathways leading to decreased metabolism after TBI. The search for reliable serum biomarkers for TBI has been ongoing, with great potential for enhancing diagnosis, understanding the mechanisms of injury, monitoring disease progression, identifying complications, predicting outcomes, and discovering new treatments (Mondello and Hayes, 2015). The use of NMR spectroscopy to

identify brain biomarkers in TBI models, such as in a closed head injury mouse model (Bahado-Singh, 2016), has also been explored. Given that TBI damage progresses over time, serial measurements of metabolite levels are essential for tracking the extent of injury, monitoring therapeutic responses, and making accurate prognoses. Blood-based biomarkers offer a distinct advantage, as they can be repeatedly measured at relatively low cost and effort, which is why they are the focus of significant research interest (Neher, 2014). In our study, we employed NMR-based metabolomics to identify potential serum and urine biomarkers for various severities of TBI.

Histological and Neurobehavioral changes in experimental TBI

Experimental models of traumatic brain injury (TBI) are crucial for understanding the neurological, cognitive, and pathological changes that occur in human patients. Komoltsev et al. (2013) utilized a lateral fluid percussion injury (FPI) model in rodents to investigate the effects of TBI on behavior and neuronal health. Their findings revealed early memory deficits, observed through new object recognition tests (NORT), linked to elevated corticosterone levels on day 3 post-injury. Rats also displayed mild behavioral impairments, including delayed spatial memory deficits in the Barnes maze, which persisted for up to three months. Changes in stress reactivity were noted, including a reduced corticosterone response to restraint stress and altered reactions to forced swim stress. Additionally, TBI resulted in astroglial changes, including increased GFAP staining, which suggested activated astrocytes and potential morphological alterations observed three months after injury (Komoltsev et al., 2013). In a study by Hylin et al. (2013), mild TBI (miTBI) was induced using 1.0 and 1.5 atm overpressure FPI. Both injury intensities led to acute, transient suppression of neurological functions without visible brain contusion. However, the 1.5 atm injury caused temporary motor disturbances and significant impairments in spatial learning and short-term memory. This injury also resulted in a marked reduction in cerebral perfusion at the injury site, accompanied by a robust inflammatory response, evidenced by increased GFAP and Iba1 immunoreactivity in the corpus callosum and thalamus. Further, a significant reduction in fractional anisotropy (FA) values in the cingulum, along with increased silver impregnation, indicated axonal injury in this region as well as in the corpus callosum and internal and external capsules.

In a study using a controlled cortical impact (CCI) model, rats showed higher mean kurtosis and mean diffusivity values in the ipsilateral perilesional cortex and hippocampus, with decreased FA values in the corpus callosum. This was accompanied by increased GFAP and Iba1 staining, and reduced NeuN and MBP staining in all ipsilateral regions, suggesting considerable neural damage. No significant differences were observed in the contralateral regions for diffusional kurtosis imaging parameters or immunohistochemical staining. Behavioral tests, such as the Morris water maze, revealed longer platform crossing times in the probe test, indicating deficits in cognitive function (Wang et al., 2018). Johnstone (2015) induced moderate lateral FPI in adult male Sprague-Dawley rats and conducted behavioral assessments, MRI, and electrophysiological recordings from the barrel cortex 12 weeks post-TBI. The results showed sensorimotor deficits, cognitive impairments, and anxiety-like behaviors, alongside significant atrophy in the barrel cortex and other brain regions. Diffusion tensor imaging (DTI) revealed increased FA, axial diffusivity (AD), and tract density, suggesting long-term recovery of neuronal responsiveness through structural reorganization. Stemper (2015) used a rodent model of rotational acceleration-induced miTBI to examine the effects of injury magnitude and duration on behavioral and neuroimaging outcomes. While no significant locomotor or cognitive deficits were observed in the Composite Neuroscore (CN) and Morris Water Maze (MWM), the Elevated Plus Maze (EPM) test showed that increased injury duration led to more significant activity and exploratory behaviors. Ex-vivo DTI analysis indicated that changes in FA in the amygdala were associated with both the magnitude and duration of the injury, with extended injury duration resulting in FA changes at the interface between gray and white matter.

Repeated Traumatic Brain Injury

A history of traumatic brain injury (TBI) significantly increases the likelihood of sustaining subsequent head injuries, with individuals being 2 to 5 times more likely to experience another TBI (Guskiewicz et al., 2007; Emery et al., 2011). This heightened risk is especially concerning for those involved in activities such as military training and sports, where repeated head impacts are common (Guskiewicz et al., 2000; Marar et al., 2012). However, accurately estimating the incidence of TBIs in these activities is challenging due to two main factors. First, many head injuries go unreported (McCrea et al., 2004), and second, current data reporting systems often categorize TBIs from sports or recreational activities under broader categories like "falls" or "struck by/against" (CDC, 2015). It is widely believed that failing to allow sufficient recovery time between head injuries may result in more severe and lasting pathological and behavioral outcomes. Individuals who have experienced multiple mild TBIs tend to perform worse on cognitive tasks compared to those who have only had a single mild TBI (Gronwall & Wrightson, 1975). A particularly severe consequence of multiple head injuries is second impact syndrome (SIS), where a second head injury occurs before the symptoms from the first injury have fully resolved, leading to dangerously high intracranial pressure and, often, significant morbidity or mortality (McCrory & Berkovic, 1998). While most individuals with mild TBI recover within a week (McCrea et al., 2005; McCrory et al., 2005; Marar et al., 2012), repeated injuries can lead to more complicated outcomes.

A study by Allen et al. (2023) investigated mild and repetitive mild TBI (rmTBI) in rats and found persistent behavioral deficits in a beam task 1 and 4 hours post-injury. The researchers observed changes in hippocampal metabolism, including increased glutamine and decreased glucose, in rats with repetitive mild TBI, while no significant effects were seen in the single mTBI group. Although there were no significant changes in lipid levels or mitochondrial function in the hippocampus, some alterations in lipids were detected in the cortex following repeated injuries. The study used an awake-closed head injury (ACHI) paradigm to model mild TBI and assessed metabolic changes in the hippocampus 24 hours after the final injury using in vivo proton magnetic resonance spectroscopy (1H-MRS). Mitochondrial bioenergetics were also measured 30 hours post-injury. Lipidomic evaluations of the hippocampus and cortex were conducted, revealing potential metabolic shifts following repetitive injuries (Allen et al., 2023).

In another study on mild and repetitive mild TBI using the fluid percussion injury (FPI) model, significant changes in righting times were observed in all injury groups compared to sham rats 1 and 2 days after injury. Behavioral testing in the beam walking task revealed notable differences in latencies between groups over a 5-day period. Spatial learning and memory were assessed using the Morris water maze (MWM) task from days 9 to 13 post-injury, with significant differences in spatial learning latencies between the groups and the sham group. Histopathological examination, using Cresyl violet staining, showed cortical thinning, hemorrhaging, and tissue damage in several brain regions, with the most severe damage observed in the qFPI group. Additionally, microglial activity in the cortex and thalamus increased, and changes in the subcortical white matter were detected. GFAP levels were elevated in the subcortical white matter and thalamus, but remained unchanged in the hippocampus and cortex following injury (Fronczak et al., 2022).

Campos-Pires et al. (2023) examined blast-induced unconsciousness in rats and found significant changes in the righting reflex and latency to fall in the accelerating RotaRod test on days 1, 15, and 22 following the blast. Weight loss was observed up to day 22 post-injury. Neuronal loss was found in specific brain regions, such as the motor cortex, somatosensory cortex, auditory cortex, and amygdala, following repeated blast exposure. However, no significant changes in astrocyte activation were detected in these regions.

Finally, Bielanin et al. (2024) reported on a study of repetitive mild TBI (rmTBI) in a mice model using the controlled cortical impact (CCI) method. They found that rmTBI mice exhibited poorer motor function in the rotarod test at days 10 and 13 post-injury compared to sham mice. Behavioral testing at day 40 post-injury revealed worsened performance in the Y-maze novel spatial recognition test, with significant reductions in differentiation and recognition indices, indicating impaired spatial memory. The study also found increased expression of Na+/H+ exchanger protein (NHE1) in reactive astrocytes, microglia, and oligodendrocytes across vari-

ous brain regions, including the cortex, corpus callosum, and hippocampus. This upregulation of NHE1 was linked to increased oxidative stress, axonal damage, and gliosis following rmTBI.

Brief History of NMR

Nuclear magnetic resonance (NMR) was independently discovered in 1946 by Felix Bloch and Edward Purcell, who demonstrated that atomic nuclei with a magnetic moment can interact with electromagnetic radiation when exposed to a magnetic field. This discovery led to the development of NMR principles and mathematical models, which laid the foundation for the technique. Bloch and Purcell, along with their teams, showed that by applying a magnetic field and radiofrequency pulses, atomic nuclei could be manipulated and their emitted signals analyzed to gather information about molecular properties.

In the 1950s, Erwin Hahn furthered the technology by introducing continuous wave NMR, allowing measurement of nuclear relaxation times. The 1960s and 1970s saw Richard Ernst and colleagues introduce Fourier transform (FT) techniques, which greatly improved the speed, resolution, and sensitivity of NMR spectroscopy. Alongside this, Ernst and Jean Jeener proposed two-dimensional NMR techniques, such as COSY and NOESY, which enabled detailed structural analysis by identifying atomic connections and spatial arrangements. In the early 1970s, Paul Lauterbur and Peter Mansfield pioneered the use of NMR for imaging. Lauterbur introduced the concept of using magnetic field gradients for spatial encoding, while Mansfield developed image reconstruction techniques, leading to the birth of magnetic resonance imaging (MRI), a crucial tool in medical diagnostics. Advancements in NMR technology, such as stronger magnets, advanced pulse sequences, and better data processing, have allowed NMR to be applied in diverse fields, including chemistry, biochemistry, materials science, and medicine.

Physical Basis of NMR

Nuclear Magnetic Resonance (NMR) relies on the behavior of atomic nuclei in a magnetic field. The process hinges on the interaction between nuclear spins and an external magnetic field, providing insights into the chemical and physical properties of molecules.

Nuclear Spin: Nuclei possess intrinsic angular momentum, known as spin, which is vital for NMR. Nuclei with odd numbers of protons or neutrons have a non-zero spin, making them suitable for NMR. Common nuclei studied include hydrogen (^1H), carbon-13 (^13C), and phosphorus-31 (^31P).

Magnetic Moment: The spinning nucleus generates a magnetic moment, which is a vector indicating the strength and direction of the magnetic field.

Zeeman Effect: When exposed to a magnetic field, the nuclear magnetic moment aligns either parallel (low energy) or antiparallel (high energy) to the field, resulting in a small energy gap between these two states, known as Zeeman splitting.

Resonance: A second magnetic field, a radiofrequency (RF) field, is applied to induce resonance, exciting the nuclear spins and transitioning them between energy states.

Larmor Frequency: The resonance frequency is determined by the Larmor equation, which links it to the external magnetic field and the gyromagnetic ratio of the nucleus.

58 **Relaxation:** After excitation, nuclear spins return to equilibrium via two processes: longitudinal relaxation (T_1) and transverse relaxation (T_2). T_1 represents the recovery of alignment with the magnetic field, while T_2 is the loss of transverse magnetization, leading to signal broadening.

NMR Signal: Once the spins relax, they emit a signal detectable by an NMR coil, which is processed to yield an NMR spectrum that provides molecular structure and dynamics information.

Net Magnetization

In magnetic resonance, the hydrogen nucleus (^1H) is commonly used to gather information in biological tissues due to its prevalent presence in the body. When placed in a magnetic field, the individual hydrogen protons align and precess at a frequency proportional to the field strength. The energy difference between protons aligned with or against the field is governed by the Zeeman interaction and described by the Boltzmann distribution.

The application of an RF pulse at the Larmor frequency can excite the protons, causing them to flip between energy states. The manipulation of these magnetic moments forms the basis of MR techniques.

Relaxation

81 Relaxation in NMR refers to the processes by which nuclear spins return to their equilibrium states after being disturbed by an RF pulse. These processes are:

Longitudinal Relaxation (T_1): Involves the return of spins to their original alignment with the magnetic field, typically via energy exchange with the surrounding environment.

Transverse Relaxation (T_2): Refers to the decay of the transverse magnetization, caused by interactions among spins and field inhomogeneities.

Both T_1 and T_2 times affect the quality and resolution of NMR signals, and their characteristics depend on the molecular environment.

Free Induction Decay (FID)

The FID is the signal generated immediately after the application of an RF pulse, as nuclear spins precess and induce voltage in the NMR coil. The signal decays due to relaxation processes, and its decay is governed by T_2. Fourier Transform (FT) is then applied to convert the FID from a time-domain signal into a frequency-domain spectrum, revealing valuable information about the chemical environment of the sample.

Chemical Shift (δ)

Chemical shifts arise due to variations in the magnetic field around a nucleus, primarily influenced by the surrounding electrons and other nuclei. Each proton experiences a unique local magnetic field, which shifts the resonance frequency. This shift is measured relative to a reference compound and is expressed in parts per million (ppm), enabling comparison across different NMR experiments.

Signal Intensity

The intensity of NMR signals correlates with the concentration of the observed nuclei. The area under a peak reflects the number of contributing nuclei, although relaxation effects can influence the measured intensity.

Spin-Spin Coupling (Multiplicity)

Spin-spin coupling refers to the splitting of NMR signals due to interactions between adjacent nuclei. The number of peaks and their spacing depend on the number of neighboring nuclei. This coupling provides insights into molecular structure by revealing the number and type of nearby protons.

NMR Sequences for Biological Samples and In-Vivo MR Spectroscopy

Various pulse sequences are used in NMR for biological samples, with each chosen based on the type of data required:

Carr-Purcell-Meiboom-Gill (CPMG): This sequence is used to measure T_2 relaxation times, ideal for analyzing serum samples and detecting smaller metabolites.

ZGPR (Zero-Go-Pulse-Relaxation): A basic sequence often used to study water-solvent peaks in biological tissues.

Nuclear Overhauser Effect Spectroscopy (NOESY): This technique enhances the detection of metabolites by analyzing small changes in electron spin populations, commonly used for urine sample analysis.

NMR Metabolomics in TBI

Nuclear Magnetic Resonance (NMR) metabolomics has emerged as a valuable tool in traumatic brain injury (TBI) research, providing insights into the metabolic alterations associated with TBI.

NMR metabolomics enables the comprehensive analysis of metabolites present in biological samples, such as blood, cerebrospinal fluid, or brain tissue. By comparing the metabolic profiles of TBI patients with healthy individuals, researchers can identify specific metabolic changes associated with TBI. This approach helps in understanding the underlying metabolic pathways and dysregulations involved in TBI pathology. It allows the identification of potential biomarkers for TBI diagnosis, prognosis, and treatment monitoring. By examining the concentrations of specific metabolites or metabolic patterns, researchers can discover biomarkers that reflect the severity of injury, predict patient outcomes, or monitor the effectiveness of therapeutic interventions. These biomarkers can aid in early detection, personalized treatment, and assessment of TBI progression. NMR metabolomics data can be used to investigate the perturbations in metabolic pathways following TBI. By integrating metabolomics data with other omics approaches, such as genomics and proteomics, researchers can gain a comprehensive understanding of the molecular mechanisms underlying TBI. This information helps in identifying key metabolic pathways involved in TBI pathophysiology and potential therapeutic targets. TBI often leads to secondary injury mechanisms, including neuroinflammation, oxidative stress, and altered energy metabolism. NMR metabolomics

provides a means to assess these secondary injury processes by monitoring changes in metabolite levels associated with inflammation, oxidative damage, and energy metabolism. This knowledge can contribute to the development of targeted interventions to mitigate secondary damage and improve patient outcomes. Therapeutic Intervention and Monitoring: NMR metabolomics can be used to evaluate the effects of therapeutic interventions in TBI. By analyzing metabolic changes pre- and post-treatment, researchers can assess the efficacy of interventions and gain insights into the mechanisms of action. This information is valuable for optimizing treatment strategies and monitoring the metabolic response to therapy.

NMR metabolomics has the potential to enhance our understanding of TBI by providing valuable insights into the metabolic alterations associated with the injury. By identifying biomarkers, elucidating metabolic pathways, and assessing treatment responses, NMR metabolomics contributes to the development of personalized and targeted approaches for TBI diagnosis, prognosis, and therapeutic interventions.

Chapter 2

Effect of closed head injury in hippocampal metabolome of rats at acute time-point

2.1 Introduction:

75 Road accidents, falls, and assaults are the most common causes of blunt traumatic brain injury (TBI), which is highly prevalent in civilian populations. Survivors of TBI often experience long-term personality changes and impairments in cognitive and motor functions, highlighting the urgent need for novel pharmacological treatment options.

Research has shown that TBI involves multiple pathological mechanisms, including oxidative stress, excitotoxicity, membrane disruption, neuronal injury, energy failure, and mitochondrial dysfunction. Additionally, altered metabolite concentrations in the hippocampus have been linked to dysregulation of energy metabolism and excitatory neurotransmission. Metabolomics, which provides a comprehensive fingerprint of biochemical processes, is a valuable tool for studying complex diseases like TBI, offering insights into the underlying metabolic disruptions and potential therapeutic targets.

Xu et al. (2011) observed lower levels of total creatine ratios of N-acetylaspartate, glutamate, myo-inositol, phosphocholine, glycerophosphocholine, and taurine on the ipsilateral side of the brain after 2 and 4 hours of injury in an MRS study on the cci model of tbi. At 4 hours after TBI, there were significant decreases in myo-inositol and taurine, as well as minor reductions in NAA. Another study on the FPI model reported a decrease in glutamate, ascorbate, phosphocholine, glycerophosphocholine, and N-acetylaspartic acid (NAA) levels in the cortex and hippocampus after 1 h of injury (Viant et al,2005). Singh et al, 2017 reported alterations in the concentration of hippocampal neurometabolites at 4hr and day1 after injury in mild and moderate tbi rats. They reported decreased level of baa, lactate, succinate while an increase in the levels of acetate, myo- inositol, taurine, gaba, glutamate. Importantly, TBI-induced impairment in the neuronal metabolism is critical to the changing energy needs of the brain. A continuous energy flow, or adenosine triphosphate (ATP) production, is required to maintain basic neurological function and to also prevent the propagation of secondary injury cascades. Pandya et al. (2019) conducted a study on severe penetrating ballistic like brain injury model at acute timepoint and they reported decreased mitochondrial function and significant decrease in the level of total flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD) in frontal cortex and striatum of injured rats compared to sham after 24hrs of injury. Another study on moderate lateral fluid percussion injury reported significant decrease in cerebral blood flow at 6 and 24hrs after injury and decreased mitochondrial function after 6 hrs of injury (Jiang et al, 2000). Rana et al. (2020) found that altered metabolites in the hippocampus and frontal cortex are related to energy metabolism dysregulation and neurotransmitter metabolism at day 1, day 3, and day 7 of blast-induced traumatic injury using untargeted 1H-NMR. An experiment on the controlled

13 cortical impact (CCI) model of TBI using LCMS metabolomics reported alterations in arginine and proline metabolism as well as taurine and hypotaurine metabolism on day 3 post-injury in the rat hippocampus. Another study using LCMS reported alterations in the purine metabolism pathway in the cortical tissue of CCI rats at the sub-acute time point ().

Graded traumatic brain injury (TBI) in animal models is a research approach used to mimic the different levels of injury severity observed in human TBI cases. This technique allows researchers to study the effects of TBI across a spectrum of injury severities, ranging from mild to moderate to severe, and provides valuable insights into the underlying pathophysiological mechanisms and potential therapeutic interventions. The present study is designed to understand the pathophysiology of diffuse graded tbi on the metabolic homogeneity of the hippocampus at acute time point using 1H-NMR metabolomics.

To generate diffuse injury in TBI, we used a modified version of Marmarou's weight drop paradigm and 1H-NMR spectrometry to investigate metabolic changes in hippocampus regions following graded injury. Using 1H-NMR metabolomics, this study may help to understand the metabolic alterations and pathophysiology of hippocampal tissues after 24hrs of closed head injury in the hippocampus.

2. 2 Material and method

2.2.1 Chemicals

Saline (0.9% NaCl), Ketamine (Psychotropics India Limited, India), and Xylazine (Indian Immunological Limited, India) , 4% formalin, 4% paraformaldehyde fixative, Phosphate-buffered saline (pH 7.4), dH2O, sodium chloride (NaCl), potassium dihydrogen phosphate (KH2PO4), , acetonitrile (Sigma Aldrich, Germany), potassium chloride (KCl), trimethylsilylpropanoic acid (TSP) (Eurisotop, France), sodium hydrogen phosphate (Na2HPO4) (Merck, Germany) Thioflavin S, deuterium oxide (D2O) (Sigma Aldrich, Germany), and liquid nitrogen were used.

2.2.2 Study model:

Total sixty-five (n=65) male Sprague-Dawley rats of age 10-12 weeks were acquired from the institutional experimental facility. The weight of animals ranges from 250-280 gm each. Prior to experiment the animals were housed in controlled conditions at least for a week at 12-hour day- night cycle, 22±2 ^{0}C temperature and 50± 10% of relative humidity with constant supply of food and water *ad libilum*. All the experiments in this study were done according to the ethical guidelines of the institutional animal ethical committee.

2.2.3 Injury Model:

Animals were randomly divided into injury groups (control – 5; each injury group- 10). To develop the injury models an in- house modified version of Marmarou's impact acceleration device was used. For injury a brass rod weighted 450gms and 15cm height with 2.5 cm diameter was freely dropped from different heights ranges 25cm to 150cm on the brain. Prior to injury the rats were anesthetized, and a stainless-steel button was placed on the sagittal suture to create a diffuse injury to the brain. Following injury, the animals were kept warm on a bed till they regained consciousness.

2.2.4 Sample collection and processing:

After 24 hours post-injury, the animals were anesthetized, euthanized, and perfused with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA). After euthanasia, the brains were carefully extracted from the skull and dissected on ice. The hippocampus was isolated and immediately snap-frozen in liquid nitrogen before being stored at -80°C. Metabolites were extracted by mixing acetonitrile and water in a 1:1 (v/v) ratio. The tissues were homogenized using a handheld homogenizer (IKA T-10, Germany) at a low temperature to prevent metabolic degradation, with 5 mL of acetonitrile/water mixture per gram of frozen tissue. The homogenate was then centrifuged at 12,000 rpm at 4°C for 20 minutes. The supernatant was separated and stored at -80°C until lyophilization. After freeze-drying, the resulting powdered samples were stored at -20°C until further analysis by NMR spectroscopy. The whole brain was preserved in 4% formaldehyde for subsequent histological examination.

2.2.5 NMR data acquisition and processing:

The ^1H NMR spectra were recorded using a 600.33 MHz Bruker BioSpin spectrometer (Switzerland). The lyophilized samples were reconstituted in a mixture of 600 µL D2O and 0.5 mM trimethylsilylpropanoic acid (TSP). The solution was then centrifuged at

12,000 rpm at 4°C for 10-15 minutes. Following centrifugation, 580 µL of the supernatant was carefully transferred into 5 mm NMR tubes for spectral analysis. NMR spectra were acquired using a water-saturation NOESYPR1d pulse program at 298 K with the pulse sequence RD-90 -t- 90 -tm- 90 -acq. A total of 64 scans, including four dummy scans, were performed with a spectral width of 9009 Hz, an acquisition time of 3.63 s, and a relaxation delay of 4 s. After acquisition, the raw data were processed using Bruker TOPSPIN 3.5 software. Phase and baseline corrections were applied manually, and the peaks were integrated. The total sum of integrals for each spectrum was then utilized to normalize the peaks, with TSP serving as the reference for peak integration.

Histological staining

The fixed brain tissues were embedded in paraffin wax following processing. Following microtome the silane-coated silted were used to mount the 5 µm thick coronal slices. The tissue sections underwent deparaffinization with xylene and were rehydrated through a graded ethanol series before being washed in water.

For staining, a 1% thioflavin S solution was prepared by mixing it with 100% alcohol in a 1:1 ratio. The slides were incubated with the thioflavin S solution in the dark for one hour. After staining, the sections were examined under a fluorescence microscope (LMI BM-Prime, London, England), and images were captured using a 20X objective lens.

2.3 Statistical analysis:

Metabolic alterations between groups were compared using one-way analysis of variance (ANOVA). For posthoc analysis, the LSD test was used. $p< 0.05$ was considered statistically significant (SPSS version 20).

2 Results:

The metabolic profiles of the hippocampus were examined to determine the blunt-induced modification in the brain after 24 hours of injury. A total of 18 metabolites were detected. These metabolites are involved in a wide range of metabolic processes, including energy metabolism, ketone body metabolism, osmoregulation, neurotransmission, and membrane integrity.

2.1. Metabolic changes in hippocampal tissues after graded injury.

In our samples, we observed changes in neurometabolites (GABA, glutamine, and glutamate). All these three neurometabolites were down-regulated in hippocampal tissues on 24hrs after injury at all injury heights. Although significant downregulation was observed in glutamate levels at 25cm, 50cm, 100cm, 125cm, and 150cm injury groups. Whereas glutamine levels were notably low in 25cm, 50cm, and 125cm injury height groups. While GABA levels were significantly downregulated at all the injury heights after injury except 150cm injury group.

The energy metabolites which were altered in our samples were lactate, succinate, and creatine. Lactate and creatine levels were significantly low in 25cm, 50cm and 25cm,50cm and 100cm injury height groups respectively. Whereas a significant increase in succinate levels in 100cm and 150cm injury groups was observed at 24hrs post-injury.

The acetate levels were up-regulated at all injury heights at 24hrs after injury.

Taurine levels in hippocampal tissues were significantly down-regulated at 25cm,50cm and 125cm injury heights after 1day of injury. The levels of myo-inositol were significantly decreased at 25cm, 50cm and 125cm injury height groups.

The tyrosine level was significantly up-regulated in the hippocampus in 25cm injury height group. The NAA (an amino acid derivative) levels in hippocampal tissues were significantly down-regulated at all injury heights except 125cm group after 24hrs of injury. BAA levels were significantly higher in 25cm, 50cm,100cm and 150 cm injury groups after 24hrs of injury in compared to controls.

The choline level in 25cm, 50cm and 150cm injury groups were significantly lower after injury in compared to control rats.

2.2 Alteration in hippocampal tissue metabolism over the spectrum of injury severity.

In our samples, we observed changes in neurometabolites (GABA, glutamine, and glutamate). After 24 hours of injury, all three neurometabolites were down-regulated in hippocampal tissues in all injury groups. Glutamate and Glutamine levels were significantly levels were significantly lower in the groups with mild and severe injuries. Following damage, GABA levels were significantly decreased in mild and moderate injury groups.

Lactate, succinate, and creatine were the energy metabolites that were altered in our samples. Lactate levels were considerably lower in the mild injury group. While creatine levels were considerably lower in the mild and severe injury groups. There was a significant rise in succinate levels in the severe damage group.

At 24 hours following damage, acetate levels increased in all injury groups.

After 1 day of damage, taurine and myo inositol levels in hippocampus tissues were considerably reduced in mildly injured rats.

After 24 hours of injury, NAA (an amino acid derivative) levels in hippocampus tissues were significantly lower in mild and severely injured rats. Whereas BAA levels were significantly higher in the mild and severe injury groups compared to controls.

Level of choline in mild and severely injured rats was noticeably lower compared to control rats.

The tyrosine level was significantly up-regulated in the hippocampus in mild injury group compared to control.

Neurite plaques

Thioflavin S staining were performed to stain neurofibrillary tangles. We observed neurite plaques at all the injury heights except 25cm injury group rats. The number of neurite plaques was found to be in increasing order with injury severity. The maximum number of plaques was observed in 150cm injury height group after injury.

2.3 Discussion:

This study explores the impact of various diffuse injury levels on animal survival and neural metabolism in hippocampal tissue at an acute time point using NMR metabolomics. After 24 hours post-injury, significant changes were observed in the levels of neurotransmitters, osmolytes, and energy metabolites in the hippocampus compared to control animals. Immunohistochemistry and thioflavin staining were also conducted across all injury severities. The data revealed a notable reduction in glutamate, GABA, glutamine, acetate, NAA, and BAA levels on day 1 post-injury, which may reflect disruptions in energy homeostasis. Given that the hippocampus consists primarily of glutamatergic neurons (approximately 90%), the observed decrease in glutamate could result from altered glutamate homeostasis following the injury [Briend et al., 2020; Piao et al., 2019]. In normal brain physiology, the continuous transport of glutamine from astrocytes to neurons for glutamate and GABA production is essential for maintaining metabolic stability. [Albrecht et al, 2010]. A significant decrease in GABA level was observed on day 1 of injury in almost all the injury severity groups.

Down-regulation in GABA levels suggest alteration in GABA production as an effect of injury. While significant decrease in the levels of glutamine in mildly injured groups may influnced by the disturbance in glutamate- glutamine cycle. The glutamate glutamine cycle is a critical process for the compartmentalization of glutamate and glutamine between neurons and glia, and during glutamatergic transmission, neurons release glutamate into the surrounding extracellular space (Petroff, 2007) (Sowers et al, 2021). Additionally, the level of NAA was significantly down regulated and acetate levels were notably high in hippocampal tissues after injury at almost all the injury heights. NAA acts as an acetate reservoir in the brain after injury to maintain acetyl-CoA levels [Moffet et al, 2013]. This acetyl-CoA then enters the TCA cycle for energy production [Akram, 2014]. Di Pietro et al. (2014) reported a decrease in whole brain tissue NAA levels in rats following mild and severe diffuse TBI using hplc [Pietro et al, 2014]. A NMR study on blast-induced repetitive mild TBI reported a decreased level of NAA in hippocampal tissues after 24 hours of injury [Kumari et al, 2023]. This suggests NAA can participate in energy production with acetate as an energy substrate after TBI. Decrease in NAA level and increase in acetate levels in almost all the injury groups may due to high energy demand of the hippocampal tissues after injury.

BAA is crucial for neurotransmitter synthesis and energy metabolism, and its levels decrease one day post-injury compared to controls [60]. Similarly, our findings show a reduction in BAA levels in hippocampal tissue at 1 day post-injury (PI). This decline in BAA suggests that the tissue experiences a high energy demand shortly after injury. Taurine, a neuromodulator that regulates dopamine in the brain [Kontro et al., 1987], plays a role in the reward system. It is known to enhance dopamine release, and we observed lower taurine levels in both mild and severe injury groups compared to controls. Additionally, choline levels were reduced, particularly in the mildly injured rats. Choline is an essential phospholipid for cell membranes, necessary for synthesizing phosphatidylcholine and sphingomyelin, and for acetylcholine production, which is critical for cholinergic neurotransmission. A loss of choline in the central nervous system (CNS) can impair acetylcholine release, potentially leading to deficits in spatial cognition and memory within the hippocampus (Zeisel et al., 2003; Zeisel, 1992). The observed decrease in choline following TBI suggests a disruption in neuronal membrane integrity, which could contribute to altered cholinergic neurotransmission post-injury. Following TBI, cells' energy demands to maintain vital cellular activities increase dramatically. This imbalance in metabolism and energy dysfunction can alter overall metabolic processes (Carpenter et al., 2015) and increase lactate production for energy (Patet et al., 2015; Van Hall et al., 2009). In our study, we found that lactate levels were downregulated in all mildly injured rats compared to controls. The reduction in lactate levels shortly after injury highlights its critical role in ATP production. Creatine, another essential energy metabolite, also plays a vital role in tissues with high energy requirements, such as the brain and muscles. As a key ATP reserve, creatine is crucial for energy storage and release. Our study observed a decrease in creatine levels in mildly injured rats compared to controls (Andres et al., 2008; Brosnan and Brosnan, 2007). Additionally, Andres et al. (2008) reported that in CNS diseases, creatine metabolism and the creatine kinase/phosphocreatine system are compromised, as the brain requires high amounts of ATP to maintain membrane potentials and signaling capacity. Finally, we observed significantly higher succinate levels in the hippocampus of severely injured rats. This increase may be linked to mitochondrial damage, which triggers the production of reactive oxygen species (ROS). Under aerobic conditions, intracellular succinate is rapidly metabolized into fumarate. Under anaerobic conditions, elevated levels of extracellular succinate bind to and activate the G protein-coupled succinate receptor GPR91 on stellate cells and macrophages in the liver, which triggers inflammation (Littlewood-Evans et al., 2016; Mills et al., 2021). Hypoxia also increases succinylation (Gibson et al., 2015; Chen et al., 2017). It is also reported that in metabolic function, succinate accumulation induces mitochondrial respiration and changes the metabolic profile (Ehinger et al., 2016; Ives et al., 2020; Zhang et al., 2020; Avram et al., 2021). Huang et al. (2022) reported accumulation of succinate in the neuronal stem cells of ischemic rats (Huang et al. 2022).

BAA works as a nitrogen doner in the glutamate synthesis, even around 25% of nitrogen bases accept nitrogen from leucine (Yudkoff, 1997). High level of BAA and low level of glutamate in injured hippocampal tissues may due to the interruption in glutamate synthesis. In addition to this thioflavin staining were done to observe the amyloid beta (Aβ) fibril formation after injury. We observed amyloid plaques at all the heights after injury. An ELISA study on TBI reported accumulation of Aβ fibrils in mice cortical tissues after 1,3 and 7 days of injury (Washington et al. 2014). Chen et al (2004) reported Aβ plaques in swine white matter after rotational head injury at all the timepoints (i.e.from day3 PI to 6 month PI). We also found our results in line with these studies.

2.4 Conclusion:

The current study uses NMR metabolomics to investigate the effects of a range of diffuse closed head injuries on hippocampus metabolism at an acute time point. TBI-induced metabolic changes are visible in changed levels of neurotransmitters, energy metabolites, and neuromodulators in hippocampus tissues. Furthermore, neurite plaques were discovered at all damage heights investigated, however the patterns of change varied depending on the injury category. This study gives important pathological insights into diffused closed head injury in people and contributes to our understanding of the tbi pathophysiology.

Chapter 3

Acute and sub-acute metabolic change in different brain regions induced by moderate blunt TBI

3.1 Introduction:

Brain injury can lead to significant metabolic changes in the body, both acutely and subacutely. A brain injury disrupts the normal functioning of the brain, which can impact various metabolic processes throughout the body.(Bartnik et al., 2007) During the acute phase of a brain injury, there is a surge in metabolic activity as the body tries to repair and recover from the damage. This higher metabolic workload is mostly sustained by glucose and other nutrients obtained from the bloodstream. The interaction of disturbed intracellular bioenergetic pathways is expected to play a role in degenerative brain disorders. These metabolic alterations can cause brain edema, bleeding, and ischemia, which are all dynamic states that persist even after the initial damage. Furthermore, the severity of the brain injury can determine the intensity of the metabolic changes. In cases of mild brain concussion, there may be a brief increase in glucose metabolism in certain areas of the cerebral hemispheres and the brain stem.(Jalloh et al., 2014) In contrast, severe head injuries are associated with a consistent decrease in cerebral oxygen metabolic rate (Sunami et al., 1989). This decrease in cerebral oxygen metabolic rate can lead to further complications and impairments in brain function. Additionally, it is important to note that the pathophysiological mechanisms of brain injury are complex and can vary depending on the type of injury.(Vink et al., 1994)

Rana et al. (2020) found that altered metabolites in the hippocampus and frontal cortex are related to energy metabolism dysregulation and neurotransmitter metabolism at day 1, day 33,and day 7 of blast-induced traumatic injury using untargeted H1-NMR [6]. An experiment on the controlled cortical impact (CCI) model of TBI using LCMS metabolomics reported alterations in arginine and proline metabolism as well as taurine and hypotaurine metabolism on day 3 post-injury in the rat hippocampus [7]. Another study using LCMS reported alterations in the purine metabolism pathway in the cortical tissue of CCI rats at the sub-acute time point [8]. McGuire et al. (2019) reported metabolic changes in the hippocampus, thalamus, frontal cortex, striatum, and brainstem after the fluid percussion injury (FPI) model after 60 days of injury. It was found that levels of creatine, glutamine, glutamate, lactate, myo-inositol, and taurine in hippocampal tissues were altered. They also reported notable changes in the levels of creatine and glutamine in the thalamus[9]. A study on mice's brains after 2 and 7 days of excitotoxic neuronal injury found that distinct brain regions (cortex, hippocampus, striatum, midbrain, cerebellum, and brainstem) show variable vulnerability to injury and highlight the spatial role of injury-induced mechanisms [10]. Another study on the FPI model reported a decrease in glutamate, ascorbate, phosphocholine, glycerophosphocholine, and N-acetylaspartic acid (NAA) levels in the cortex and hippocampus after 1 h of injury [5]. Importantly, TBI-induced impairment in the neuronal metabolism is critical to the changing energy needs of the brain. A continuous energy flow, or adenosine triphosphate (ATP) production, is required to maintain basic neurological function and to also prevent the propagation of secondary injury cascades. A study on TBI patients using micro dialysis showed an elevated lactate/pyruvate ratio in white matter over the first 3–7 days after injury [11]. Along with this, it has been reported that TBI leads to a prolonged glucose depression that can induce conditions like Alzheimer’s disease as glucose deprivation promotes amyloidogenesis and tau pathology and affects synaptic function [12]. Therefore, injury-induced metabolic alterations in the brain are important to understand to prevent long-term neurological deficits.

Because of the distinct tissue composition of each brain region, the metabolome of each region differs, leading to differential responses to injury. The majority of the neurons in the hippocampus are pyramidal. Nakatomi et al. (2002) reported regeneration of

hippocampal pyramidal neurons in ischemic rats[13]. The thalamus is made up of excitatory projection neurons and inhibitory interneurons, while the stratum is made up of medium-spiny neurons and interneurons. A study on Parkinson's disease reported altered medium spiny neurons [14]. A mouse cci model of TBI study reported a notably lower proportion of inhibitory input neurons after 24 hours of injury [15]. Another study on a mixed diffuse and focal injury reported a change in inhibitory neurons after 8 weeks of injury [16].

The current study is designed to understand the differential effect of diffuse brain injury on the metabolic homogeneity of the regions distant from the site of impact (hippocampus, thalamus, and striatum) at acute, early sub-acute and sub-acute time points using 1H-NMR metabolomics.

For TBI, we used a modified version of Marmarou's weight drop model to induce diffuse injury and 1H-NMR spectrometry to assess the metabolic change in tissues following injury. This study may help to understand the metabolic changes and pathophysiology of TBI in the hippocampus, thalamus, and striatum after day 1 and from the site of impact (hippocampus, thalamus, and striatum) at acute, early sub-acute and sub-acute time points using 1H-NMR metabolomics. For TBI, we used a modified version of Marmarou's weight drop model to induce diffuse injury and 1H-NMR spectrometry to assess the metabolic change in tissues following injury. This study may help to understand the metabolic changes and pathophysiology of TBI in the hippocampus, thalamus, and striatum after days 1, 3, and day 7 of injury. This study may help in better understanding the metabolic cascade of pathological events in different brain regions following trauma.

3.2 Material and method

3.2.1 Chemicals

Saline (0.9% NaCl), Ketamine (Psychotropics India Limited, India), and Xylazine (Indian Immunological Limited, India) , 4% formalin, 4% paraformaldehyde fixative, Phosphate-buffered saline (pH 7.4), dH2O, sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH2PO4), sodium hydrogen phosphate (Na2HPO4) (Merck, Germany), acetonitrile (Sigma Aldrich, Germany), trimethylsilylpropanoic acid (TSP) (Eurisotop, France), deuterium oxide (D2O) (Sigma Aldrich, Germany), and liquid nitrogen were used.

3.2.2 Study model:

All experiments in this work were conducted in accordance with the Institutional Animal Ethical Committee's ethical guidelines (INM/IAEC/2019/09/Ext1). To evaluate the effect size, we determined the ɳ2 value for each metabolite in all three brain areas (supplementary table 1). We obtained twenty (n = 20) male Sprague-Dawley rats aged 10 to 12 weeks from the Institute's Experimental Animal Facility.These rats weighed an average of 250-280 g each. The animals were housed in a well-ventilated room with regulated circumstances, including a temperature of 22±2ºC, relative humidity of 50±10%, and a 12-hour day-night cycle. The rats were constantly given adequate amounts of food and water. Rats were divided into two groups: sham controls (n=5) and rats with TBI (n=15). Out of 15 rats, brain tissue samples were collected at 24hr, 3day and 7 days (n=5 at each time point).

3.2.3 Injury Model:

The head injury animal model was developed using the in-house modified Marmarou's impact acceleration model [17]. Rats were anaesthetized, and a stainless-steel button was placed on the skull. The button was placed between the bregma and lambda on the

sagittal suture. The stainless-steel button was placed to create a global injury to the brain. For injury, a brass rod of 450g was freely dropped from 75cm height. After injury, rats were placed on a warm bed until they regained consciousness (Figure 1).

3.2.4 Sample collection and processing:

At each time-point after injury, the animals were perfused with PBS followed by PFA. Following perfusion, the brains were excised from cranium and then hippocampus, thalamus and striatum were separated on ice and snap frozen in liquid nitrogen and then stored at -80^{0}C. The acetonitrile extraction method was used for the extraction of metabolites. A handheld homogenizer (IKA T-10, Germany) was used to homogenize the tissue in a (1:1, v/v) acetonitrile/water mixture of 5 ml per 1 g of frozen tissue. The homogenized tissue mixture was centrifuged for 20 min at 12,000 rpm at 4 ºC. The supernatant was collected and stored at -80^0 C before lyophilization. The tissue samples were lyophilized following extraction and stored at -80^{0}C till spectra acquisition. During the processing, some of the samples were lost. (Supplementary table 2).

3.2.5 NMR data acquisition and processing:

1H NMR spectra were acquired utilizing 600.33 MHz (Bruker, BioSpin, Switzerland) spectrometer. The lyophilized examples were reconstituted in a combination of 600 µl D2O and 0.5 mM trimethylsilylpropanoic acid (TSP). The mixture was then centrifuged at 12000 rpm at 40°C for 10-15 minutes. After centrifugation, 580 µl of the supernatant was separated and transferred into 5 mm NMR tubes for the acquisition of NMR spectra. A water saturation NOESYPR1d pulse program was used for the acquisition of NMR spectra at a temperature of 298 K, and with the pulse sequence RD-90 -t- 90 -tm- 90 -acq. Total number of scans (ns= 64), including four dummy scans, were performed, with the spectral width 9009 Hz, the acquisition time 3.63 s, and the relaxation delay 4 s. After spectra acquisition, the Bruker TOPSPIN 3.5 software was used to process the raw NMR spectra. Manual changes were made to correct the phase and baseline correction and then pecks were integrated (Figure 2). TSP was used as reference for integration. After integration, the pecks were normalised by dividing the total sum of integrals for each spectrum.

3.3 Statistical analysis:

Metabolic alterations between groups were compared using one-way analysis of variance (ANOVA). For posthoc analysis, the Bonferroni test was used. $p< 0.05$ was considered statistically significant (SPSS version 20). Pathway analysis was done using MetaboAnalyst 5.0 software.

3.4 Results:

The metabolic profiles of hippocampus, thalamus, and striatum tissue were evaluated to assess the blunt-induced alteration in the brain after 1 day, 3 days, and 7 days of injury. A total of 18 metabolites were observed. These metabolites are involved in a variety of metabolic processes like energy metabolism, ketone body metabolism, osmoregulation, neurotransmission, and as associates in different metabolic pathways and membrane integrity (Figure 3).

One-way ANOVA

Neurometabolites in different regions were studied at each time-point. Temporal alterations were observed in the metabolic levels (Figure 3). In addition to metabolite alterations in each region with time, we also observed significantly altered metabolites between different regions at the baseline study (Table 1).

3.4.1 Change in metabolic levels among different brain regions in control

At baseline, we observed increased levels of NAA, myo-inositol, creatine, glutamate, and lactate in the hippocampus compared to both the thalamus and striatum; however, these metabolites showed no change between the thalamus and striatum. Choline, alpha-KG, GABA, acetate, and branched amino acid levels were significantly lower in hippocampal tissue compared to both the striatum and thalamus. Significantly increased levels of aspartate and tyrosine were observed in the thalamus compared with both the hippocampus and the striatum. Taurine levels were highest in striatal tissue, followed by the hippocampus, and then the thalamus.

3.4.2 Temporal changes in metabolites within a region after injury

In our samples, we found changes in GABA and glutamate. Glutamate levels were downregulated in hippocampal tissues on day 3 after injury. TBI group rats had significantly lower GABA levels in thalamus at day1 and in striatum at days 1 and 3, respectively.

The energy metabolites that were altered in our samples were lactate, succinate, and creatine. In the hippocampal region, significantly decreased levels of lactate and creatine were observed at day 3 PI. Whereas a significant increase in succinate levels was observed in the hippocampus at day 3 PI. No significant change was observed in the levels of these three metabolites in the thalamus and striatum at any time point after injury.

On day 1, the acetate levels in the thalamus region were significantly lower. The level of alpha KG showed a down-regulation at day 1 and day 3 PI in striatal tissues after injury.

Taurine levels in the striatum were significantly downregulated at days 1 and 3 PI. The levels of myo-inositol were significantly decreased at day 3 in the hippocampus, while the thalamus and striatum showed significantly increased myo-inositol levels at day 1.

The tyrosine level was significantly up regulated in the hippocampus at day 7 PI. The NAA (an amino acid derivative) levels in hippocampal tissues were downregulated at days 3 and day7 PI. Although, it reached statistical significance at day 3 PI. BAA showed no significant changes in the hippocampus at any time after injury. BAA levels were significantly lower in the thalamus and striatum on day 1 after injury compared to controls.

The choline level in the thalamus was significantly lower after injury at all the time points, while in the striatum it was significantly downregulated at day 1 and day 3 PI compared to control rats.

3.4.3 Pathway analysis:

Most of the observed metabolites participate in alanine, aspartate, and glutamate metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; glutamine and glutamate metabolism; and taurine and hypotaurine metabolism. Out of these four pathways, the alanine, aspartate, and glutamate metabolism pathways were found to be altered in all three regions, with the maximum impact value among all pathways (0.71). The alanine, aspartate, and glutamate metabolism pathways were found to be altered in all three regions at different time points; they showed alteration in the hippocampus at days 3 and day 7 PI, in the thalamus at days 1 and 7 PI, and in the striatum at day 1 PI. Phenylalanine, tyrosine, and tryptophan biosynthesis were found to be altered in the hippocampus at days 3 and 7 PI, in the thalamus at day 1 PI, and in the striatum at day 3 PI. Taurine and hypo-taurine pathways were found altered only in the striatum at day 1 and day 3 PI (Table 2).

3.5 Discussions:

The present study demonstrates the effect of moderate diffuse TBI on the neural metabolism of different brain regions at acute and sub-acute time points using NMR metabolomics. The effect of injury on brain regions was non-uniform. The levels of neurotransmitters and osmolytes were altered in all three regions, whereas the levels of choline were altered in the thalamus and striatum. In addition to neurotransmitters and osmolytes, energy metabolites were altered in the hippocampus. While acetone levels were changed only in the thalamus. The level of taurine was found to be altered only in striatal tissues. Alanine, aspartate, and glutamate metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; glutamine and glutamate metabolism; and taurine and hypotaurine metabolism are important pathways that change after injury.

Alterations in metabolic pathways are the result of collective metabolic changes rather than a single metabolic alteration. Several metabolites are involved in alanine, aspartate, and glutamate metabolism, as well as glutamine and glutamate metabolism. These metabolites function as a neurotransmitter or neuromodulator and as an energy metabolite. Alteration in these metabolic pathways is responsible for an imbalance between excitatory and inhibitory neurotransmitters and perturbs energy metabolism [18]. Succinate, oxo-glutarate, pyruvate, and fumarate are metabolites that participate in the above metabolic pathways as well as the tricarboxylic acid cycle (TCA cycle) [19]. As a result, alterations in these metabolites may affect the TCA cycle. Phenylalanine is a precursor of tyrosine, and tyrosine participates in dopamine, epinephrine, and norepinephrine [20,21]. Perturbation in phenylalnine, tyrosine, and tryptophan biosynthesis may influence neurotransmitter production and ultimately cause cognitive decline [22]. 3-(4-Hydroxyphenyl) pyruvate is another intermediary metabolite that participates in phenylalnine, tyrosine, and tryptophan biosynthesis. 3-(4-hydroxyphenyl) pyruvate changes into fumarate in the cytoplasm and enters mitochondria to participate in the TCA cycle (Bénit et al., 2014). Taurine and hypotaurine metabolism start with the conversion of l-cysteine to taurine, and then taurine transforms into taurocholate and 5-glutamyl taurine.

Metabolic alteration among brain regions in controls

Metabolic alterations were observed among brain regions in control animals. We found an increased level of myo-inositol, creatine, glutamate, NAA, and lactate in the hippocampus compared to the thalamus and striatum. While taurine level in hippocampus was found significantly lower than striatum and thalamus. Most of these metabolites are involved in energy generation, indicating that energy demand in the hippocampus is higher than in other regions of the brain. A study on the energy efficiency of different brain regions also reported that the hippocampus needs more energy for its function than the thalamus [23]. Metabolically, hippocampal tissues act differently compared to the other two regions. This may be due to the unique tissue architecture and functions of different brain regions. Neurogenesis is the process of generating new neurons. The dentate gyrus of the hippocampus is one of the important sites for neurogenesis. Growing knowledge of neurogenesis suggests that neurometabolites, energy metabolites, neuromodulators, and osmolytes play important roles in this process. Glutamate, an excitatory neurotransmitter in mature neurons, can influence immature neural cell proliferation and differentiation [24]. GABAergic mechanisms control differentiation and the timing of synaptic integration in the sub-granular zone [25].Neurogenesis is known to be energetically demanding, and studies have revealed that lactate, rather than glucose, is favored as a metabolic substrate in neurons to support their activity when faced with high energy demands [26]. Lactate plays a crucial role in modulating molecular pathways associated with neurogenesis, such as angiogenesis, neuronal excitability, and plasticity. These pathways collectively contribute to the survival and development of newly formed neurons [27,28]. Non-neural cells in the neurogenic niche, such as astrocytes, endothelial cells, oligodendrocytes, and microglia, which direct the process of neurogenesis, are also influenced by lactate in their metabolic and signaling pathways. Additionally, NAA is one of the most concentrated metabolites in the brain; studies suggest that NAA works as an acetate reservoir in the brain, and the reduced level of NAA is considered parallel to low ATP levels [29]. Creatine is an amino acid that is mostly found in muscle and the brain and helps in

energy production. Leem et al. (2018) reported that creatine and exercise prevent a decrease in neurogenesis in mice's brains. GABA, aspartate, choline, acetate, and BAA were found to increase in thalamic tissues compared to the hippocampus, followed by the striatum[30]. The thalamus contains a large number of gabargic neurons and interneurons[31,32]. A study reported the highest gabaergic TCA cycle flux in the thalamus and hypothalamus [33]. Acetate, aspartate, and branched-chain amino acids are used by the brain for energy metabolism [34–36]. Although cholinergic neurons are distributed to almost all parts of the brain, a major population is present in the striatum [37]. In cholinergic neurons, acetylcholine is formed from choline and acetyl coenzyme A (acetyl coA) [38]. While the thalamus contains cholinergic axons and dense cholinergic innervation, which contain a high amount of acetylcholine, striatal cholinergic neuronal activity plays a crucial role in motor control, memory, social behavior, and behavioral flexibility. Striatum contains a relatively high amount of taurine [39]. Taurine is a neuromodulator, and it regulates dopamine in the brain [40]. Striatum contains a population of dopaminergic neurons. Dopamine is a part of the reward circuitry in the brain. Studies report that taurine increases the release of dopamine in the brain. We also observed a relatively high level of taurine in the control striatum.

Metabolic alteration in the hippocampus following TBI

TBI studies report that it induces neurogenesis in the brain. Neurogenesis requires a lot of energy; the brain prioritizes energy homeostasis maintenance [41]. To meet the high energy demands of neurogenesis, neuronal stem cells switch their metabolism from glycolysis to oxidative phosphorylation [42].

The significant decreased levels of glutamate, creatine, lactate, and NAA at day 3 after injury in the hippocampus of injured rats may be due to altered energy homeostasis. As the hippocampus contains 90% of glutamatergic neurons, a decrease in glutamate levels could be due to altered glutamate homeostasis after injury [43,44]. Physiologically, transportation of glutamine from astrocytes to neurons for the production of glutamate, or GABA, takes place in continuation; this balance is crucial for metabolic stability inside the brain [45]. Glutamate to alpha keto-gluterate conversion is a reversible reaction where it enters the TCA cycle, and succinate is an intermediate of the TCA cycle [46]. Thus, down-regulation of glutamate and up-regulation of succinate at day 3 PI may indicate the preference of the brain for energy production. A decrease in the level of glutamate and increased levels of succinate at day 3 PI in hippocampal tissues implies neuronal damage and a preference for energy production. At day 7 PI, both metabolites start to come across the control, which may be due to recovery after injury. In addition to this, the level of NAA was significantly down regulated in hippocampal tissues after 3 days of injury. NAA acts as an acetate reservoir in the brain after injury to maintain acetyl-CoA levels [47]. This acetyl-CoA then enters the TCA cycle for energy production [48]. Di Pietro et al. (2014) reported a decrease in whole brain tissue NAA levels in rats following mild and severe diffuse TBI using hplc [49]. A NMR study on blast-induced repetitive mild TBI reported a decreased level of NAA in hippocampal tissues after 24 hours of injury [50]. This suggests NAA can participate in energy production with acetate as an energy substrate after TBI. A decrease in the level of NAA at day 3 PI may be due to tissue damage and high energy demand after injury. We observed an increase in the NAA at day 7 PI, which can be an indication of recovery after injury. Additionally, the decrease in lactate and creatine levels at day 3 PI may be due to neurogenesis and high energy demand, respectively. Also, myo-inositol levels were down-regulated in hippocampal tissues at 3 days PI. Myo-inositol is a known osmolyte, which maintains cell volume and fluid balance in the body. Barkai et al. (1978) reported decreased myo-inositol levels in affective disorders [51]. A study of major depressive disorder patients reported decreased myo-inositol levels in the hippocampus [52]. Notably, depression is a common outcome of TBI [53]. The initial decrease and then increase in myo-inositol levels indicate the fluid imbalance and their recovery, respectively. Additionally, tyrosine levels were also found to increase after 7 days of injury. Tyrosine is important for neurotransmitter production, and the level of tyrosine directly affects its level in tissues [21]. A HPLC analysis of the

hippocampus of PTSD rats showed a decrease in the level of monoamines after 1 hour of stress, while an increase in monoamine levels was observed after 7 days of stress induction [54].

Effect of TBI on the thalamus at acute and sub-acute time points

GABA, acetate, and BAA were down-regulated, while myo-inositol levels were up-regulated in the thalamus after 1 day of injury. The thalamus contains interneurons, which are inhibitory and release gaba[55]. A decrease in gaba levels may be due to damage to GABAergic neurons in the thalamus [56]. Zhang et al. (2017) reported a decrease in GABA levels in the ventrobasal thalamus of chronic inflammatory pain rat models using HPLC[57]. A significant decrease in GABA level was observed on day 1 of PI. Down-regulation in GABA levels suggest gabaergic neuronal damage as an effect of injury, while almost partially normal levels of GABA at day 7 PI indicate improvement in neuronal damage. Acetate plays an important role in energy production and acetylcholine synthesis[58]. As an effect of TBI, the brain's energy demands increase, and to fill this energy demand, acetate is transported into astrocytes for energy production [47]. Another fate of acetate after TBI is the formation of acetylcholine; as reported previously, the level of acetylcholine increases after brain injury [59]. We observed a significant decrease in acetate levels in the injury groups after 1 day of PI compared to control rats. BAA plays an important role in neurotransmitter synthesis and energy metabolism. The level of BAA is found to decrease after 1 day of injury compared to control [60]. Similarly, we also found a decreasing level of BAA in the thalamus at acute time (1 day PI). Decreasing levels of BAA and acetate after injury indicate the high energy demand of the thalamus at acute time point. In contrast, myo-inositol levels were found to increase in thalamic tissues on day 1 of PI. Choline levels were significantly down regulated in the thalamus after injury at all-time points compared to controls. Thalamus contains cholinergic axons and dense cholinergic innervation [61]. Reports suggest TBI can generate acute and chronic effects on the cholinergic system [62]. Previous studies have reported altered levels of acetylcholine in the brain following TBI. Studies have reported a decreasing trend in acetylcholine moving from acute to chronic time points [63]. Our results of a temporally decreased level of choline in thalamus tissue are in line with the previous observations. A significant decrease in myo-inositol levels at the acute time point and choline levels at all the time points in PI may be due to neuronal tissue damage and irregular cell volume.

Effect of TBI on the striatum at acute and sub-acute time points

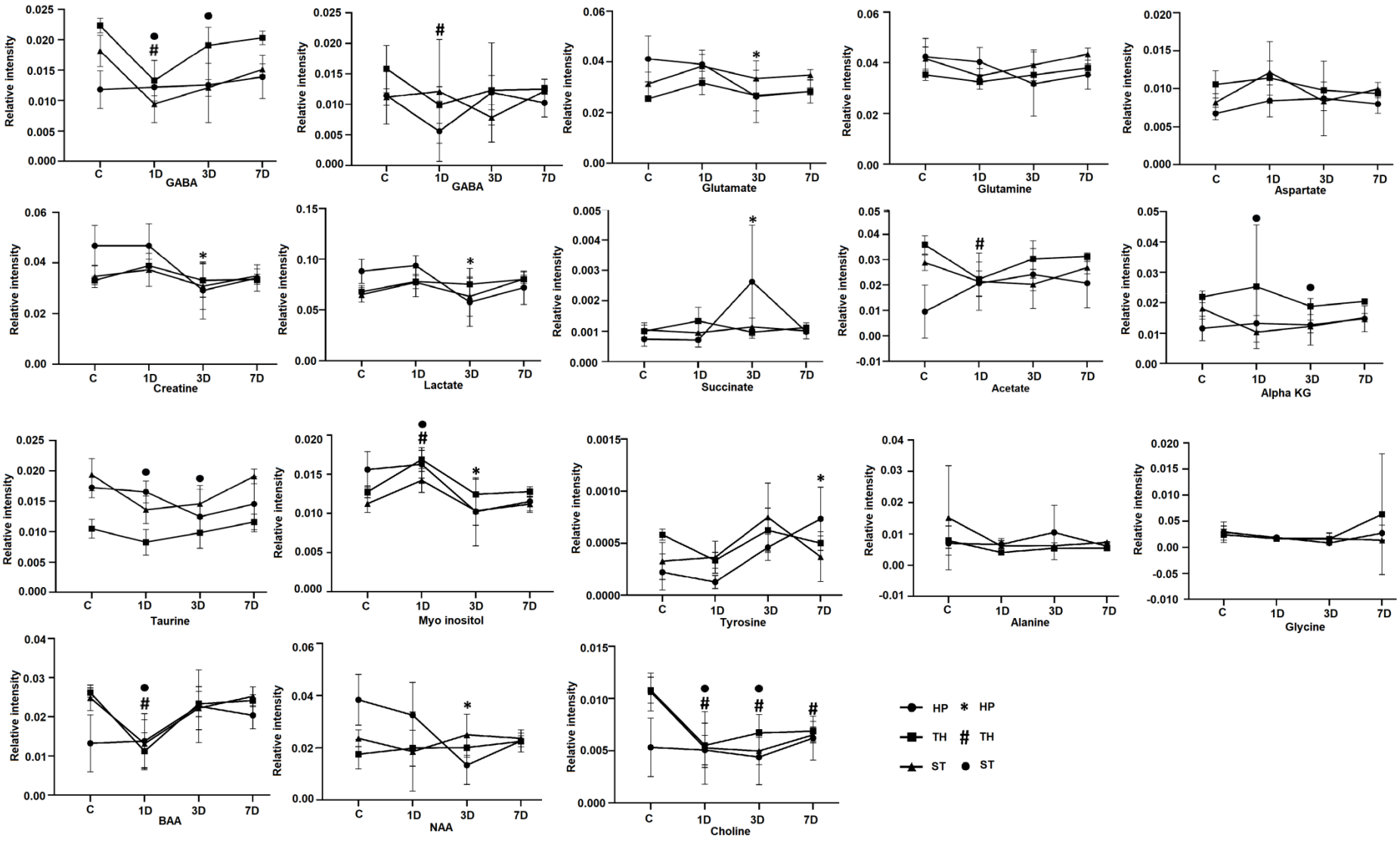
In the striatum, GABA and alpha-ketoglutarate levels were found to be downregulated at days 1 and day 3 PI. The down regulation of GABA levels in the striatum may be affected by gabaergic neuronal damage, as the striatum contains 95% of gabaergic neurons in rodents [64]. Alpha-KG is an intermediate in the TCA cycle and provides energy for cell processes [65]. A decrease in alpha-KG levels indicates altered energy metabolism in the striatum. A decreased level of alpha-KG may influence the downregulation of GABA. Alpha-KG serves as the metabolic precursor of GABA and glutamate[66]. Decreased levels of GABA and alpha-KG at day 1 and day 3 PI show striatal neuronal damage and energy demand to recover the damage. BAA plays an important role in neurotransmitter synthesis and energy metabolism; it acts as a nitrogen source for glutamine production inside the brain [60]. Aquilani et al. (2005) reported that short-term supplementation of BAA can enhance the cognitive ability of severe TBI patients[67]. A significant decrease in striatal BAAs was observed at day 1 PI. A decrease in BAA levels at day 1 PI and an almost normal level of it implies its role in energy metabolism and neurotransmitter synthesis. Taurine is an antioxidant and has neuroprotective properties [68]. A study on neuronal damage rats reported that taurine treatment increases the survival of rats following damage. Taurine was also found to increase the survival of the rats and reduce striatal lesions after damage induced by 3-nitropropionic acid (3-NP) in the striatum [69]. Therefore, this suggests that decreased taurine levels at day 1 and day 3 PI in the striatum could progress into oxidative damage. The initial decrease (at day 1 and day 3 PI) in the level of taurine indicates striatal damage. While a partial recovery in taurine

levels at day 7 PI may be due to the initiation of striatal damage repair, a decrease in choline levels at day 1 and day 3 PI was also observed in the striatum. Choline is an acetylcholine precursor, and acetylcholine synthesis takes place in cholinergic neurons. The concentration of choline regulates the concentration of acetylcholine in the striatum [70]. A study on the FPI model reported increased acetylcholine concentrations following the jury at early time points (4 hr and 12 hr) [71]. The decrease in choline levels at day 1 and day 3 PI could be due to cholinergic neuron damage.

3.6 Conclusion:

Our study reports changes induced after diffuse moderate blunt injury at acute and sub-acute time points using NMR metabolomics in different brain regions. Moderate TBI induced metabolic alteration is evident in altered level of neurotransmitter, energy metabolites and neuromodulator in all brain regions at different timepoints. In addition to this, evidence of metabolic dysregulation was found in all brain regions studied, the patterns of change observed in each brain region were regionally different. These results suggest that TBI can cause widespread and persistent metabolic changes. This study provides useful pathological insights into closed head injury in humans and expands our knowledge of pathophysiology of TBI.

Figure 1: Line graph of all selected metabolites in hippocampus, thalamus and striatum at all three time-points. P- Value < 0.05, considered significant.



Chapter 4

Acute metabolic alterations in the hippocampus are associated with decreased acetylation after blast induced TBI

4.1 Introduction

Blast explosion and consequent blast related concussion is linked to destructive alteration in the brain leading to behavioral and cognitive impairments in the affected individuals (Clark et al. 2018) Importantly, soldiers deployed in Iraq war diagnosed with blast concussion showed symptoms of post-traumatic stress disorder (PTSD) and depression (Hoge et al. 2015).

Explosive detonation causes concussive blast waves, which are related with cerebrovascular injury as well as disturbance of th e blood-brain barrier. Low level blast exposure induces structural and functional changes in the brain vasculature including alterations in the arterial smooth muscle layer (Gama Sosa et al. 2019). Changes in the cerebral blood flow and damage associated with blast wave results in oxidative stress and dysfunctional cerebral metabolism in the exposed rats (Rana et al. 2020). Mild blast concussion may have serious outcomes if exposed repetitively, as a single insult increases cerebral vulnerability such that rats re-exposed to blast waves show increased oxidative stress, vascular pathologies and inflammation (Kamnaksh et al. 2014). On the other hand, military soldiers who experience minor and temporary symptoms of blast TBI are redeployed to their services, whe re they may sustain repeated mild injuries from re-exposure to blast waves.

A repeated mild injury further possess the individual at a risk of severe ongoing secondary injury mechanisms in the central nervous system (CNS) along with long term outcomes like neurodegenerative changes and chronic traumatic encephalopathy (Stern et al. 2011). At the molecular level it has been found that blast induced repetitive mild TBI is accompanied by a complex pathology involving changes in the cerebral metabolism with increased vascular and oxidative damage (Kamnaksh et al. 2014). Cerebro-cerebellar hypometabolism is found as a major contributing factor leading to chronic post concussive symptom in Iraq combat veterans with repetitive blast-trauma (Peskind et al. 2011). Similarly, Prins et al. (2013) reported a decrease in cerebral glucose metabolism in both parietal cortex and hippocampus in a subpopulation of children and young adults who experienced repetitive mild TBI (rm TBI).

Crucially, changes in brain metabolism can affect cellular gene expression via the mechanism of epigenetic modifications. Histone modifying enzymes employ cellular metabolites to change histone modifications, which rewires gene expression (Katada et al. 2012). By controlling the amounts of acetyl CoA through the tricarboxylic cycle (TCA cycle), the content of glucose in the cell has been shown to affect the activity of histone acetyltransferase (HATs). Similarly, the methylation pattern of the cell is affected by the concentration of the co-factor FAD/FADH2 in the cell as well as the levels of SAM (S-adenosylmethionine) and SAH (S-adenosylhomocysteine) (Weinhold 2006). Furthermore, following moderate controlled cortical impact (CCI), a reduction in histone methylation and H3 acetylation is seen, particularly in the hippocampus region (Gao et al. 2006).

A large number of preclinical investigations on traumatic brain injury (TBI) have used male test subjects. Increasing the amount of sex-balanced studies may result in more reliable human clinical trials and a better knowledge of disease causes. This study was specifically designed to evaluate metabolic changes in women's health. It seeks to investigate how repeated blast concussions alter the metabolism of exposed female rats. Furthermore, the study investigates the relationship between metabolic and epigenetic changes caused by repeated blast injury.

4.2 Materials and method

4.2.1. Chemicals

Acetonitrile (Sigma Aldrich, Germany), deuterium oxide (D_2O) (Sigma Aldrich, Germany), formalin, ketamine (Psychotropics India Limited, India), mylar membrane, potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO4), sodium chloride (NaCl), sodium hydrogen phosphate (Na_2HPO4) (Merck, Germany), trimethylsilylpropanoic acid (TSP) (Eurisotop, France) and xylazine (Indian immunologicals Limited, India).

4.2.2 Study Design

All experiments adhered to the ethical guidelines outlined by the Institutional Animal Ethical Committee (INM/IAEC/2019/09/Ext1). The study aimed to investigate differences in neurometabolite concentrations between sham and blast-exposed groups. The null hypothesis (H_0) posited no significant differences between these groups, while the alternative hypothesis (H_a) indicated otherwise. With an α level of 0.05 and 80% test power, a two-tailed test determined a minimum sample size of 5 animals per group. Twenty female Sprague Dawley rats (10–12 weeks old, weighing 250–280 g) were sourced from the Experimental Animal Facility of the institute. Animals were randomly assigned into groups of five and housed in a controlled environment (temperature 22 ± 2ºC, humidity 50 ± 10%) with a 12-hour light/dark cycle, providing ad libitum access to food and water.

4.3 Blast Exposure

A compression-driven gas shock tube was employed to generate shock waves, based on the protocol described by Mishra et al. (2016). Rats were divided into four groups: sham, mild TBI, moderate TBI, and repetitive mild TBI (rmTBI), with each group comprising five animals. Animals were anesthetized with 3.5% isoflurane in zero air (19.9–21.9% O_2) and positioned horizontally on a platform within the shockwave tube. The mild and moderate TBI groups were subjected to single shock waves of 100 kPa and 190 kPa, respectively. The rmTBI group received three 100 kPa shock waves at intervals of 0 minutes, 30 minutes, and 24 hours. Sham animals underwent identical procedures without exposure to shock waves. At 24 hours post-injury, rats were perfused sequentially with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) to minimize blood contamination during tissue extraction. The hippocampus was isolated from one hemisphere, snap-frozen in liquid nitrogen, and stored at –80ºC for subsequent analyses. The other hemisphere was fixed in 4% formalin for histological examination.

4.3.1 Immunohistochemistry (IHC) Analysis

Fixed brain tissues were processed and embedded in paraffin wax. Sections of 5 µm thickness were prepared using a microtome and mounted on silane-coated slides. Sections were deparaffinized in xylene and rehydrated through a graded ethanol-to-water series. After rinsing with PBS, tissues underwent antigen retrieval by incubation in heated sodium citrate (85ºC) for one hour. To suppress endogenous peroxidase activity, sections were treated with hydrogen peroxide (H_2O_2) for 15 minutes, following the protocol of the Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC Kit (ab64264). After rinsing with PBS, sections were blocked with a protein-blocking buffer for 10 minutes and incubated overnight at 4ºC with primary antibodies in a humidified chamber. Specific antibodies used included GFAP (MA5-12023, Thermo) for astrocytic activation and AH3 (MBS9126034, My Biosource) for histone

acetylation quantification. Tissues were washed with PBS and incubated for 10 minutes with the secondary antibody, followed by a 10-minute treatment with streptavidin-peroxidase. Sections were then exposed to DAB (3,3´-diaminobenzidine) for visualization, counterstained with hematoxylin for 30 seconds, air-dried, and cover-slipped. Slides were examined under a microscope (LMI BM-Prime, London, England) at 20× magnification, and images were captured. GFAP-positive and AH3-positive cells were manually counted in the dentate gyrus (DG) region of the hippocampus for mild, moderate, and repetitive TBI groups. Cell counts were performed using ImageJ software (Bethesda, USA), with regions of interest (ROIs) selected in each DG area based on the methodology described by Gao et al. (2006). Positive cell counts were normalized per mm^2.

4.4 Sample Preparation for nmr and SpectraAcquisition

Metabolites were extracted from tissue samples using the acetonitrile extraction method. Frozen tissue samples were homogenized in a 1:1 acetonitrile-water mixture (5 mL per 1 g of tissue) using a handheld homogenizer (IKA T-10, Germany) under cold conditions to prevent metabolic degradation. The homogenized solution was centrifuged at 12,000 rpm at 4°C for 20 minutes. The resulting supernatant was collected and stored at –80°C until lyophilized. Lyophilized samples were then stored at –20°C for subsequent analysis.

Proton nuclear magnetic resonance (^1H NMR) spectra were acquired using a 600.33 MHz NMR spectrometer (Bruker, Biospin, Switzerland). For spectral acquisition, the lyophilized samples were reconstituted in 600 µL of a solution containing deuterium oxide (D_2O) and 0.5 mM trimethylsilylpropanoic acid (TSP). The mixture was centrifuged at 12,000 rpm at 4°C for 10–15 minutes, and 580 µL of the supernatant was transferred into 5-mm NMR tubes.

The NMR spectra were obtained using a water-saturation NOESYPR1d pulse program with the following parameters: pulse sequence (RD-90°-t-90°-tm-90°-acq), 64 scans (including 4 dummy scans), spectral width of 9009 Hz, acquisition time of 3.63 seconds, and relaxation delay of 4 seconds. All spectra were recorded at a temperature of 298 K.

The raw NMR spectra were processed using TOPSPIN 3.5 software (Bruker). Baseline and phase corrections were manually adjusted for each spectrum. Peak integration was performed manually, and peaks were identified by referencing the Human Metabolome Database (HMDB) and prior literature. TSP was used as the internal reference for peak integration. The integrated peak data were normalized to the total sum of integrals for each spectrum.

5.5 Concentration of metabolites

Concentration of metabolites in tissues were calculated using the following formula [Equation 1 (Tyagi et al, 2011)]:

$$C(x) = C(TSP) \times \frac{N(TSP) \times I(X) \times V(s)}{N(x) \times I(TSP) \times W(s)} \qquad \textbf{Equation 1}$$

Where,

C (x)- concentration of metabolite,

C(TSP)- Concentration of TSP,

I_X – Intensity of metabolite

N_X - Number of protons in integrated signal,

N_{TSP}-Number of protons in TSP,

W(S) - Weight of wet tissues in grams,

V(S) - Volume of sample in NMR tube.

The concentrations of all metabolites, excluding branched-chain amino acids (BAAs), are detailed in Supplementary Table 2. Quantification of BAA concentrations was not performed due to overlapping peaks of valine, leucine, and isoleucine. Instead, their relative intensities are provided in Supplementary Table 2.

4.6 Statistical Analysis

Metabolic and immunohistochemical differences between groups were evaluated using one-way analysis of variance (ANOVA) for comparison. Tukey's HSD test was conducted for post-hoc analysis, with statistical significance set at $p < 0.05$. All statistical analyses were performed using SPSS software (version 20).

To explore the relationships among metabolites in each injury group, Pearson's correlation coefficients were calculated for the 19 identified metabolites across all samples using MetaboAnalyst software.

4.7 Results

Metabolic profiles of hippocampal tissue were analyzed to identify blast-induced alterations 24 hours post-injury. Nineteen metabolites involved in various metabolic pathways were identified. These included metabolites associated with energy metabolism (lactate, succinate, creatine, alpha-ketoglutarate), ketone body metabolism (acetate), osmolytes (myo-inositol, taurine), neurotransmitter amino acids (glutamine, glutamate, aspartate, GABA), branched-chain amino acids (valine, leucine, isoleucine), and other amino acids (alanine, glycine, tyrosine). Additionally, N-acetyl aspartate (NAA) and membrane-associated metabolites (phosphocholine/choline, phosphoethanolamine (PE), ethanolamine) were identified.

4.7.1 Neurometabolic alteration among the groups

Significant changes in the concentrations of N-acetyl aspartic acid (NAA), glutamate, acetate, phosphocholine/choline (PC/choline), phosphoethanolamine (PE), ethanolamine, and creatine were observed only in the repetitive TBI (rmTBI) group compared to the sham group 24 hours post-injury. Acetate levels were significantly increased, while NAA, glutamate, PC/choline, PE, ethanolamine, and creatine were notably decreased in the rmTBI group. Additionally, lactate, alanine, and branched-chain amino acids (BAAs) displayed significant changes across all TBI groups when compared to the sham. Lactate levels were elevated, whereas alanine and BAA levels were reduced in all injury groups. (Figure 2)

4.7.2 Correlation among the neurometabolites

To explore the relationships among altered metabolites post-injury, a correlation heatmap was generated using MetaboAnalyst software. NAA was positively correlated with glutamate, creatine, ethanolamine, PE, and PC/choline, while showing a negative correlation with acetate and lactate concentrations (Figure 3).

4.7.3 Histological changes among the groups

Astrocytic activation following TBI was assessed using GFAP immunostaining. At 24 hours post-injury, the number of astrocytes in the hippocampus was significantly higher in the mild and moderate injury groups compared to the sham group (Figure 4). Although the rmTBI group showed increased astrocyte proliferation in the hippocampus, the change was not statistically significant.

To evaluate TBI-induced epigenetic changes, AH3-positive cells were examined. One-way ANOVA revealed a significant reduction in AH3-positive cells in the rmTBI group compared to the sham and mild TBI groups (Figure 5). No significant changes were observed in other groups.

4.8 Discussion

Mild traumatic brain injury (mTBI) has garnered increasing attention in both clinical and preclinical research, yielding extensive data on post-injury metabolic changes in the brain (Banoei et al., 2018). mTBI is particularly prevalent among military personnel exposed to repeated blasts during training, combat, or field trials (Hoge et al., 2015). Mild blasts are known to impair cognitive, metabolic, and behavioral functions (Arciniegas et al., 2002; Subramaniam et al., 2015; Rana et al., 2020). However, the timeline for recovery and the benchmarks for evaluating these recoveries remain under discussion.

This study assessed the metabolic changes in hippocampal tissue following different severities of injury (mild, moderate, and repeated mild blast exposures) at 24 hours post-injury. Significant alterations in the levels of NAA, acetate, PC/choline, PE, ethanolamine, glutamate, and creatine were observed exclusively in rmTBI rats compared to sham. These changes suggest that blast-induced metabolic disruptions may impact energy metabolism and epigenetic processes.

Histologically, the study found reduced acetylated cells in the hippocampus of rmTBI rats compared to sham animals. Histone acetylation is a crucial epigenetic mechanism, and the enzyme AceCS1 plays a vital role in this process. Previous studies indicate that acetate serves as an energy source under hypoxic conditions (Vazquez et al., 2016) and may trigger epigenetic changes (Gao et al., 2006). Consistent with earlier findings, this study observed increased acetate levels and reduced histone acetylation post-injury, suggesting acetate may be rerouted to energy production rather than epigenetic modification.

NAA's role in generating acetate through deacetylation by ASPA (aminoacylase II) aligns with the observed reduction in NAA levels post-rmTBI (D'Adamo Jr et al., 1972). Furthermore, decreased glutamate levels alongside reduced NAA concentration indicate impaired neurotransmission, consistent with findings from studies on Alzheimer's disease (Lin et al., 2003; Dedeoglu et al., 2004).

In astrocytes, the glutamate-glutamine cycle supports neurotransmission. This study reported decreased alanine and glutamate concentrations after rmTBI, indicating potential disruptions in this cycle. Increased GFAP-positive cells after mild and moderate injuries further underscore metabolic stress following rmTBI. Additionally, reduced PC/choline levels in rmTBI rats point to compromised membrane integrity, potentially affecting cholinergic neurotransmission (Zeisel et al., 2003).

Lactate, a critical energy substrate, was elevated across all TBI groups, reflecting increased energy demand and glycolytic activity post-injury (Dienel, 2014). Creatine, essential for ATP production, was decreased in rmTBI rats, indicating disrupted energy metabolism (Andres et al., 2008). Similarly, reduced PE and ethanolamine levels suggest their role in energy production may be impaired following rmTBI (Karagezyan et al., 1975).

The study also observed reductions in BAA concentrations across all injury groups. BAAs are vital for energy metabolism and neurotransmitter synthesis, and their depletion post-TBI underscores potential metabolic stress (Caplan et al., 2018).

The metabolic alterations specific to the rmTBI group highlight the vulnerability of the brain to secondary injury mechanisms after repeated trauma. These changes reflect impaired mitochondrial function, oxidative stress, and metabolic shifts in energy production, which could lead to long-term neurological deficits (Roberto et al., 2008).

4.9 Conclusion

This study demonstrates that blast-induced TBI leads to significant metabolic changes at acute time points, particularly in the rmTBI group. Altered levels of NAA, glutamate, acetate, creatine, PC/choline, PE, and ethanolamine suggest a shift towards energy production at the expense of other cellular functions. Reduced acetylation in hippocampal cells post-rmTBI indicates an interplay

between metabolic and epigenetic mechanisms. The findings also reveal increased astrocyte proliferation in mild and moderate injuries, with a modest increase in rmTBI. These results emphasize the need for further studies to explore the relationship between metabolism and epigenetic regulation in TBI recovery.

Chapter 5

Closed head injury induced change in rat metabolome and gut microbiota

5.1 Introduction

Repetitive mild traumatic brain injury (rm TBI) is a condition in which a person sustains multiple mild traumatic brain injuries over time. Athletes participating in contact sports such as football, hockey, or soccer, and persons involved in activities involving a high risk of falls or head impacts are all at risk of repetitive mild TBI. Repeated mild TBIs can have a cumulative effect, which means that each consecutive damage may have a bigger influence on the brain's functioning and healing. rmTBI studies reported chronic metabolic, microstructural and pathological alterations along with cognitive decline in rodents (Boska et al; 2013). Vike et al, 2022 reported metabolic alterations in collegiate football athletes before and after sports season and they found alteration in xanthine metabolism, fatty acid metabolism, medium chain fatty acid, primary bile acid metabolism and glycolysis, gluconeogenesis, and pyruvate metabolism pathways. Another study on cci mice models reported disrupted axonal transport and accumulation of app on corpus callosum and prefrontal cortex at 48h, 1w, 4w and 10w post rmtbi. Same study highlights an increase in app/beta- actine levels using western blotting at 8h, 48h and 1w after injury. Ravdin et al, 2003 examine the cognitive change for a month in professional boxers and found significant cognitive decline in them. Along with metabolic changes traumatic brain injury has been found to have a notable impact on the composition and functioning of the gut microbiome. More than 10^{13}-10^{14} gut microorganisms from over 1000 different known bacterial species exist in the human gut, which outnumber the somatic cells in the human body, and the bacterial genome is 100-fold greater than those in the human genome The gut microbiota has a close relationship with the central nervous system (CNS). Studies have demonstrated that traumatic brain injury can alter the diversity and abundance of gut microbial species, leading to dysbiosis and the development of virulent phylogenetic changes within the intestinal commensal microbiota. This disruption of the gut microbiome following traumatic brain injury is thought to be a result of the bidirectional communication between the brain and the gut. This communication is mediated by various factors, including immune modulatory metabolites, gut peptides, neurotransmitters, and cytokines. Furthermore, it has been shown that the extent of the brain injury, as indicated by the volume of the lesion seen on MRI, is significantly correlated with the severity of the changes in the gut microbiome. Additionally, research suggests that damage to distant organs, such as burn injury or ischemia reperfusion, can also lead to alterations in the gut microbiome (Kosakamoto et al., 2020). Treangen et al. (2018) reported that traumatic brain injury (TBI), an acute brain injury, can lead to significant changes in the gut microbiome. Specifically, they observed a decrease in the abundance of *Lactobacillus gasseri*, *Ruminococcus flavefaciens*, and *Eubacterium ventriosum* and an increase in *Eubacterium sulci* and *Marvinbryantia formatexigens* 24 hours post-TBI. Additionally, the study demonstrated that gut microbiome dysbiosis induced by TBI persisted for up to 7 days and was correlated with lesion volume. Similarly, cerebral ischemic stroke, another form of acute brain injury, has been shown to induce gut microbiota dysbiosis, which is associated with injury severity and outcomes. These alterations in the gut microbiome not only worsen the pathological outcomes of TBI but also contribute to sterile inflammation. The gut microbiome—a complex community of microorganisms residing in the gastrointestinal tract—plays a critical role in regulating human health and disease. It has been increasingly recognized that the gut microbiome can impact brain function and normal brain growth (Hassani et al., 2022). Emerging evidence suggests that the gut microbiome may have an impact on neurological disorders such as Alzheimer's disease, Parkinson's disease, and traumatic brain injury. Specifically, studies have shown that the gut microbiome is involved in the regulation of pain perception and can contribute to the occurrence of neurologic disorders such as anxiety and autism. Moreover, alterations in the gut microbiota have been linked to inflammatory cascades and increased risk of developing post-traumatic stress disorder following traumatic brain injury (Li et al., 2022). Research has demonstrated that there is a direct connection between gastrointestinal function and the brain, as seen in cases of traumatic brain injury (Ye et al., 2021).

The current study aimed to investigate TBI-induced alterations over time, from acute to chronic, following mild and rmTBI. The study used 1H-NMR metabolomics to examine metabolic changes in serum. Fecal 16S rRNA sequencing were used to investigate the potential processes that alter following injury the gut microbiome.

5.6 Results:

Fecal Microbiome changes using 16S rRNA sequencing post blunt impact TBI

The gut microbiome composition was observed using 16S rRNA sequencing which showed changes in both mild and repetitive mild injured rats. The α diversity was expressed as Chao, Shannon, Simpson and Fisher indexes. The β diversity was evaluated using ordination analysis. The α- diversity showed an increase in richness within the mild group while rmTBI group showed decrease level of richness. At the phylum level, the Firmicutes and Bacteroidetes were decreased in rmTBI group. While phylum firmicutes was downregulated in mild TBI rats compared to sham. Mild injured rats showed increased Proteobacteria abundance.

The genus abundance showed decrease in *Agathobacter, Faecalibacterium, Holdemanella, Lactobacillus, Oribacterium, Rikenellaceae-RC9, Ruminococcaceae_UCG-008, Ruminococcaceae_UCG-014* and *Subdoligranulum* in mild and rmTBI groups. While *Anaerostpes, Fusicatenibacter, Marvinbryantia, Parabacteroides, Ramboustia* and *Treponema* showed increase in mild and rmTBI rats compared to controls. *Alloprevotella, Bacteroides, Blacutia, Candidatus_Saccharimonas, Dorea, Prevotella_9, Prevotellaceae_NK3B31 group, Pygmaiobacter, Ruminococcaceae_UCG-002, Ruminococcus_2, Solobacterium and Succini vibrio* showed increased abundance after mild injury only. On the other hand, Lachnospiraceae*_ND3007_group, Lachinospiraceae_NK3A20_group, Lachnospiraceae_ UCG_002, Oscillibacter, Sellimonas* and *Solobacterium* showed increased abundance only in rmTBI rats compared to control.

The EdgeR analysis showed significant alterations in the genus Blautia, *Lachnospiraceae_ND3007, Lachnospiraceae_ UCG_002, Lactobacillus, Ruminococcaceae_UCG-014* and *Sellimonas* from the phylum Firmicutes. From the Bacteroidetes phylum, genus *Alloprevotella, Parabacteroides, Prevotella_9, Pygmaiobacter* and *Rikenellaceae-RC9* showed significant change.

Alloprevotella, Parabacteroides, Prevotella_9, Pygmaiobacter and *Candidatus_Saccharimonas* abundance was high in mild TBI rats, whereas *Lachinospiraceae_NK3A20_group, Lachnospiraceae_ UCG_002* and *Sellimonas* abundance was downregulated in rmTBI rats. While, *Rikenellaceae-RC9* and *Ruminococcaceae_UCG-014* abundance showed downregulation in both mild and rmTBI rats. (Figure:)

Metabolic alterations in serum after blunt TBI

We observed significant decrease in formate and lactate at all the time-points after injury. while leucine, isoleucine, valine, alpha-glucose and beta-glucose ratio showed significant decrease at sub- acute and chronic time-point in mild and rmTBI groups. Additionally, the level of NAA was significantly low in both mild and rmTBI groups at all the timepoints except acute mild injury group in compare to control rats. (Figure:)

5.7 Discussion:

The findings of this chapter reveal the metabolic and gut microbiota changes caused by mild and repeated mild diffused closed head injury over time. Diffuse traumatic brain injury (TBI) has a major impact on systemic metabolism, namely cerebral glucose metabolism, and can cause a variety of metabolic and neurological abnormalities. Experimental TBI models in rats have demonstrated unique temporal patterns of brain metabolite alterations at acute, sub acute and chronic timepoints after injury.

Along with the previous studies we also observed Significant changes in the serum metabolites as early as 4 hr after injury and persisted as long as 30day after injury. We also found notable change in urine metabolites at 7day and 30day PI. Changes in gut microbiome were also observed at 30 day following injury as compared with sham. Significant changes in the GI microbiome were evident after TBI as compared with sham rats, with varying trends among the phylogenetic families.

5.7.1 Systemic Change in serum metabolome following diffused closed head injury:

Serum metabolomics revealed a varied shift in metabolites following mild and repeated mild injuries. Both mild and repetitive mild injuries in rats resulted in decreased energy metabolites, including lactate:pyruvate and α:β glucose ratios. Following injury, the branched chain amino acids (BCAAs) valine, iso leucine, and leucine were reduced, along with other amino acids such as glutamine, naa, alanine, and taurine, at both the sub-acute and chronic time points.

Both injury groups showed a decrease in their α:β glucose ratio at sub-acute and chronic time points. Decreased alpha and beta glucose ratio values in serum indicate impaired glucose transport and metabolism, poor glucose transport across the blood-brain barrier, resulting in cerebral metabolic distress and increased mortality following severe brain damage.

Kurtz et al. (2013) conducted a 300-day observational study on 46 consecutive comatose patients with subarachnoid or intracerebral hemorrhage, traumatic brain injury, or cardiac arrest who underwent cerebral microdialysis and intracranial pressure monitoring, and found reduced serum and brain glucose ratios as well as impaired glucose transport. The study also suggests that decreased glucose transport and the blood-brain barrier are related with cerebral metabolic distress and higher mortality following severe brain injury.

Another study observed tbi patients for 6 months after injury using intracerebral microdialysis, electroencephalography, and measurements of brain tissue oxygen levels and jugular venous oxygen saturation in 30 patients with traumatic brain injury and reported persistent decrease in extracellular blood glucose. (In our study we also observed significant decrease in blood glucose levels following injury at sub-acute and chronic timepints. A spike in the lactate-to-pyruvate ratio in serum is a typical metabolic disruption seen in animal investigations. This ratio is a sign of metabolic failure, which is frequently associated with mitochondrial malfunction and hypoxia. A microdialysis investigation on the ballistic-like penetration model of tbi indicated a rise in lactate to pyruvate ratio in rats after 2 hours after injury, and the increase maintained until the 14th day PI.

Another microdialysis study on tbi rats reported significantly higher level of lactate to pyruvate ratio in injured rats after 24hrs of injury Similarly, we also observed an increase in lactate to pyruvate ratio after injury but significant increase was observed at chronic timepoint only.

N-Acetylaspartate (NAA) is a neuron-specific metabolite, and its decrease is a sign of neuronal death. A microdialysis and HPLC investigation on severe TBI patients found an early increase and subsequently decline in NAA levels till 70 hours PI compared to non-TBI individuals. Visser et al. (2023) did a study using magnetic resonance spectroscopy (MRS) or diffusion tensor imaging (DTI) and found a lower level of total naa in moderate TBI patients at the subacute time point compared to control persons.

Our results also compliment these studies, as we observed the similar change in injured rats at sub-acute and chronic timepoints following injury. TBI pathophysiology has implications for injury progression and outcome. Identifying metabolic cascades that are altered after acute human mild and severe TBI, due to breakdown of branched-chain amino acids (BAAs; i.e., valine, isoleucine, and leucine), leads to glucose and energy metabolism, as well as neurotransmitter synthesis and availability. Previous research has also shown that baa supplementation can help the cognitive recovery of TBI patients.

Jeter et al, 2013 reported decreased levels of all three BCAAs in patients with mild TBI relative to healthy volunteers after 24hrs of injury. another study on severe tbi patients reported decreased levels of baa in plasma after 14days of injurySimilar to these results we also found significant decrease in baa levels of injured rat serum.

5.7.2 Gut microbiome changes after mild and rm TBI

The gut microbiome was sequenced in this study, and we discovered that injured rats had lower alpha and beta diversity than sham animals. The structure of a microbial community can be summarized using alpha diversity measures based on its evenness (distribution of group abundances), richness (number of taxonomic groups), or both (Willis, 2019). Alpha metrics that are frequently employed include Shannon's indices (Lemos et al., 2011; Magurran, 2013), Chao1 (Chao, 1984), observed quantity of amplicon sequence variants (ASVs) (Callahan et al., 2017), and phylogenetic diversity (PD) (Faith, 2006). By taking into account sequence abundances or just the existence or absence of sequences, beta diversity metrics provide a summary of which samples differ from one another. Curtis and Bray, 1957. Alpha diversity was also found to have decreased in TBI rats in previous investigations (Taraskina A et al. ; 2022). The phylums of Firmicutes, Bacteriodetes, and Proteobacter showed the maximum alteration. Houlden et al. demonstrated that TBI alters the caecal gut microbiota composition and revealed a connection between the severity of TBI and modifications in the microbiome, particularly in the composition of Firmicutes, Proteobacteria, and Bacteroidetes. The study postulated that these findings might be related to changes in brain function and the stress response brought on by tissue damage. Research revealed that while the phylum Proteobacteria is not abundant in a healthy human gut, a rise in Proteobacteria indicates gut dysbiosis. In comparison to sham, we also noticed a rise in Proteobacteria following moderate TBI (Shin, Na-Ri et al; 2015). Nicholson et al. (2019) highlighted a significant drop in alpha diversity in moderate TBI rats, as well as a significant decrease in relative abundance at the phylum level in Firmicutes on day 1 after TBI. Specifically, family Lachnospiraceae levels were suppressed after 2 hours to 7 days of PI, although lactobacillace levels showed no significant impact. Our findings also revealed a decrease in Lachnospiraceae levels in tbi rats. Zhanfeng et al. (2022) showed higher Bacteroidetes and lower Firmicutes levels in TBI patients. Bacteroidetes levels were found to be higher in the mild tbi group, while Firmicutes levels were lower in the tbi groups, consistent with previous findings. Furthermore, the mild and rm tbi injured groups showed gut microbiota changes consistent with TBI pathology. A distinguishing feature between mild and rm tbi was increased Protedobacter abundance in mild, which may hinder gut function.

5.8 Conclusion

The present study observed changes in the gut microbiota following injury as well as systemic alterations in metabolism following mild and repeated TBI. Both injury groups revealed a lower alpha:beta glucose ratio and a higher lactate:pyruvate ratio. Signs of oxidative stress and reduced energy metabolites in urine were also indicated by the urine metabolomics of injured rats. Additionally, the gut microbiome showed reduced levels of Firmicutes and Bacteroidetes following injury, as well as altered β diversity and decreased α diversity in rm tbi rats following both traumas. Therefore, the study suggests that while gut dysbiosis and systemic changes are caused by both mild and repeated injury, a different metabolic phenotype with increased inflammation and oxidative stress can be observed by more severe injury.

Chapter 6

Behavioral changes induced by blunt and blast TBI

6.1 Introduction

Traumatic brain injury can induce significant behavioral changes in both humans and animals, particularly at chronic time points after the initial injury. These changes can manifest in a variety of ways, including alterations in mood, cognition, and social functioning. Previous studies have shown that individuals with traumatic brain injury often experience changes in mood and behavior. For instance, they may exhibit symptoms of depression, such as persistent feelings of sadness or loss of interest in previously enjoyable activities. In addition, individuals with traumatic brain injury may experience heightened levels of anxiety, resulting in increased worry and fear. Furthermore, impulsiveness and difficulty with concentration and memory are common behavioral changes observed in both human and animal subjects with traumatic brain injury. These changes in behavior can have a profound impact on the individual's overall quality of life.

TBI patients are not only at risk for cognitive impairments, but also for developing long-term psychiatric conditions. For example, there is an increased risk for the development of dementia, and even Alzheimer's disease in individuals who have experienced a traumatic brain injury. The exact mechanisms underlying these behavioral changes in traumatic brain injury are not yet fully understood. However, it is believed that the physical damage to the brain, as well as changes in neurotransmitter function and neural connectivity, play a role in these behavioral changes. Furthermore, the social and functional implications of these behavioral changes cannot be ignored. Individuals with traumatic brain injury often experience difficulties in their relationships and social interactions. These behavioral changes can lead to decreased participation in employment and social activities, as well as increased dependence on others for everyday functional activities. In a study blaze et al. 2020 reported anxiety like behavior in rats after blast induced TBI after 1 to 1.5 months of injury. Another TBI study conducted by Arulsamy et al, 2019 using Marmarou’s weight drop model reported significant cognitive decline in moderate to severe injury groups after 12 months of injury, although they did not observe any notable change in mild and repetitive mild injury groups at the same time after injury .Although in a lifelong study (24 months) after diffuse blunt TBI on mice reported impaired working memory, decreased spatial memory and vestibule-motor vestibulomotor deficits in rmTBI and mild TBI injury groups compared to sham animals.

To study the behavioral change in animals we developed two set of injury models based on injury types one is diffused blunt injury and the other is blast induced tbi. Each injury group was divided into three injury group’s based on injury frequency i.e control, mild and repetitive mild.

6.2Materials:

Rats

70 % ethanol

Balance beam (2 cm wide × 100 cm long; typically, plastic/polycarbonate with a smooth, no-grip surface that is easily cleaned)

Score sheet

Cotton swabs

6.3Method:

Animal models

Two types of injury groups were created based on injury type (1) Blunt trauma Using modified Marmarou's weight drop model, and (2) Blast trauma using compression driven shock wave tube.

6.3.1Blunt trauma

Three groups—Control, Mild TBI, and rmTBI—were randomly allocated to thirty female Sprague-Dawley (SD) rats weighing 200–250 g. Rats were put on a foam platform and exposed to a free-falling brass rod that was 25 cm above the midpoint of the sagittal plane of the brain for both mild and rmTBI. At 24-hour intervals, the rmTBI group experienced three impacts. Injury induction occurred following a seven-day acclimatization period, and the institutional ethical council authorized all experimental techniques. A regular pellet meal (M/S Golden Feeds, Meharuli, Delhi, India) and unlimited water were given to the rats, who were kept in a 12-hour light/dark cycle at 23±1°C.

6.3.2Blast exposure.

A shock wave was generated using a compression-driven gas shock tube, as previously described (Mishra et al., 2016). Male SD rats were randomly assigned to three groups: sham, mild TBI, and rmTBI, with eight rats in each. Rats were sedated with 3.5% isoflurane and zero air (having approximately 19.9-21.9% oxygen) and put horizontally on a platform within the shockwave tube (Mishra et al., 2016). The mild and moderate TBI groups received a single shock wave dose of 100 kPa. The rmTBI group received three 100 kPa shock wave exposures at 0, 10, and 24 hour intervals. The same treatment was performed on sham rats, but they were not exposed to any injuries.

6.4 Behavioral paradigm

All behavioral assessments were administered between 10:00 a.m. and 3:30 p.m. during the light phase of the cycle. Rats were tracked using automated video tracking software (ANYmaze™, Stoelting Co., USA), with the researcher blind to the experimental groups during testing and analysis.

6.4.1 The Neurobehavioral Severity Scale (NSS)

The NSS was used to examine balance, motor coordination (including movement and posture), as well as sensory and motor reflexes at acute, sub-acute, and chronic stages. Evaluations included balance, landing, tail raise, dragging, righting reflex, ear reflex, eye reflex, sound reflex, tail pinch, and hind paw pinch. The NSS employs a distinct three-point Likert scale, with "0" for normal responses, "1" for incomplete or compromised responses, and "2" for absent responses.

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6.4.2 Rotating pole:

The rotating pole test was performed on days 1, 5, and 15 post-injury to assess coordination and movement integration (Ohlsson and Johansson, 1995). The pole was 2.54 cm in diameter and 1.5 m in length, rotating at 4 turns per minute. The pole was divided into three sections, and the time taken for rats to move from one end of the pole to the other was recorded. Falls and reversals were also noted. Rats were trained for two days, followed by a baseline measurement. After injury, three measurements were taken per rat, and the average was calculated.

6.4.3 Open Field Test (OFT)

As previously mentioned, the OFT was used to evaluate emotional, exploratory, and locomotor activity in a new setting (Kumar et al., 2013). The device was 80 cm x 80 cm x 40 cm and was constructed of plexiglass. A 15-W CFL lamp was positioned 60 cm above the center of the arena floor, which was divided into 16 identical squares. The rats were given five minutes to investigate after being put in a corner that faced the wall. Time spent in the center, average speed, distance traveled, and latency to reach the center were among the behavioral metrics that were noted.

6.4.4 Novel Object Recognition Test (NORT)

In line with the protocol described in an earlier study, the NORT was carried out in the OFT arena (Kumar et al., 2013). There were two stages of the test. In the first, known as the acquisition or learning trial, the rats were given five minutes to investigate two similar objects before going back to their own cage. Following a 5-minute rest, a test trial was conducted where a new object was used in place of a well-known one. The discriminatory ratio was determined by recording the amount of time spent with the known and novel objects during the three-minute test trial.

The Discriminatory Ratio (DR) was calculated as follows:

DR = (Total time spent with the novel object) / (Total time spent with both objects).

6.5 Statistical analysis:

Behavioral changes were compared among the control group, the mild TBI group, and the rmTBI group using a one-way analysis of variance (ANOVA). Graphs and statistical analysis were done using graph pad prism version 5.0 software.

6.6 Results:

6.6.1 Behavioral change after repetitive TBI

NSS

Blunt TBI

Balance, motor coordination, as well as motor and sensory reflexes in rats was observed at acute, sub-acute and chronic time-points. We found decreased motor coordination and reflexes in both injury groups in compare to sham. RmTBI rats showed significant change in all the time-points in compare to sham rats. While, miTBI rats showed significant change in acute and sub-acute time-points in compare to sham rats.

Blast TBI

Balance, motor coordination, as well as motor and sensory reflexes in rats was observed at acute, sub-acute and chronic time-points after blast injury. We found decreased motor coordination and reflexes in both injury groups in compare to sham. miTBI rats showed significant change at acute time-point in compare to sham rats. While, rmiTBI rats showed significant change in chronic time-point in compare to sham rats.

Rotating pole:

Blunt TBI

We observed significant change after rmTBI at acute time point. The coordination and movement were found to decrease in rmTBI rats compare to sham and mildTBI rats.

Blast TBI

We observed significant change after rmTBI at all three time-points. The coordination and movement were found to decrease in rmTBI rats compare to sham and mildTBI rats at acute and sub-acute time-point. While, the significant change were observed in rmTBI rats compare to sham rats only.

NORT and OFT

Blunt TBI

We observed behavioral change following mild and rmtbi at day30 PI. We found decrease in locomotor activity (total distance travelled and average speed) in mild and rmTBI injury groups respectively compare to sham. While anxiety level was found to increase in mildTBI animals compare to rmTBI injury animals.

NORT results reveled that after injury animals were found to spend more time with novel objects compare to sham, rmTBI groups spent the maximum time with novel object compare to mildTBI and sham group animals. Discriminatory ratio did not show much visible change between injury groups after injury.

We did not observe any significant change in NORT and OFT test of blunt injury groups at day 30 PI.

Blast TBI

We observed behavioral change following mild and rmtbi at day30 PI. We found significant decrease in locomotor activity (total distance travelled and average speed) in mild and rmTBI injury groups respectively compare to sham. While, anxiety level was found to be in increasing order in mildTBI animals and rmTBI injury animals respectively.

NORT study (time spent with novel object and discriminatory ratio) results showed a decrease in the time spent with novel object and discriminatory ratio in **injury animals compare to sham at day30 PI. Although, this decrease was not significant.**

6.7 Discussion:

Behavioral assessment is important for the tbi studies as it help to understand the neuronal and functional change due to the injury. We conducted neurobehavioral assessments in both blunt and blast traumatic injury groups. Following injury NSS and rotating pole were performed at acute, sub- acute and chronic timepoint, while NORT and OFT was performed at chronic timepoint after injury. The NSS of the blunt rmTBI group showed a significant change at all time points following injury, whereas blunt miTBI rats

showed significant changes at acute and sub-acute timepoints only. In blast NSS we observed significant change in rmTBI rats at chronic timepoint while miTBI rats were significantly changed at acute timepoint only. Rotating pole studies were performed to assess the change in coordination and movement. We observed significant alteration in blunt rmTBI at acute timepoint only. While, in blast TBI groups we significant change were recorded at all three timepoints in rmTBI rats compared to sham. We did not find any significant change in blunt TBI rats at chronic timepoint after injury. While, blast tbi rats showed significant change in OFT test in miTBI and rmTBI rats at chronic timpoint. Blast tbi rats did not showed any significant change in NORT test.

6.7.1 Alteration in NSS after blast and blunt TBI

21 **NSS** is used to evaluate behavioral and sensorimotor effects of tbi on rats that are thought to be the result of changes in neurological functioning (Shohami et al., 1995; Hamm, 2001; Mahmood et al., 2001). We observed significant change in blunt rmTBI rats at acute, sub-acute and chronic timepoints compared to control rats while miTBI rats showed significant changes at acute and sub-acute timepoints compared to control rats. Additionally, the blast tbi rats also showed increase in NSS score at all the timepoints after injury compared to sham but it was significantly upregulated in miTBI rats at acute timepoint and in rmTBI rats at chronic timepoint only. A recent study on moderate to severe closed head injury and decompressive craniectomy model reported increased nss score after injury at day1, day3, day7, day14 and day28 PI. They also mentioned the significant change in day1, day3, day7 and day28 rats after closed head injury only, compared to sham (Szczygielski et al, 2023). Szczygielski et al, (2016) in a different study reported significant increase in the NSS score in closed head injury rats after 24hrs of injury compared to sham. Addtitinally, Yarnell et al (2012) reported increased NSS score in mild to moderate blast affected male and female rats at day4 and day8 after injury. Our results also showed the similar changes following TBI.

6.7.2 Alteration in rotating pole after blast and blunt TBI

Roatating pole experiment was performed to evaluate coordination and movement integration ability of rats following TBI (Ohlsson and Johansson, 1995). We found significant decrease in blunt rmTBI only at acute timepoint following injury. While, blast TBI rats showed significant decrease in their score at all three timepoints after TBI. A recent study on closed head injury model reported significant decrease in rotarod score of rmTBI rats after injury (Khan et al, 2023). Mattiason et al (2000) reported significant decrease in the rotating pole score of injured rats at day2 and day7 after fpi injury. Hoover et al (2004) reported significant decrease in rotating pole scores following FPI rats after day3,1week, 2week, 3week and 4 weeks of injury. Divani et al (2015) reported decrease in rotarod scores after injury at 3hrs, 6hrs, 24hrs, 72hrs and 168hrs after blast injury but significant decrease was recorded at 3hrs PI. Therefore, our study is also in line with previous experiments.

6.7.3 Alteration in cognitive function after blast and blunt TBI

NORT and OFT were done to assess the effect of blunt and blast TBI on the cognitive function of rats. NORT is used to evaluate the memory function of animals following TBI. We did not observe any significant change in NORT after blast and blunt injury at chronic timepoint. OFT or 54 open field test was done to see the change in locomotor activity and anxiety level of the rats following injury at chronic timepoint. No significant change was found in blunt TBI rats at chronic timepoint. While Blast TBI rats showed significant decrease in their locomotor activity in mild and repetitive mild TBI rats at chronic timepoint. Broussard et al (2017) reported significant decrease in oft(distance travelled) after 5day of rmTBI impact injury mice compared to control.

6.8 Conclusion

In conclusion we found that rm and mi tbi significantly affect neurobehavior functions such as NORT, OFT, NSS and rotating pole at acute, sub- acute and chronic timepoints. We observed altered NSS after blunt tbi at all timepoints after injury. While, significant change in rotating pole scores were observed at acute timepoint after blunt rmTBI. After blast injury mi TBI rats showed significant increase in NSS score. While rmTBI rats showed significant change at chronic timepoint. Rotating pole score showed significant decrease in rmTBI rats only after blast induced tbi at all three timepoints. While OFT study showed significant change following blast tbi in both mi and rmTBI groups at chronic timepoint.

Summary

The present thesis entitled Assessment of alterations in neurometabolites and brain microstructure after repetitive mild traumatic brain injury and its association with the behavioral outcome. The present study was intended to explore the histological and metabolic basis of said alterations in the clinical subjects using pre-clinical models. The work under this thesis has focused on TBI which is the leading cause of death and disability in adult population in developing countries. Cerebral and biofluid metabolic profile has been studied to infer a metabolic signature using most reliable/significant set of metabolites altered. Different behavioral aspects of miTBI and rm TBI has been studied.

In chapter 3, NMR metabolomics was used in this study to standardise the classification of diffuse TBI in rats and its effect on the hippocampal metabolome. Ten-week-old Sprague-Dawley rats were used for the experiments; each group contained 10 animals. A laboratory-made modified Marmarou‘s weight drop model (Marmarou et al. 1994) was used in this study. TBI was created using a trauma apparatus consisting of a 450-gram metal rod that fell freely through a PVC tube. The brass rod was threaded so that it could be freely lowered from a predetermined height through a 2-metre vertical segment of PVC tubing held in place by an iron support. A stainless-steel button was placed on the anaesthetized animal skull to create a diffuse impact on the brain. The button was placed between the bregma and lambda on the sagittal suture. The rats were placed on a foam bed placed on the base of the iron stand. From a height of 25 cm and 50 cm (mild TBI), 75 cm (moderate TBI), 100 cm, 125 cm, and 150 cm (severe TBI) above the sagittal midline of the rat brain, a 450 gm (1 cm diameter, blunt tip) brass rod was dropped freely. PBS and PFA were employed to anesthetize and perfuse the animals. After a day of wounding and perfusion, the animals were put to death. After being separated, the hippocampus was liquid nitrogen-snapshot frozen. Tissue samples were then kept for later use at -80 °C. Metabolites were extracted using acetonitrile extraction. Tissues were homogenised in acetonitrile and water (v/v; 1:1). Tissue homogenate was then centrifuged, and the supernatant was collected and lyophilized. For histological studies, whole brains were stored in 4% formalin. Thioflavin S staining was done to observe amyloidosis in the brain after injury. A total of 18 metabolites were identified and observed for metabolic change in the hippocampal tissues after 24 hours of injury. Myo-inositol, choline, taurine, creatine, GABA, glutamate, glutamine, NAA, and lactate were significantly down-regulated after injury. While BAA, acetate, and succinate showed significant upregulation in their levels after injury. An increasing number of beta plaques was observed with injury severity in rats after 24 hours of injury.

In chapter 4, We examined the temporal effects of moderate closed head injury on the hippocampus, thalamus, and striatum. Four groups of five rats each were randomly selected from a total of twenty rats. A modified Marmarou's weight-drop mechanism was used to injure the animals, and samples were taken one, three, and seven days after the injury (PI). After processing the materials, nuclear magnetic resonance (NMR) spectra were acquired for examination. The findings showed metabolic alterations that were specific to time and place. Myo-inositol, creatine, glutamate, succinate, lactate, and N-acetylaspartic acid (NAA) levels in hippocampus tissues changed at 3 days post-infection (PI), whereas tyrosine levels changed at 7 days PI. In the thalamus and striatum, branched-chain amino acids (BCAA) and myo-inositol levels were altered at 1 day PI. Striatal tissues exhibited significant alterations in taurine, gamma-aminobutyric acid (GABA), choline, and alpha-ketoglutarate (alpha-KG) levels at 1 and 3 days PI. In the thalamus, acetate and GABA levels were altered at 1 day PI, and choline levels were consistently altered at all-time points post-injury. These

metabolite alterations may result from disruptions in their associated biochemical pathways. The findings suggest that neurotransmitter and energy metabolism pathways are affected in the hippocampus, thalamus, and striatum following traumatic brain injury (TBI).

In Chapter 5, We investigated metabolic and histological changes in the hippocampus of rats 24 hours after blast-induced damage. Blast-induced traumatic brain injury (BI-TBI) is common among both military personnel and civilians in conflict zones. Individuals in these places may be subjected to repeated blast wave exposures, which can cause cognitive impairments and metabolic abnormalities. The rats were classified into four groups: (i) sham, (ii) mild TBI (miTBI), (iii) moderate TBI (moTBI), and (iv) repetitive mild TBI (rmTBI). Each group experienced varying amounts of blast exposure. Hippocampal tissue samples were taken 24 hours after damage for proton nuclear magnetic resonance spectroscopy (^1H NMR) and immunohistochemistry examination. The metabolic profile of hippocampus tissues indicated significant alterations in glutamate, N-acetylaspartic acid (NAA), acetate, creatine, phosphoethanolamine (PE), ethanolamine, and phosphatidylcholine (PC)/choline levels with these alterations being most pronounced in the rmTBI group. IHC results showed a decrease in acetyl histone 3 (AH3)-positive cells in the rmTBI group compared to other TBI groups and sham rats, indicating potential epigenetic changes due to repeated blast exposure. Furthermore, astrogliosis was noted in the hippocampus of miTBI and moTBI rats, while no significant changes were observed in the rmTBI group.

In chapter 6, The study was aimed to investigate TBI-induced alterations over time, from acute to chronic, following mild and rmTBI. The study used 1H-NMR metabolomics to examine metabolic changes in tissue, serum, and urine. IHC and faecal 16S rRNA sequencing were used to investigate the potential processes that alter following injury, histological changes, and the gut microbiome. Repetitive mild traumatic brain injury (RM TBI) is a condition in which a person sustains multiple mild traumatic brain injuries over time. Athletes participating in contact sports such as football, hockey, or soccer and persons involved in activities involving a high risk of falls or head impacts are all at risk of repetitive mild TBI. Repeated mild TBIs can have a cumulative effect, which means that each consecutive injury may have a bigger influence on the brain's functioning and healing. A study reported metabolic alterations in collegiate football athletes before and after sports season, and they found alteration in xanthine metabolism, fatty acid metabolism, medium-chain fatty acid, primary bile acid metabolism and glycolysis, gluconeogenesis, and pyruvate metabolism pathways. The gut microbiota has a close relationship with the central nervous system (CNS). Studies have demonstrated that traumatic brain injury can alter the diversity and abundance of gut microbial species, leading to dysbiosis and the development of virulent phylogenetic changes within the intestinal commensal microbiota. This disruption of the gut microbiome following a traumatic brain injury is thought to be a result of the bidirectional communication between the brain and the gut. Thirty female SD rats weighing 200 to 250 g were randomly assigned to one of three groups: control, mild TBI, or rmTBI. For mild and rmTBI, rats were placed on a foam bed and subjected to a free-falling brass rod was dropped from a height of 25 cm, on the sagittal midway of the animal skull. Rm TBI rats were subjected to three impacts at 24-hour intervals. Serum (4 hours, 5 days, and 30 days) and faecal samples (0 days) w were collected for NMR and 16Sr RNA analysis. The α-diversity showed an increase in richness within the mild group, while the rmTBI group showed a decrease in richness. At the phylum level, the firmicutes and bacteria decreased in the rmTBI group. While phylum firmicutes was downregulated in mild TBI rats compared to sham, Other bacterial phylum, including Spirochaetes, Proteobacteria, and Patescibacteria, were also altered after injury. Mildly injured rats showed increased proteobacteria abundance. While serum metabolites ratio of alpha and beta glucose showed a significant increase at the sub-acute and chronic time- points in the mild and rmTBI groups. Baa showed decreases at sub-acute and chronic time- points in the mild and rmTBI groups. Lactate and pyruvate ratio levels were upregulated at chronic timepoints in both mild and repeated mild rats. Additionally, there was a significant decrease in VLDL/LDL at all the time points except for the for the mild 4 hours after injury.

In chapter 7, we focused in the tbi induced significant behavioural changes inrodents, particularly at chronic time points after the initial injury. These changes can manifest in a variety of ways, including alterations in mood, cognition, and social functioning. Research has shown that individuals with traumatic brain injuries often experience changes in mood and behavior.To study the behavioural change in animals, we developed two sets of injury models based on injury types: one is diffused blunt injury, and the other is blast-induced BI. Each injury group was divided into three groups based on severity, i.e., control, mild, and repetitive mild. Thirty female SD rats weighing 200 to 250 g were randomly assigned to one of three groups: control, mild TBI, or rmTBI. For mild and rmTBI, rats were placed on a foam bed and subjected to a free-falling brass rod from a height of 25 cm, above the sagittal halfway point of the rat brain. Rm TBI rats were subjected to three impacts at 24-hour intervals. In the case of blunt TBI NSSR scoring, we observed decreased motor coordination and reflexes in both injury groups in comparison to sham. RmTBI rats showed a significant change in all time points compared to sham rats. While miTBI rats showed significant changes in acute and sub-acute time points in comparison to sham rats, while in the rotating pole experiment, blunt TBI rats showed significant change after rmTBI at the acute time point. The coordination and movement were found to decrease in rmTBI rats compared to sham and mildTBI rats. Additionally, we did not observe any significant changes in the NORT and OFT tests after blunt trauma.

On the other hand, in blast TBI rats, we found decreased motor coordination and reflexes in both injury groups compared to sham. miTBI rats showed a significant change at the acute time point in comparison to sham rats. While rmiTBI rats showed a significant change in chronic time points compared to sham rats, In the rotating pole experiment, we observed a significant change after rmTBI at all three time points. The coordination and movement were found to decrease in rmTBI rats compared to sham and mildTBI rats at acute and sub-acute time- points. While a significant change was observed in rmTBI rats compared to sham rats only, We observed behavioural change following mild and rmtbi at day 30 PI. We found a significant decrease in locomotor activity (total distance travelled and average speed) (OFT) in the mild and rmTBI injury groups, respectively, compared to sham. While anxiety levels were found to be in increasing order in mildTBI animals and rmTBI injury animals, respectively, The NORT study (time spent with novel objects and discriminatory ratio) results showed a decrease in the time spent with novel objects and discriminatory ratio in injury animals compared to sham at day 30 PI. Although this decrease was not significant.