Cardiovascular changes in urethane anesthetized Wistar rats after the traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) leads to cardiovascular complications and negative impact on the recovery of these patients. There are not much published reports regarding the alterations in brain areas controlling cardiovascular function in TBI. In this study, we investigated the effects of severe, moderate and mild TBI on systemic blood pressure, heart rate, greater splanchnic nerve activity (GSNA) and baroreflex. The experiments were done in anesthetized, male Wistar rats. Fluid percussion injury (FPI) was applied to induce TBI. Application of severe, moderate and mild FPI resulted in 100%, 40% and 10% mortality, respectively. In moderate and mild FPI, apnea was observed for 38.2 ± 17.3 and 14.8 ± 3.6 sec, respectively, however, no apnea was seen in severe FPI treated rats, since most of the rats in this group died just after the injury. The decreases in MAP and HR at 30 minutes, 1, 2, 4 and 6 hours after the moderate and mild FPI were significantly attenuated when compared with sham control rats. The GSNA and baroreflex sensitivity were significantly attenuated after moderate and mild FPI. In these rats, microinjections of L-glutamate and gabazine into medullary areas elicited altered cardiovascular responses. These results indicate that both moderate and mild FPI elicit cardiovascular changes.

KEYWORDS: Traumatic Brain Injury, Blood Pressure, Heart Rate, Sympathetic Nerve Activity and baroreflex

Citation:
INTRODUCTION

Traumatic brain injury (TBI) alters the cardiovascular functions such as, changes in systemic baseline blood pressure (BP) and heart rate (HR; Dixon et al. 1987; McIntosh et al. 1989; McMahon et al. 2008) however not much has been published regarding changes in BP, HR and greater splanchnic nerve activity (GSNA). TBI induced brain lesions may contribute changes in central cardiovascular regulation resulting into severe fatalities. The cardiovascular reflex mechanisms are also changed after TBI (Anderson et al. 1990; Hilz et al. 2016). Although, TBI results in long-term motor, cognitive and behavioral dysfunction, in the present studies we focused on changes after the TBI in central cardiovascular regulation especially on BP, HR, GSNA and baroreflex. Morbidity and mortality after TBI may increase after the TBI-induced consequences on cardiovascular function. Previous studies have shown that in rats, after the moderate TBI, a decrease in BP was observed (Gabrielian et al. 2011; McMahon et al. 2011), however, others have stated not much changes in BP (Rogatsky et al. 2003; Lafrenaye et al. 2012; Lafrenaye et al. 2014) after the TBI. It has been reported that in early hours after the TBI, hypotension was responsible for mortality and reduced autonomic functions (Manley et al. 2001; Brenner et al. 2012). Hypotension and hypoxia accompanying TBI lead to ischemic brain damage, increased mortality and morbidity. Even brief episodes of hypotension are reported to increase mortality eightfold in TBI patients (Goldstein et al. 1998; Manley et al. 2001; Andrews et al. 2002; Bratton et al. 2007; Schirmer-Mikalsen et al. 2007).

One of the main neural mechanism that maintains spontaneous changes in BP is baroreflex and weakening of this mechanism may lead to severe consequences. The key component of cardiovascular homeostasis depends on arterial baroreflex (Spyer, 1984). Changes in cardiovascular regulation and baroreflex have been shown after the acute TBI (Brenner et al. 2012), however, more studies are needed to confirm these results. In humans, after mild TBI cardiovascular abnormalities and baroreflex were found to be impaired due to autonomic modulation and dysfunction (Goldstein et al 1998; Hilz et al 2011; Hilz et al 2016; Sykora et al 2016). During the early stages of hemorrhagic shock, the arterial baroreflex triggers HR increase and sympathetic activity to enhance the supply of major tissues. TBI is associated with disturbed baroreflex function resulting in high mortality (Anderson, 1990).

In the present study, we also focused on the cardiovascular changes induced by microinjections of L-Glutamate (L-GLU) and gabazine (GABA-A antagonist) in the medullary cardiovascular areas e.g. medial subnucleus of nucleus tractus solitarius (mNTS), caudal ventrolateral medullary depressor area (CVLM) and rostral ventrolateral medullary pressor area (RVLM) after the fluid percussion injury (FPI) in these rats and the neurons in these areas contain glutamate and gamma aminobutyric acid (GABA) as a major neurotransmitters controlling cardiovascular regulation (Persson, 1980; Willette et al. 1983b; Willette et al. 1984; O'Dell et al. 2000; Sapru, 2002). L-glutamate (L-GLU) stimulates, while GABA, inhibits neuronal cell bodies (Hess and Murata, 1974). Baseline BP, HR, GSNA and cardiovascular reflex functions at normal levels are balanced by the actions of these neurotransmitters in these medullary areas. TBI-induced changes in the neurons of these medullary areas containing these neurotransmitters may affect the function of afore-mentioned medullary cardiovascular regulation which may result in changes in the baseline BP, HR, GSNA and baroreflex. A balance of the actions of these neurotransmitters in these medullary areas may affect the function of afore-mentioned neurons containing these neurotransmitters may compromise the function of afore-mentioned medullary cardiovascular regulatory areas resulting in decreases in the baseline BP, HR and GSNA. The role of L-GLU and GABA after the TBI has been reported by several
investigators (O'Dell et al. 2000; Guerriero et al. 2015). Glutamate and GABA are very crucial for maintaining normal neurologic function. On one hand, after the TBI, immediate glutamate release produced metabolic changes which are responsible for excitotoxicity (Hayes et al. 1992) eventually leading to neuronal injury and finally, the cell death, on the other hand, after the TBI loss of GABA-generating cells disrupts the balance of excitation and inhibition leading to further cell injury and apoptosis. These excitation and inhibition imbalances may lead to cell damage via mitochondrial irregularities, shearing of axons (O’Dell et al. 2000; Guerriero et al. 2015). After the TBI, the cell membrane is swollen and ruptured causing a release of neurotransmitters into the extracellular space which results in Na+/K+ pump failure (Bullock et al. 1998; Yokobori and Bullock, 2012).

Since, no information is available in the published literature regarding FPI-induced alterations after the microinjections of L-GLU and gabazine (GABA-A receptor antagonist) into the medullary cardiovascular regulatory areas in rats after the FPI, these two neurotransmitters were selected for these studies because we have previously reported blockade of GABA receptors in the RVLM abolishes the cardiovascular responses to baroreflex stimulation (Kawabe et al. 2007) and the involvement of RVLM in the maintenance and reflex regulation of blood pressure (Willette et al. 1983b; Willette et al.1984). Assuming the difficulty of the pathophysiology of injuries, it is important to gain more knowledge about this imbalance of L-GLU and GABA to find a therapeutic and neuroprotective goal.

**MATERIALS AND METHODS**

**General procedures**

Adult male Wistar rats (Charles River Laboratories, Wilmington, MA, USA), weighing 300-350 gm, were used in this study. The animals were housed under controlled conditions with a 12h: 12h light: dark cycle. Food and water were available to the animals ad libitum. The experiments were designed according to the NIH "Guide for the Care and Use of Laboratory Animals" and approved by the Institutional Animal Care and Use Committee of this institution. All the procedures used in the present study are reported in publications (Sapru, 2002; Chitravanshi et al. 2009; Chitravanshi and Sapru, 2011; Gupta et al. 2012).

For applying the FPI, a hub on the rat's head was made 24 hours before the injury by giving pentobarbital anesthesia (50 mg/kg, i.p.). Under aseptic conditions, a hole (4.8 mm diameter) was drilled in the parietal bone (1 mm caudal and 1.5 mm lateral to the bregma) on one side, a sawed-off hub of an 18-guage hypodermic needle was fixed in the hole using krazy glue and dental cement, a cotton ball was placed inside the hub to prevent the entry of dust particles and the rat was allowed to recover from anesthesia for 24 hours. An antibiotic (cefazolin, 30 mg/kg, twice a day) and an analgesic (one dose of slow release buprenorphine, 1 mg/kg) was administered subcutaneously and food and water were allowed ad libitum. On the day of experiment (24 hours later), since the cannulation of femoral artery and vein needed for observing the changes in MAP and HR, the rats were first anesthetized with inhalation of isoflurane (2-3% in 100% oxygen), using a vaporizer (Fluotec-3, Cyprane Ltd., UK). The trachea was cannulated and the rats were artificially ventilated using a rodent respirator (model 683, Harvard Apparatus, Holliston, MA, USA). The femoral vein and artery on one side were cannulated. Rats were administered urethane (1.2-1.4 gm/kg, i.v.), using a solution containing 800 mg/ml of urethane, which was injected intravenously (i.v.) in 6-9 aliquots (each 0.05-0.1 ml containing 40-80 mg of urethane) at 2 min intervals. Isoflurane inhalation was discontinued after the administration of 4-5 aliquots of urethane, however, the rats were still artificially ventilated for the rest of the experiment. Administration of the total anesthetic dose of urethane was completed within 12-18 min. The adequate depth of
anesthesia was indicated by the absence of an increase in blood pressure (BP) and/or withdrawal of the limb in response to hind paw pinch. The depth of anesthesia was periodically checked until the end of the experiment. The rectal temperature was maintained at 37 ± 0.5°C using a rectal probe (RET-1) connected to temperature controller (model TCAT-2A) supplied by Physitemp Instruments, Clifton, NJ, USA. The rats were placed in a prone position with bite bar 3.3 mm below the interaural line in a stereotaxic instrument (David Kopf Instruments, Tajunga, CA, USA). The BP and heart rate (HR) recordings before the FPI were stored on a computer hard drive using 1401 plus A/D converter and Spike 2 software (Cambridge Electronic Design Ltd., Cambridge, UK). The rats were then connected to a fluid-percussion assembly via the previously placed needle-hub, and a single, brief (20 msec), unilateral pressure wave (severe, moderate or mild FPI) was delivered to the exposed dura through the needle hub. The pressure pulse was measured extracranially via a pressure transducer. In the rat model, severe, moderate and mild brain injury can be elicited by the application of 2.8-3.6, 1.5-2.2 and 0.1-1 atm pressure pulses (20 msec), respectively (Santhakumar et al. 2001; Mangat, 2012). FPI applied in anesthetized rats has been reported to reproduce many of the features of head injury in humans. The neurological and behavioral features of FPI are similar to those observed in human head injury cases (McIntosh et al. 1989; Manley et al. 2001; Rogatsky et al. 2003; McMahon et al. 2008; McMahon et al. 2011; Brenner et al. 2012). In our experiments, anesthetized rats were used because invasive surgery was needed prior to the application of brain injury to cannulate the femoral artery, vein and recording of the greater splanchnic nerve activity (GSNA). The procedures used for these are reported in published literature (Sapru, 2002; Chitravanshi et al. 2009; Chitravanshi and Sapru, 1996; Santhakumar et al. 2001; Gupta et al. 2012).

**GSNA recording**
Rats were placed in a prone position in a stereotaxic instrument with a spinal clamp on the 10th thoracic vertebra. They were paralyzed with decamethonium (3 mg/kg i.v. initial dose, supplemented with 0.5 mg/kg i.v. as needed; Chitravanshi and Sapru, 1996) and artificially ventilated with room air. The depth of anesthesia was ascertained before the administration of neuromuscular blocker by pinching one of the limbs and testing the corneal reflexes. The effects of neuromuscular blocker lasted for 20-30 minutes. The end-tidal CO2 was maintained between 4-5%. The greater splanchnic nerve (GSN) on one side was exposed retroperitoneally, sectioned as it joins the celiac ganglion, and a small segment was desheathed and placed on a bipolar platinum-iridium electrode. The desheathed nerve segment was embedded in Kwik-Sil and the electrical activity was recorded as published earlier using Spike 2 program (CED, UK; Kawabe et al. 2006; Chitravanshi and Sapru, 2011). After completion of the experiment, the noise level of GSNA was determined by sectioning the GSN centrally and was subtracted from the GSNA amplitude. In this nerve recording experiments, the possibility of recording from afferent fibers is eliminated since the nerve is sectioned distally.

**Microinjections**
Multi-barrel glass micropipettes (tip size 20 µM) were used to microinject L-GLU, gabazine, India ink and aCSF into the mNTS, CVLM and RVLM. The detailed techniques and coordinates for these areas are mention in the previous reports (Willette et al. 1983a; Kawabe et al. 2006; Kawabe et al. 2007; Chitravanshi and Sapru, 2011).

**Baroreflex testing**
Intravenous injections of phenylephrine (PHE; 10 µg/kg, i.v.) were administered sequentially and reflex changes in HR and GSNA were monitored (Chitravanshi et al. 2009). Intravenous injection of PHE causes increase in BP and reflex decreases in HR and GSNA.

**Fluoro-Jade C staining**
After the experiments, the rats were perfused and the sections were cut in cryostat (Leica, CM 1900) and stained with Fluoro-Jade C. to visualize the degenerating cells in the brain (Gupta et al. 2012).

**Retrograde tracing of medullary projections to Hippocampus area**

These experiments were done under aseptic conditions. The rats were anesthetized with intraperitoneal (i.p.) injections of pentobarbital sodium (50 mg/kg) and fixed in a prone position in the stereotaxic instrument. All the surgical instruments were sterilized using an autoclave. The coordinates for approaching to hippocampus from the bregma were rostro-caudal -3.6 mm, medio-lateral 4.4 mm and 3.5-3.7 mm deep from the surface. A microinjection of Fluoro-Gold (FG, 4%, 2 nl) was made into the hippocampus area (n = 3). After completing the microinjection, the exposed brain surface was covered with a small piece of absorbable gelatin sponge (Surgifoam, Ethicon Inc., Somerville, NJ, USA) and the skin over the wound was sutured. After the recovery from pentobarbital anesthesia, the rats survived for 14 days. During the recovery period, after injections of FG, the rats were administered an antibiotic (cefazolin, 30 mg/kg) subcutaneously twice a day for 3 days and one dose of a slow release dosage form of an analgesic (buprenorphine SR, 1 mg/kg). Fourteen days after the microinjection of FG into the hippocampus area, the rats were deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and perfused first with heparinized normal saline, which was followed by 2% paraformaldehyde solution. The brains were removed and placed in 2% paraformaldehyde for 48 hours. On completion of the fixation procedure, one side of the brain surface was marked by a shallow cut and serial sections of the hypothalamic area were cut (40 μm) in a vibratome (model 1000 Plus, The Vibratome Company, St. Louis, MO, USA). The microinjection sites of FG in the hippocampus area and the retrogradely labelled cells in the mNTS and RVLM were visualized under a microscope (model AX70, Olympus Provis, Middlebush, NJ, USA) using an ultraviolet filter (excitation, 323 nm; emission, 408 nm). The images of the sections were captured, 1 μm apart, using a laser scanning confocal microscope (AIR confocal microscope, Nikon Instruments Inc., Melville, NY, USA) and compared with a standard atlas (Paxinos and Watson, 2007). Retrograde tracings of multisynaptic projections from the mNTS to the hippocampus area were also shown by other studies (Castle et al. 2005).

**Drugs and chemicals**

Decamethonium, Gabazine, L-glutamate monosodium, isoflurane, phenylephrine and urethane. All of the solutions for the microinjections were freshly prepared in aCSF (pH 7.4). Isoflurane was purchased from Piramal Critical Care (Bethlehem, PA, USA). All other drugs were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

**Statistical analyses**

The integrated signals of GSNA obtained before and after the FPI were averaged over a period of 60 - 90 sec. The changes in GSNA were expressed as percentage of activity. Student’s paired t-test was used for comparison of BP, HR, GSNA and baroreflex in following experiments: 1) cardiovascular changes before and after the moderate and mild FPI in different groups, 2) changes in baroreflex before and after moderate and mild FPI, and 3) changes in MAP and HR before and after the moderate and mild FPI. Group values of BP, HR, GSNA and baroreflex were expressed as mean ± standard error. In all cases, the differences were considered significant at p < 0.05.

**RESULTS**

A total number of 107 rats were used to study the changes in the BP, HR, GSNA and microinjections of L-GLU and gabazine into mNTS, CVLM and RVLM after the application of severe, moderate and mild FPI. All the experiments were done in urethane anesthesia except placement of a hub on the head of rat 24 hours before the experiment and retrograde tracing of medullary projections to...
Hippocampus area where only pentobarbital was used (50 mg/kg, i.p.).

**Figure 1.** Cardiovascular changes induced by application of FPI: decreases in PAP, MAP and HR. A: Severe FPI. The mortality in these rats was 100% within 2-3 min of the FPI application. B: Moderate FPI. The mortality in these rats was 40% within 6-8 min of FPI application. C: Mild FPI. The mortality in these rats was 10% within 10-12 min of FPI application. Top panel: PAP (mmHg), 2nd panel: MAP (mmHg), 3rd panel: HR (bpm; Time scale = 2 min). **Abbreviations:** PAP: pulsatile arterial pressure; MAP: mean arterial pressure; HR: heart rate; bpm: beats/min (for this and other panels).

**Effects of severe, moderate and mild FPI on urethane anesthetized Wistar rats**

The application of FPI in adult male Wistar rats was done under urethane anesthesia and the rats were artificially ventilated before and after the FPI until the end of the experiments. The experiments included the following groups of rats: severe FPI (2.8-3.6 atmospheres; atm; n = 6), moderate FPI (1.5-2.2 atm; n = 6) and mild FPI (0.1-1 atm; n = 6). The mortality was 100% in rats with severe FPI; mean arterial pressure (MAP) and heart rate (HR) decreased to zero level within 2-3 min (Fig. 1A) and further experiments for monitoring MAP, HR and GSNA could not be carried out in this group. In rats with moderate FPI, mortality was 40% within 6-8 min; a tracing of the decrease in MAP and HR is shown in Fig. 1B. The mortality in rats with mild FPI was 10% within 10-12 min; a tracing of the decrease in MAP and HR in these rats is shown in Fig. 1C. Immediate apnea was elicited by moderate and mild FPI which lasted for 38.2 ± 17.3 sec and 14.8 ± 3.6 sec, respectively. The onset times for movement responses to toe pinch were 7.1 ± 1.6 min and 5.3 ± 0.9 min in moderate and mild application of FPI,
respectively. In severe FPI, frequent seizures were followed by death whereas, in moderate and mild FPI treated rats the seizures were occasionally present.

**Changes in MAP and HR in rats treated with moderate FPI at 30 min, 1, 2, 4 and 6 hours.**
In these experiment changes in MAP and HR were recorded in sham control (n = 6) and moderate FPI (n = 6) at the following time points: 30 min and 1, 2, 4, and 6 hours.

**Changes in MAP after moderate FPI:**
The decreases in MAP (mmHg) values after 30 min in sham control rats and acute FPI were 0.5 ± 0.5% and 16.8 ± 3.1% (p < 0.001), respectively (Figs. 2A-B). The decreases in MAP (mmHg) values after 1 hour in sham control rats and acute FPI were 1.8 ± 1% and 18.7 ± 2.6% (p < 0.001), respectively (Figs. 2C-D). The decreases in MAP (mmHg) values after 2 hours in sham control rats and acute FPI were 4.2 ± 1.5% and 43.1 ± 2.1% (p < 0.001), respectively (Figs. 2E-F). The decreases in MAP (mmHg) values after 4 hours in sham control rats and acute FPI were 9.8 ± 0.7% and 61 ± 2.8% (p < 0.001), respectively (Figs. 2G-H). The decreases in MAP (mmHg) values after 6 hours in sham control rats and acute FPI were 10.4 ± 0.9% and 63.2 ± 3% (p < 0.001), respectively (Figs. 2I-J).

**Changes in HR after moderate FPI:**
The decreases in HR (bpm) values after 30 min in sham control rats and acute FPI were 0.2 ± 0.2% and 7.8 ± 1.7% (p < 0.001), respectively (Figs. 2K-L). The decreases in HR (bpm) values after 1 hour in sham control rats and acute FPI were 1.1 ± 0.3% and 11.3 ± 2.9% (p < 0.01), respectively (Figs. 2M-N). The decreases in HR (bpm) values after 2 hours in sham control rats and acute FPI were 2.3 ± 0.5% and 14.2 ± 4.1% (p < 0.05), respectively (Figs. 2O-P). The decreases in HR (bpm) values after 4 hours in sham control rats and acute FPI were 3.8 ± 0.7% and 15.7 ± 4.3% (p < 0.05), respectively (Figs. 2Q-R). The decreases in HR (bpm) values after 6 hours in sham control rats and acute FPI were 5.7 ± 1.1% and 18.5 ± 4.4% (p < 0.05), respectively (Figs. 2S-T).

**Changes in MAP and HR after mild FPI at 30 min, 1, 2, 4 and 6 hours**
In this group of rats (n = 6), changes in MAP and HR were recorded at the following time points: 30 min, and 1, 2, 4, and 6 hours after injury. The decreases in MAP (mmHg) in response to mild FPI were 14.5 ± 3.3%, 17.7 ± 2.5%, 37.4 ± 4.3%, 59.1 ± 3.1% and 61.9 ± 2.4%, respectively. The decreases in HR (bpm) elicited by mild FPI at corresponding
time points were 5.5 ± 2.1%, 9.1 ± 2.8%, 10.6 ± 4.1%, 12.4 ± 4% and 15.6 ± 3.9%, respectively. The decreases in MAP were significantly different in all the groups when compared with sham controls (p < 0.001 - p < 0.0001) (Fig. 3A). The decreases in HR were significantly different in all the groups when compared with sham controls (p < 0.05 - p < 0.01). (Fig. 3B).

Changes in baseline greater splanchnic nerve activity (GSNA) after moderate and mild FPI

In this group of rats (n = 6), the control baseline GSNA was 16 ± 2.1 (µV). After the application of moderate FPI the GSNA activity was 9.3 ± 0.8 (µV) eliciting a decrease of 6.7 ± 2.7 (µV) in GSNA activity. In the other group of rats (n = 6), the control baseline GSNA was 13.9 ± 1.9 (µV). After the application of mild FPI the GSNA activity was 9.1 ± 0.7 (µV) eliciting a decrease of 4.8 ± 0.7 (µV) in GSNA activity. In both the groups the differences before and after the FPI were significantly different (p < 0.01).

Figure 3. Bar diagram showing effects on MAP and HR in sham and mild FPI treated rats: 30 minutes, 1 hour, 2 hours, 4 hours and 6 hours. A: Changes in MAP. B: Changes in HR. (*p< 0.05 - ****p<0.0001.

Changes in reflex HR induced by moderate FPI in responses to baroreflex activation

In these rats (n = 6), baroreceptors activation was done by injections of PHE (10 µg/kg, i.v.) and an increase in MAP elicited. PHE-induced reflex decreases in HR (bpm) after 30 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 3.5 ± 0.5%, 1.5 ± 0.1%, 1.1 ± 0.2% and 0.3 ± 0.3%, respectively (Fig. 4A). PHE-induced reflex decreases in HR (bpm) after 40 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 4.6 ± 0.6%, 2.8 ± 0.1%, 2.3 ± 0.5% and 0.7 ± 0.4%, respectively (Fig. 4B). PHE-induced reflex decreases in HR (bpm) after 50 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 7.9 ± 0.8%, 4.2 ± 0.6%, 3.7 ± 0.5% and 1.2 ± 0.6%, respectively (Fig. 4C). PHE-induced reflex decreases in HR (bpm) after 60 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 11.8 ± 1.6%, 6.1 ± 1.2%, 5.6 ± 0.3% and 2.6 ± 0.9%, respectively (Fig. 4D). Thus, baroreflex sensitivity was attenuated after the moderate FPI. (*p< 0.05; **p<0.01).

Effects on greater splanchnic nerve activity (GSNA) induced by baroreflex activation after the moderate FPI

The baseline reduction in GSNA (µV) in response to PHE-induced reflex decreases after 30 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 45.9 ± 6.5%, 30.5 ± 2.1% and 22.9 ± 2.1% and 20.3 ± 2.4%, respectively (Fig. 4E). The baseline reduction in GSNA (µV) in response to PHE-induced reflex decreases after 40 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 64.8 ± 7%, 45.6 ± 5%, 32.8 ±
5.1% and 31.1 ± 6%, respectively (Fig. 4F). The baseline reduction in GSNA (µV) in response to PHE-induced reflex decreases after 50 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 73.9 ± 6%, 57.5 ± 4%, 53.7 ± 5.5% and 44.7 ± 5.4%, respectively (Fig. 4G). The baseline reduction in GSNA (µV) in response to PHE-induced reflex decreases after 60 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 77.5 ± 2.5%, 69.8 ± 2%, 66.9 ± 3.2% and 63.6 ± 2.1%, respectively (Fig. 4H). Intravenous injections of PHE elicited a transient reflex inhibition of GSNA. Thus, baroreflex sensitivity was attenuated after the moderate FPI. (*p<0.05; **p<0.01). A typical tracing showing effects of moderate FPI on baroreflex is shown in Figs. 4I-4L. Linear regression curve showing baroreflex responses before and after moderate FPI is presented in Fig. 4M.

![Figure 4. Effects of moderate FPI on baroreflex by intravenous (i.v.) administration of phenylephrine (PHE). A-D: Reflex changes in HR before and after moderate FPI at different levels of increase in MAP. E-H: Reflex changes in GSNA before and after moderate FPI at different levels of increase in MAP. I-L: A typical tracing showing reflex changes induced by PHE (10 µg/kg i.v.) in HR and GSNA before and after the moderate FPI (Time scale = 2 minutes). M: Baroreflex function curves. Linear regression curves showing the control baroreflex responses before moderate FPI (solid squares and straight line), after 30 min of moderate FPI (solid triangles and straight line), after 60 min of moderate FPI (solid circles and straight line) and after 120 min of moderate FPI (open squares and straight line). The r² values for the regression curves of the bradycardic reflex were 0.9501, 0.9916, 0.9887 and 0.9066 for control (before FPI), 30 min, 60 min and 120 min after FPI, respectively. (*p<0.05 - ***p<0.001).](image)

**Changes in reflex HR induced by mild FPI in responses to baroreflex activation**

In these rats (n = 6), baroreceptors activation was done by injections of PHE (10 µg/kg, i.v.) and an increase in MAP elicited. PHE-induced reflex
decreases in HR (bpm) after 30 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 3.9 ± 0.8%, 1.7 ± 0.3%, 1.1 ± 0.2% and 1.4 ± 0.2%, respectively (Fig. 5A). PHE-induced reflex decreases in HR (bpm) after 40 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 6.9 ± 0.9%, 4.1 ± 0.8%, 2.5 ± 0.5% and 2.6 ± 0.4%, respectively (Fig. 5B). PHE-induced reflex decreases in HR (bpm) after 50 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 10.9 ± 0.8%, 6.6 ± 1%, 5.2 ± 1% and 4.9 ± 0.6%, respectively (Fig. 5C). PHE-induced reflex decreases in HR (bpm) after 60 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 14.6 ± 1.4%, 8.8 ± 0.9%, 7.4 ± 1.1% and 7.2 ± 0.7%, respectively (Fig. 5D). Thus, the baroreflex sensitivity was attenuated after the mild FPI. (*p< 0.01; **p<0.001).

Figure 5. Effects of mild FPI on baroreflex by intravenous (i.v.) administration of phenylephrine (PHE). A-D: Reflex changes in HR before and after mild FPI at different levels of increase in MAP. E-H: Reflex changes in GSNA before and after mild FPI at different levels of increase in MAP. I-L: A typical tracing showing reflex changes induced by PHE (10 µg/kg i.v.) in HR and GSNA before and after the mild FPI (Time scale = 2 minutes). M: Baroreflex function curves. Linear regression curves showing the control baroreflex responses before mild FPI (solid squares and straight line), after 30 min of mild FPI (solid triangles and straight line), after 60 min of mild FPI (solid circles and straight line) and after 120 min of mild FPI (open squares and straight line). The r² values for the regression curves of the bradycardic reflex were 0.9983, 0.9978, 0.9750 and 0.9231 for control (before FPI), 30 min, 60 min and 120 min after FPI, respectively. (*p< 0.05 - ***p<0.001).
**Effects on greater splanchnic nerve activity (GSNA) induced by baroreflex activation after the mild FPI.**

The baseline reduction in GSNA (µV) in response to PHE-induced reflex decreases after 30 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 25.2 ± 2.2%, 18.4 ± 2.7%, 20.9 ± 2.7% and 21.1 ± 5 %, respectively (Fig. 5E). The baseline reduction in GSNA (µV) in response to PHE-induced reflex decreases after 40 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 35.9 ± 3.4%, 28.9 ± 2.2%, 25 ± 2.9% and 23.6 ± 4.8%, respectively (Fig. 5F). The baseline reduction in GSNA (µV) in response to PHE-induced reflex decreases after 50 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 44.5 ± 2.8%, 40.6 ± 1.9%, 34.8 ± 3.9% and 29.5 ± 4.5%, respectively (Fig. 5G). The baseline reduction in GSNA (µV) in response to PHE-induced reflex decreases after 60 mmHg increase in MAP before FPI and after FPI at 30, 60 and 120 min were 54.1 ± 3.1%, 48.8 ± 2.6 %, 43.6 ± 2.9% and 40.1 ± 3.5%, respectively (Fig. 5H). Intravenous injections of PHE elicited a transient reflex inhibition of GSNA. Thus, the baroreflex sensitivity was attenuated after the mild FPI. (*p<0.05; **p<0.01). A typical tracing showing the effect of mild FPI on baroreflex is shown in Figs. 5I-5L.

**Linear regression curve showing baroreflex responses before and after mild FPI is presented in Fig. 5M.**

**Effect of moderate and mild FPI on cardiovascular responses induced by unilateral microinjections of L-glutamate (L-GLU) and gabazine.**

In this group of experiments, changes in MAP and HR were recorded in mNTS (n =6), CVLM (n = 6) and RVLM (n = 6) of sham control rats in response to the microinjections of L-GLU and gabazine. In another group of experiments, changes in MAP and HR were recorded in mNTS (n =6), CVLM (n = 6) and RVLM (n = 6) of rats treated with moderate FPI in response to the microinjections of L-GLU and gabazine. In the last group of experiments, changes in MAP and HR were recorded in mNTS (n =6), CVLM (n = 6) and RVLM (n = 6) of rats treated with mild FPI in response to the microinjections of L-GLU and gabazine. There were no changes in MAP and HR after the microinjections of aCSF which was used as a vehicle to dissolve the drugs.

**Effect of L-GLU on MAP in mNTS**

In these groups of rats, the decreases in MAP (mmHg) elicited by microinjections of L-GLU (5mM, 50nl) into mNTS of sham and moderate FPI treated rats were -40.8 ± 1.3 % and -31.3 ± 3.2 % (Figs. 6A-B), respectively. The decrease in MAP (mmHg) elicited by microinjections of L-GLU (5mM, 50nl) into mNTS of mild FPI treated rats was -28.1 ± 2 % (Fig. 6C). The differences in MAP (mmHg) elicited by microinjections of L-GLU into mNTS between sham and moderate FPI and sham and mild FPI treated rats were -9.5 ± 3.7% and -2.8 ± 0.9%, respectively (not shown). In these rats, only the decreases in MAP between sham and moderate FPI were significantly different (p < 0.05).

**Effect of gabazine on MAP in mNTS**

The decreases in MAP (mmHg) elicited by microinjections of gabazine (2mM, 50nl) into mNTS of sham and moderate FPI treated rats were -35.7 ± 3.1 % and -4.8 ± 13.4 % (Fig. 6 D-E), respectively. The decrease in MAP (mmHg) elicited by microinjections of gabazine (2mM, 50nl) into mNTS of mild FPI treated rats was -15.1 ± 6.5 % (Fig. 6F). The differences in MAP (mmHg) elicited by microinjections of gabazine into mNTS between sham and moderate FPI and sham and mild FPI treated rats were -30.8 ±15.1 % and -20.5 ± 5.8 %, respectively (not shown). In these rats, the decreases in MAP between sham and moderate FPI were significantly different (p < 0.01) and in mild FPI (p < 0.05).

**Effect of L-GLU on HR in mNTS**

The decreases in HR (bpm) elicited by microinjections of L-GLU (5mM, 50nl) into mNTS of sham and moderate FPI treated rats were -20.7 ± 2.7 % and -11.4 ± 3% (Fig. 6G-H), respectively. The decrease in HR (bpm) elicited by microinjections of L-GLU (5mM, 50nl) into mNTS of mild FPI treated
The differences in HR (bpm) elicited by microinjections of L-GLU into mNTS between sham and moderate FPI and sham and mild FPI treated rats were -9.2 ± 3.7 % and -8.7 ± 4.3 % respectively (not shown). In these rats, only the decreases in HR between sham and moderate FPI were significantly different (p < 0.05).

**Effect of gabazine on HR in mNTS**

The decreases in HR (bpm) elicited by microinjections of gabazine (2mM, 50nl) into mNTS of sham and moderate FPI treated rats were -20.4 ± 2.7 % and -9.2 ± 2 % (Fig. 6J-K), respectively. The decrease in HR (bpm) elicited by microinjections of gabazine (2mM, 50nl) into mNTS of mild FPI treated rats was -12.7 ± 3 % respectively (Fig. 6L). The differences in HR (bpm) elicited by microinjections of gabazine into mNTS between sham and moderate FPI and sham and mild FPI treated rats were -11.2 ± 3.4 % and -7.8 ± 2.2 % respectively (not shown). In these rats, only the decreases in HR between sham and moderate FPI were significantly different (p < 0.01). Thus, the responses elicited by microinjection of L-GLU and gabazine into the mNTS were attenuated after the moderate and mild FPI. A typical tracing of L-GLU and gabazine-induced responses from mNTS after the moderate FPI is shown in Figs. 6M–N, respectively.
Figure 7. Effects of moderate and mild FPI on L-glutamate (L-GLU) and gabazine induced responses in CVLM. A-C: Changes in MAP induced by L-GLU in sham, moderate and mild FPI treated rats. D-F: Changes in MAP induced by gabazine in sham, moderate and mild FPI treated rats. G-I: Changes in HR induced by L-GLU in sham, moderate and mild FPI treated rats. J-L: Changes in HR induced by gabazine in sham, moderate and mild FPI treated rats. M: A typical tracing showing changes induced by L-GLU (5 mM; 50 nl) from CVLM in MAP and HR (Time scale = 2 minute). N: A typical tracing showing changes induced by gabazine (2 mM; 50 nl) from CVLM in MAP and HR (Time scale = 5 minutes; Note: no changes were seen by microinjections of aCSF into the NTS; *p < 0.05 - **p < 0.01).

**Effect of L-GLU on MAP in CVLM**

In these groups of rats, the decreases in MAP (mmHg) elicited by microinjections of L-GLU (5 mM, 50 nl) into CVLM of sham and moderate FPI treated rats were -37.6 ± 1.7 % and -25.5 ± 3.9 % (Figs. 7A-B), respectively. The decrease in MAP (mmHg) elicited by microinjections of L-GLU (5 mM, 50 nl) into CVLM of mild FPI treated rats was -32.1 ± 3.4 % (Fig. 7C). The differences in MAP (mmHg) elicited by microinjections of L-GLU into CVLM between sham and moderate FPI and sham and mild FPI treated rats were -11.9 ± 4.1 % and -5.2 ± 2.5 %, respectively (not shown). In these rats, only the decreases in MAP between sham and moderate FPI were significantly different (p < 0.05).

**Effect of gabazine on MAP in CVLM**

The decreases in MAP (mmHg) elicited by microinjections of gabazine (2 mM, 50 nl) into CVLM of sham and moderate FPI treated rats were -41.7 ± 6.8 % and -27.6 ± 2.3 % (Fig. 7D-E), respectively. The decrease in MAP (mmHg) elicited by microinjections of gabazine (2 mM, 50 nl) into CVLM of mild FPI treated rats was -31 ± 3.9 % (Fig. 7F). The differences in MAP (mmHg) elicited by microinjections of gabazine into CVLM between sham and moderate FPI and sham and mild FPI treated rats were -14.1 ± 8.5 % and -10.7 ± 8.6 %, respectively (not shown). In these rats, only the decreases in MAP between sham and moderate FPI were significantly different (p < 0.05).

**Effect of L-GLU on HR in CVLM**

The decreases in HR (bpm) elicited by microinjections of L-GLU (5 mM, 50 nl) into CVLM of
sham and moderate FPI treated rats were -27.4 ± 4.3 % and -10.9 ± 3% (Fig. 7G-H), respectively. The decrease in HR (bpm) elicited by microinjections of L-GLU (5mM, 50nl) into CVLM of mild FPI treated rats was -12.3 ± 3% (Fig. 7I). The differences in HR (bpm) elicited by microinjections of L-GLU into CVLM between sham and moderate FPI and sham and mild FPI treated rats were -16.5 ± 5.8% and -5.2 ± 5.3%, respectively (not shown). In these rats, the decreases in HR were significant between sham and moderate FPI and also sham and mild FPI (p < 0.01).

**Effect of gabazine on HR in CVLM**

The decreases in HR (bpm) elicited by microinjections of gabazine (2mM, 50nl) into CVLM of sham and moderate FPI treated rats were -38.5 ± 6.1% and -18.1 ± 3.7 % (Fig. 7J-K), respectively. The decrease in HR (bpm) elicited by microinjections of gabazine (2mM, 50nl) into CVLM of mild FPI treated rats was -27.3 ± 6.1 % (Fig. 7L). The differences in HR (bpm) elicited by microinjections of gabazine into CVLM between sham and moderate FPI and sham and mild FPI treated rats were -20.4 ± 5.3% and -11.2 ± 8.4 %, respectively (not shown). In these rats only the decreases in HR between sham and moderate FPI were significantly different (p < 0.01).

Thus, the responses elicited by microinjection of L-GLU and gabazine into the CVLM were attenuated after the moderate and mild FPI. A typical tracing of L-GLU and gabazine-induced responses from CVLM after the moderate FPI is shown in Figs. 7M-N, respectively.
Effect of L-GLU on MAP in RVLM

In these groups of rats, the increases in MAP (mmHg) elicited by microinjections of L-GLU (5mM, 50nl) into RVLM of sham and moderate FPI treated rats were 22.3 ± 2.1% and 14.6 ± 3% (Figs. 8A-B), respectively. The increase in MAP (mmHg) elicited by microinjections of L-GLU (5mM, 50nl) into RVLM of mild FPI treated rats was 19.9 ± 2.3% (Fig. 8C). The differences in MAP (mmHg) elicited by microinjections of L-GLU into RVLM between sham and moderate FPI and sham and mild FPI treated rats were 7.8 ± 3% and 2.4 ± 1.3%, respectively (not shown). In these rats, only the increases in MAP between sham and moderate FPI were significantly different (p < 0.05).

Figure 9. Fluoro-Jade C staining of the degenerating cells in hippocampus area. A: Showing site of FPI (Scale = 1 mm). B: Sham rat showing no fluorescent activity (Scale: 50 µm). C: Degenerating cells showing Fluoro-Jade C staining after the FPI in hippocampus area (Scale: 1 mm). D: Higher magnification of cells shown in panel C (Scale = 500 µm).
**Effect of gabazine on MAP in RVLM**
The increases in MAP (mmHg) elicited by microinjections of gabazine (2mM, 50nl) into RVLM of sham and moderate FPI treated rats were 27.1 ± 2.5% and 18.3 ± 3.6% (Fig. 8D-E), respectively. The increase in MAP (mmHg) elicited by microinjections of gabazine into RVLM of mild FPI treated rats was 23.4 ± 2.9% (Fig. 8F). The differences in MAP (mmHg) elicited by microinjections of gabazine into RVLM between sham and moderate FPI and sham and mild FPI treated rats were 8.8 ± 1.6% and 3.7 ± 0.8%, respectively (not shown). In these rats, the increases in MAP were significant between sham and moderate (p<0.05) and sham and mild FPI (p<0.05).

**Effect of L-GLU on HR in RVLM**
The increases in HR (bpm) elicited by microinjections of L-GLU (5mM, 50nl) into RVLM of sham and moderate FPI treated rats were 7.7 ± 0.7% and 2.9 ± 0.2% (Fig. 8G-H), respectively. The increase in HR (bpm) elicited by microinjections of L-GLU into RVLM of mild FPI treated rats was 4.7 ± 0.9% (Fig. 8I). The differences in HR (bpm) elicited by microinjections of L-GLU into RVLM between sham and moderate FPI and sham and mild FPI treated rats were 4.5 ± 0.7% and 2.4 ± 0.9%, respectively (not shown). In these rats, the increases in HR between sham and moderate FPI were significantly different (p < 0.0001) and sham and mild FPI (p < 0.05).

**Fluoro-Jade C staining**
In the sham rat, no fluorescent activity of Fluoro-Jade C was observed in the hippocampus area where the hub was placed (Fig. 9A-B). The degenerating cells are shown in hippocampus area after the FPI (Fig. 9C). A higher magnification of these degenerating cells are shown in Fig. 9D. The Fluoro-Jade C labelled neurons were confined to hippocampus area and were not seen in any other cardiovascular areas in the medulla and the hypothalamus.

**Identification of projections from the mNTS and RVLM to Hippocampus**
Unilateral microinjections of Fluoro-Gold (FG; 4%; 2 nl) were made into the hippocampus area (n = 3) (Fig. 10A), which resulted in retrograde labelling of mNTS (Fig. 10B) and RVLM cells (Fig. 10C-D), bilaterally with ipsilateral preponderance. The presence of retrogradely labelled FG neurons in mNTS and RVLM showed that there are a direct projections from these medullary areas to hippocampus area.
**Figure 10. Retrograde labelling of projections from the NTS and RVLM to Hippocampus.** A: Injection site of Fluoro-gold in the hippocampus at ~3.6 mm rostro-caudal from bregama, medio-lateral 4.4 mm from midline and 3.5-3.7 mm deep from the surface (Scale: 50 µm). B: Fluoro-gold containing cells of NTS (Scale: 50 µm). C: Fluoro-gold containing cells of RVLM (Scale: 50 µm). D: Higher magnification of cells shown in panel C (Boxed area; Scale: 50 µm).

**DISCUSSION**

The major findings of the present studies are, 1) decrease in MAP, HR and GSNA in anaesthetized rats after the moderate and mild FPI, 2) decrease in baroreflex sensitivity after the moderate and mild FPI, 3) attenuation of cardiovascular responses induced by L-GLU and gabazine from mNTS, CVLM and RVLM after the moderate and mild FPI, 4) a direct projection from mNTS and RVLM to hippocampus area, 5) apnea was present after the moderate and mild FPI however, no apnea was observed in severe FPI, since, most of the rats in this group died just after the application of FPI though they were artificially ventilated before and after the FPI, and 6) in these experiments, the Fluoro-Jade C labelled neurons were only seen in the hippocampus area however, no activity of Fluoro-
Jade C was seen in cardiovascular regions in the medulla and the hypothalamus.

In our experiments, FPI was applied in anesthetized rats because invasive surgery was needed prior to the application of brain injury (Nakamura and Sapru, 2009; Chitravanshi and Sapru, 2011). FPI applied in anesthetized rats has been reported to reproduce many of the neurological and behavioral features of head injury in humans. The pressure wave delivered in this manner simulates concussive, closed head trauma, which is the most common form of civilian head injury. The decreases in MAP and HR were significantly reduced between sham control and acute FPI treated rats. In our studies, effect of severe FPI was not studied because there was a 100% mortality in rats after the FPI. In our studies the mortality rate was higher compare to other published reports (McIntosh et al. 1989) which could be due to the rats used in our study comprising between 300-350 gm in weight when compared with 400 – 450 gm of weight used in other studies and also due to the difference in strain of rats. TBI-induced alterations in the medullary and hypothalamic neurons containing the Glutamate and GABA may compromise the function of aforementioned cardiovascular regulatory areas resulting in decreases in the baseline BP, HR and SNA. Severe and moderate FPI resulted in more mortality because hypotension and hypoxia after the TBI lead to ischemic brain damage resulting in increased mortality and morbidity. Small episodes of hypotension are also reported to increase mortality several times more after TBI (Manley et al. 2001; Andrews et al. 2002; Bratton et al. 2007; Schirmer-Mikalsen et al. 2007).

It has been reported that in early hours after the TBI, hypotension was responsible for mortality and reduced autonomic functions (Manley et al. 2001; Brenner et al. 2012). Our studies showing decreases in MAP are further supported by previous studies which have shown that in rats after the moderate TBI a decrease in BP was found (McMahon et al. 2011). In our studies, the decreases in MAP, HR, GSNA and baroreflex could be related due to focal traumatic brain injury and focal contusion or penetrating injury, which may directly damage the tissue at the site of injury that may cause local swelling, ischemia and, or hemorrhage which can cause even more diffused injury that may impact widespread areas of the brain, including axonal injury followed by a molecular level injury causing membrane swelling and potentially rupture resulting into neurotransmitter and ion release into the extracellular space and failure of Na+/K+ pump (Bullock et al. 1998; Yokobori and Bullock, 2012). The key component of cardiovascular homeostasis depends on arterial baroreflex (Spyer, 1984). In our studies, after the moderate and mild (acute) FPI, changes in cardiovascular regulation and baroreflex were elicited which are supported by other published studies (Sykora et al. 2016). One of the main neural mechanism that maintains spontaneous changes in BP is baroreflex and since our results show that the baroreflex mechanism is weakened which may lead to decrease in MAP and HR and vice versa. In humans, after mild TBI, cardiovascular and baroreflex abnormalities were found due to impaired autonomic modulation (Hilz et al. 2016; Sykora et al. 2016). In some published reports, acute TBI is shown to increase baroreflex sensitivity in the early hours (McMahon et al. 2011) however, in our studies baroreflex was attenuated after the moderate and mild FPI which is supported by others studies that after the TBI, decreased baroreflex function results in high mortality and is shown to be linked with autonomic dysfunction (Anderson et al. 1990; Goldstein et al. 1998; Sykora et al. 2016). The reflex mechanisms are changed after the TBI since the normal levels of Glutamate and gamma aminobutyric acid (GABA) in the medullary areas are altered which are involved in maintaining the cardiovascular reflex responses.

Glutamate is the primary excitatory neurotransmitter in the brain, while GABA is the principal inhibitory neurotransmitter. The balance of glutamatergic and GABAergic tone is crucial to normal neurologic function. The role of glutamate
signaling in TBI pathophysiology is twofold. Acute posttraumatic glutamate release is responsible for excitotoxicity following brain injury that leads to neuronal injury, cell death, and dysfunction of surviving neurons. Micro dialysis studies after the severe TBI have shown that extracellular glutamate after 1 hour of injury gave an immediate rise in extracellular glutamate following severe TBI (Bullock et al. 1998; Vespa et al. 1998; Chamoun et al. 2010; Folkersma et al. 2011), resulting in immediate glutamate release which sets off a different metabolic changes. In other studies, it has been shown that after the FPI glutamate is released into the extracellular space and acts on AMPA and NMDA receptors prompting Ca++ ions to flow into the postsynaptic neuron. Glutamate is then taken up into astrocytes by the glutamate transporter. TBI-induced decreases in this transporter allow excess glutamate to remain in the synapse and continue its excitotoxic actions. This action of glutamate excitotoxicity may be related with the cardiovascular changes elicited after FPI in our studies. Delayed disruption of excitatory glutamate circuits leads to deficits in cognitive and motor function, and in experience-dependent plasticity. Alternatively, GABA is produced in interneurons that modulate cortical and thalamocortical circuits that relay sensory information and play a role in coordinating motor functions, attention, and memory (Castro-Alamancos, 1997; Kandel, 2013). GABA modulates excitatory pathways in the brain and, following injury, loss of GABA-producing cells disrupts the balance of excitation and inhibition leading to further cell injury and apoptosis.

There are no reports in the published literature regarding the microinjections of L-GLU and gabazine (gamma aminobutyric acid antagonist) into the medullary areas in rats treated with FPI since these two neurotransmitters play a major role in cardiovascular hemostasis. In the present study, we have shown that cardiovascular responses to microinjections of L-GLU and gabazine are attenuated in medullary areas e.g. mNTS, CVLM and RVLM after the moderate and mild FPI when compared with sham control rats indicating that functions of neuronal groups in these areas were also altered after the FPI. These experiments were carried out in separate group of rats and were compared since for microinjections into these areas needed opening of medulla by removing the occipital bone. The possibility of damaging the surrounding tissue with the tip of micropipette are very rare since the size of the tip was 20 µM and the responses to these drugs were compared with sham rats. The role of L-glutamate and GABA after the TBI has been reported by several investigators (Guerriero et al. 2015). Magnetic resonance spectroscopy (MRS) studies demonstrate a decrease in glutamate at 2 and 4 h after a CCI model with an open skull injury (Xu et al. 2011).

The role of medulla and spinal cord has long been studied in cardiovascular regulation (Sapru, 2002). In the present studies, we have shown direct projections from mNTS and RVLM to hippocampus area. It may be possible that due to alterations in these projections, the functioning of the neurons in this pathway was attenuated or lost resulting in the decreases in MAP, HR, changes in baroreflex and attenuation of the direct microinjections responses into the medullary cardiovascular areas. This theory needs further confirmation and these projections need to be further explored. In previous studies, noradrenergic neurons in the mNTS are considered to play a role in changes in cardiovascular dyshomeostasis. Lesions of mNTS A2 neurons have shown reduced the tachycardic response to acute restraint, confirming that A2 neurons promote sympathetic activation following acute stress (Osacka et al. 2015). Studies have shown that in the localized A2 noradrenergic cell group of general visceral portion of mNTS, neurons infected with Pseudo rabies virus (PRV) were found when PRV was injected into the ventral CA1 hippocampus area via a multi-synaptic pathway of noradrenergic neurons of the locus coeruleus and cholinergic neurons of the medial septum/diagonal band. Injections of PRV into the CA1 area resulted in labelling of mNTS and in the ventrolateral reticular...
formation in the medulla oblongata via A1, A2, A5, A7, LC and sub-coeruleus neurons (Castle et al. 2005). However, in these studies the PRV was injected into the ventral CA1 area as compared to our injection site of Fluoro-Gold which was in the dorsal hippocampus area. Since, mNTS is the main vagal sensory relay nucleus of the brain which receives primary inputs from the cardiovascular, respiratory, and gastrointestinal systems, it is possible that direct projections from mNTS and RVLM (Zagon et al. 1994) to hippocampus are interrupted eliciting the attenuated responses after the FPI in cardiovascular regulation.

In conclusion, after the FPI, there are dynamic changes in excitatory–inhibitory balance that result in neuronal dysfunction and may result in long-term decreases in MAP, HR and GSNA. The decreases in MAP, HR, GSNA and baroreflex in rats treated with moderate and mild FPI could also be due to the alterations in hypothalamic and medullary neurons controlling the cardiovascular functions maintaining the systemic baseline blood pressure (BP), heart rate (HR) and greater splanchnic nerve activity (GSNA).

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Conflict of interest statement
Authors have no existing competing or conflict of interests. The funding agency had no part in designing and writing the study.

Authors’ contributions
YU and VCC contributed towards the experiments performed in the study. YU and VCC also contributed towards analyzing the data and making figures. YU and VCC contributed for writing the data. VCC contributed for all scientific ideas, manuscript writing and critical revision.

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