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Acute sleep deprivation preconditions the heart against ischemia/ reperfusion injury: the role of central GABA-A receptors

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ABSTRACT

Objective(s): Central γ-aminobutyric acid (GABA) neurotransmission modulates cardiovascular functions and sleep. Acute sleep deprivation (ASD) affects functions of various body organs via different mechanisms. Here, we evaluated the effect of ASD on cardiac ischemia/reperfusion injury (IRI), and studied the role of GABA-A receptor inhibition in central nucleus of amygdala (CeA) by assessing nitric oxide (NO) and oxidative stress.

Materials and Methods: The CeA in sixty male Wistar rats was cannulated for saline or bicuculline (GABA-A receptor antagonist) administration. All animals underwent 30 min of coronary occlusion (ischemia), followed by 2 hr reperfusion (IR). The five experimental groups (n=12) included are as follows: IR: received saline; BIC+IR: received Bicuculline; MLP+IR: received saline, followed by the placement of animals in an aquarium with multiple large platforms; ASD+IR: underwent ASD in an aquarium with multiple small platforms; and BIC+ASD+IR: received bicuculline prior to ASD.

Results: Bicuculline administration increased the malondialdehyde levels and infarct size, and decreased the NO metabolites levels and endothelial nitric oxide synthase (eNOS) gene expression in infarcted and non-infarcted areas in comparison to IR group. ASD reduced malondialdehyde levels and infarct size and increased NO metabolites, corticosterone levels and eNOS expression in infarcted and non-infarcted areas as compared to the IR group. Levels of malondialdehyde were increased while levels of NO metabolites, corticosterone and eNOS expression in infarcted and non-infarcted areas were reduced in the BIC+ASD+IR as compared to the ASD+IR group.

Conclusion: Blockade of GABA-A receptors in the CeA abolishes ASD-induced cardioprotection by suppressing oxidative stress and NO production.

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Introduction

Although insufficient sleep due to modern lifestyle is a distressing problem (1), it seems that acute sleep deprivation (ASD) protects some vital organs against ischemic injury (2, 3). In healthy individuals, cardiac autonomic control during sleep is preserved after chronic sleep restriction (4). Furthermore, sleep deprivation protects gastric mucosa against hydrochloric acid - induced gastric damage (5) and attenuates neuroinflammation and neurodegeneration following global ischemia (6). The central nucleus of amygdala (CeA) is a key area in the limbic system that links psychological conditions to the cardiovascular system. It plays an important role in regulating blood pressure through its projections to the nucleus tractus solitarius (NTS), the primary regulator of the sympathetic nervous system. Electrical stimulation of amygdala has been shown to alter the heart rate (HR), blood pressure and muscular blood flow (7). Gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the central nervous system (CNS), is highly expressed in the CeA (8). It acts through two distinct classes of receptors: ionotropic GABA-A and metabotropic GABA-B receptors. GABA is involved in mediating autonomic responses during psychological situations and the sympathetic outflow to the kidney and heart is strongly modulated by GABAergic projections (9). Imbalance in central GABAergic transmission is associated with development of cardiovascular diseases. Moreover, activation of GABA-A receptors reduces sympathetic activity and blood pressure, and attenuates cardiovascular responses to emotional

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stress (7, 9). GABA also plays a critical role in sleep homeostasis (10). Barbiturates, enhancers of GABA neurotransmission, are well-known for promoting sleep (11). Wakefulness-mediating orexin neurons in the lateral hypothalamus are potent regulators of sleep-active GABAergic neurons in anterior hypothalamus (12). Genetic deletion of GABA receptors on orexin neurons have been shown to induce fragmentation of vigilance states in mice (13). In addition, sleep deprivation causes a substantial increase in the expression of GABA-A receptors in the lateral hypothalamus, basal forebrain and perifornical hypothalamus (14-16). Corticosterone, a potent positive allosteric modulator of GABA-A receptors, has been shown to facilitate GABA neurotransmission (17), and exert anxiolytic, antidepressant and anticonvulsant effects via activation of GABA-A receptors (18).

Nitric oxide is a neuromodulator with sleep promoting action as it inhibits neuronal discharge in wake-promoting brain regions like perifornical-lateral hypothalamic area and the basal forebrain (19). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are two major cellular oxidants (20). Oxidative stress exerts subcellular effects, including oxidative modifications of nucleic acids, proteins, and lipids. Imbalance between the formation and elimination of ROS and RNS has been associated with cancers, neurological diseases, immunodeficiency, and cardiovascular diseases (21). Mitochondria, the intracellular organelles that produce metabolic energy, are the most important sources of cellular ROS generation. In addition, myocardial ischemia reperfusion injury (IRI) induces mitochondrial injury by generating oxygen free radicals (22). Due to high metabolic activity during wakefulness, there is enhanced accumulation of free radicals. Accordingly, ASD may induce oxidative stress; however, it does not cause any oxidative or structural damage in brain, liver and skeletal muscle (7, 21).

Since stimulation of GABA-A receptor activation protects the myocardium against IRI (23), and GABAergic transmission is enhanced during sleep deprivation (24), we designed this study to evaluate the cardiac preconditioning effect of ASD and assess the role of central GABA-A receptor in mediating ASD-induced cardioprotective effects.

Materials and Methods

Animals

Sixty male adult Wistar rats aged 22 weeks, weighing 250-300 g were housed in an air-conditioned colony room maintained at 22 –24 °C and 12-hr light-dark cycles. All animals had free access to food and water. The experimental protocols followed in this study conformed to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No.85-23, revised 1996) and were further approved by the institutional ethical committee of Tehran University of Medical Sciences (Tehran, Iran).

Experimental groups

The CeA of the animals were bilaterally cannulated for drug injection. The animals were allowed to recover for five days. Twenty-four hours after drug or saline administration, all of the anesthetized animals were subjected to 30 min ischemia followed by 120 min reperfusion period (IR). The animals were randomly divided into five groups (12 animals per group): (1) Ischemia-reperfusion group (IR): saline was injected into the CeA and then, IR was induced; (2) Bicuculline group (BIC+IR): bicuculline (a GABA-A receptor antagonist) was injected into the CeA, and after 24 hr, the rats were subjected to IR ; (3) Multiple large platforms group (MLP+IR): saline was injected intra-CeA and the animals were placed for 24 hr in an aquarium with large platforms followed by IR induction; (4) Acute sleep deprivation group (ASD+ IR): saline was administered intra-CeA and then, the animals were placed in an aquarium with small platforms for 24 hr for induction of ASD followed by IR; and (5) Bicuculline + acute sleep deprivation group (BIC+ASD+IR): the animals received bicuculline intra-CeA, and then, they were placed in the aquarium with small platforms for 24 hr for ASD induction followed by IR (Figure 1).

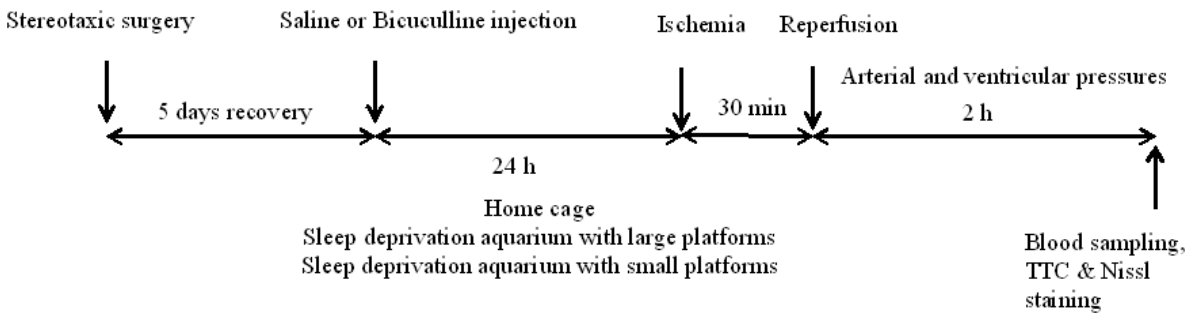


Figure 1. The experimental protocol

Stereotaxic surgery and drug administration

The rats were anesthetized using ketamine (50 mg/kg) and xylazine (5 mg/kg, IP), and placed in a stereotaxic frame (Stoelting, USA) after shaving the heads. The CeA were bilaterally cannulated using stainless steel guide cannula (23 gauge, 15 mm length) at the following coordinates with reference to the Bregma: AP:-2.8, L: ± 4.6, DV:-8.1 according to the Paxinos and Watson rat brain atlas (25). The cannula was then secured in place with dental acrylic supported by two jewelers screws anchored to the skull. Saline (0.5 µl) or bicuculline (0.1 nmol/0.5 µl dissolved in saline, Sigma) was injected bilaterally into the CeA using a 1 ml Hamilton syringe connected to a piece of polyethylene tubing (PE-10) and 27-G microinjection needle (26).

Induction of acute sleep deprivation

ASD was induced for 24 hr by placing the animals in a sleep deprivation aquarium (125 cm × 44 cm × 44 cm) comprising of 8 small circular platforms (6.5 cm in diameter). The aquarium was filled with water (25°C) approximately 1 cm below the surface of platforms, such that, upon sleeping, the animals would fall in water due to muscular atonia and would become awake. For the MLP group, the platforms were replaced by larger ones (14 cm in diameter) on which the rats could sleep without falling down. The rats were allowed to move around freely from one platform to another and had free access to food and water *ad libitum* (27, 28). The MLP model does not impose restriction of movement or social isolation (21). The electroencephalogram (EEG) recording confirmed the induction of sleep deprivation by multiple plate forms model (29).

Surgical procedure of inducing myocardial ischemia and reperfusion

Rats were anesthetized using thiopental (60 mg/kg, IP). The trachea was cannulated and ventilated using a rodent ventilator (tidal volume 2–3 ml, respiratory rate 65–70 breaths/min, Harvard rodent ventilator model 683, Holliston, MA, USA). The heart was exposed through left intercostal thoracotomy (between the fourth and fifth costal spaces) and the pericardium was cut. Next, ischemia was induced for 30 min by passing a 6/0 silk suture around approximately 1–2 mm distal to the origin of the left anterior descending coronary artery (LAD) and tightening over a pipette tip to ligate. Successful LAD ligation was characterized by ST elevation. Reperfusion was performed for 2 hr by removing the tubes and loosening the suture (30, 31).

Blood sampling

At the end of the experiments, blood samples were collected from the heart for biochemical analysis [Malondialdehyde (MDA) and NO metabolites (nitrite/nitrate)]. The samples were centrifuged at 7,000 rpm at 4 ˚C, for 20 min and the serum_was collected and stored at −70 ˚C until biochemical analysis.

Measurement of myocardial infarct size

After IR, the hearts were removed from the animals (n=4), and snap frozen at -20 ˚C for 24 hr. Then, the hearts were sliced into 2 mm transverse sections from apex to the base using stainless steel rat heart slicer matrix. The slices were then incubated in 1% 2, 3, 5 triphenyltetrazolium chloride (TTC in 0.1 M phosphate buffer, pH 7.4) for 20 min at 37 ˚C. The reaction of TTC with viable parts of tissue produced a red region in ventricle, which is distinct from the pale necrotic tissue after fixation in 10% formalin for 24 hr. The size of the total left ventricle area and the infracted area were measured by planimetry of scanned slices using Adobe Photoshop software (31).

Assessment of hemodynamic parameters

In order to record hemodynamic parameters during ischemia and reperfusion periods, a small incision was made to the right of the midline in the neck, and the right common carotid artery was exposed. The exposed artery was cannulated with a PE-50 catheter connected to the Power lab data acquisition system (AD Instrument, Australia) through a pressure transducer (AD Instrument, Australia). Heart rate (HR), rate pressure product [RPP=HR×dLVP (developed left ventricular pressure= left ventricular systolic - end-diastolic pressures)], mean arterial pressure (MAP), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were monitored and recorded by Power lab system. For measurement of dLVP, the left ventricle was catheterized (30).

Real-time polymerase chain reaction (Real-Time PCR)

Following the assessment of hemodynamic parameters, myocardial tissue samples (n=4) were immediately removed, rinsed in phosphate-buffered saline (PBS), snap frozen in liquid nitrogen, and stored at -80 ˚C. Total RNA was extracted from frozen infarcted and non-infarcted area using TRIzol (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized using a Prime Script RT reagent kit (Takara, Cat. RR037A). cDNA samples were then used as templates for quantitative reverse transcription polymerase chain reaction (qRT-PCR). Gene expression was quantified using the Rotor-Gene 6000 (Qiagen). Real-Time RT-PCR analysis was performed using qPCR Master Mix for SYBER Permix Ex Taq (Takar, Cat.RR280L). All samples were analyzed in triplicates. Hypoxanthine phosphoribosyltransferase-encoding gene (HPRT) was used as a housekeeping gene (30). The specific primer sequences are listed in Table 1.

Table 1. The sequence of primers used in real-time PCR

Gene name	Primer sequence	PCR product size (bp)
eNOS	F: 5'-CTCGAGCGGTGGACACAAG-3' R: 5'-CACAGAAAGTTTCACAGGCAGC-3'	144
HPRT	F: 5'-CTCATGGACTGATTATGGACAGGAC-3' R: 5'-GCAGGTCAGCAAAGAACTTATAGCC-3'	123

Measurement of nitrite/nitrate ratio

Based on the Griess reaction, serum levels of NO metabolites, nitrite (NO^{2-}) and nitrate (NO^{3-}) were measured as an index of NO production. The principle of the assay is based on the conversion of nitrate into nitrite by copperized cadmium granules, followed by color development by Griess reagent (sulfanilamide and N-naphthylethylenediamine) in acidic medium. The assay was calibrated with standard solutions of sodium nitrite. A 50 µl sample of serum was used to determine NOx and the results were expressed as nmol/ml. Optical densities were measured at 540 nm in a 96-well microplate reader after 3 hr incubation (32).

Assessment of oxidative stress by TBARS estimation

Measuring thiobarbituric acid-reactive substances (TBARS) in serum is a reliable fluorometric method, based on the reaction between malondialdehyde (MDA) and thiobarbituric acid. Serum MDA levels (the end product of lipid peroxidation) were assessed using a commercially available ELISA kit (ZellBio, Germany), according to the manufacturer's instructions.

Measurement of corticosterone

Serum corticosterone, as a marker for stress, was measured using a commercially available ELISA kit (ZellBio, Germany), based on the manufacturer's instructions.

Nissl assay

At the end of reperfusion, the animals (n=3) were perfused through the heart with 50-100 ml of 4% formaldehyde. Next, the animals were decapitated and the brains were removed from the skull and immersed in 4% formaldehyde at 4˚C for 3 days. The brains were sliced into 20 µm sections using a microtome. Every section was Nissl-stained with 0.1% cresyl violet. Neural loss was quantified in the amygdala in at least three sections for each rat using image-capturing software (Moticam 5 MP). The number of degenerated neurons was counted in three fields (× 40) for each section and was averaged to obtain a final number (33).

Statistical analysis

All data are presented as mean ± SEM. Normal distribution of the data was tested using Kolmogorov-Smirnov test. Moreover, homoscedasticity was determined using Bartlett's test. Accordingly, all statistical differences among the experimental groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Analyses were performed using SPSS software (Version 22, SPSS IBM, Chicago, IL) and Prism (Version 6, GraphpadSofrware, San Diego, CA). *P*-values<0.05 were considered statistically significant. Prespecified sample size from previous studies was used to obtain significant results (30, 32). None of the samples were excluded from the analysis. The animals were randomly allocated to experimental groups and no blinding was observed during the assessment of experimental outcomes.

Results

Infarct size

One-way ANOVA analysis showed significant differences in the infarct size between groups ($F_{(4, 17)}$=11.63; *P*=0.0001). The *post hoc* test confirmed reduced infarct size in ASD group compared with the IR group (31.4±3.47 vs. 42.6±1.6, *P*<0.05). Moreover, microinjection of bicuculline into the CeA before IR increased the myocardial infarct size when compared to the IR group (56.25±4.4 vs. 42.6±1.6, *P*<0.05). Intra-CeA injection of bicuculline before ASD did not alter the infarct-sparing effect of ASD as compared to ASD+IR group (37±1.47 vs 31.4±3.47); however, it significantly reduced the infarct size when compared with the BIC+IR group (37±1.47 vs 56.25±4.4, *P*<0.01) (Figure 2).

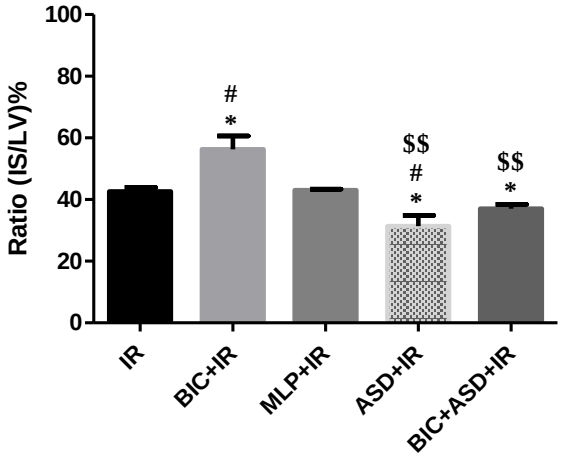


Figure 2. Myocardial infarct size expressed as a percentage of the left ventricle. Data are presented as mean± SEM (animals/group n=6). ASD: Acute sleep deprivation, BIC: bicuculline, IR: ischemia-reperfusion, MLP: Multiple large platforms. *P<0.05 vs IR, #P<0.05 vs MLP, $$P<0.01 vs BIC

Table 2. Hemodynamic parameters at the end of reperfusion

Group	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	Heart rate (beat/min)	RPP (beat.mmHg/min)
IR	125±2.5	94±2.6	109±2	265.4±3.4	25591±760
BIC+IR	147.5±6*	119±3.7*	133±5$^{**,\#\#}$	341±8.5$^{***,\#\#\#}$	25436±1257
MLP+IR	128±2	97±3.5	112±3	252.6±4	22236±1076
ASD+IR	102.5±6$^{*,\#\#,\$\$\$}$	78±3$^{*,\#,\$\$\$}$	90±2$^{**,\#\#,\$\$\$}$	211±6.7$^{***,\#\#,\$\$\$}$	25289±696
BIC+ASD+IR	115±5$^{\$}$	96±2.5$^{\$\$}$	105±3$^{\$\$\$,\&}$	266±6.6$^{\$\$\$,\&}$	23882 ±1375

ASD: Acute sleep deprivation, BIC: bicuculline, DBP: diastolic blood pressure (mmHg), IR: ischemia-reperfusion, MAP: mean arterial pressure, MLP: Multiple large platforms, SBP: systolic blood pressure (mmHg), RPP: rate pressure product. $^{*}P$<0.05, $^{**}P$<0.01 and $^{***}P$<0.001 vs IR, $^{\#}P$<0.05, $^{\#\#}P$<0.01 and $^{\#\#\#}P$<0.001 vs MLP, $^{\$}P$<0.05, $^{\$\$}P$<0.01 and $^{\$\$\$}P$<0.001 vs BIC, $^{\&}P$<0.05 vs ASD

Hemodynamic parameters at the end of reperfusion

We observed significant differences in SBP ($F_{(4, 16)}$=11.94; P=0.0001), DBP ($F_{(4, 16)}$=22.47; P =0.0000), MAP ($F_{(4, 16)}$=22.63; P=0.0000), and HR ($F_{(4, 16)}$= 60.64; P=0.0000) between the groups. Pairwise comparisons showed that the BIC+IR group had significantly higher HR, SBP, DBP and MAP as compared to the IR group. Furthermore, induction of ASD in the ASD+IR group reduced these hemodynamic parameters in comparison with the IR group. Bicuculline injection prior to ASD abolished the effect of ASD on these hemodynamic parameters. No statistically significant difference in RPP was observed between the groups (Table 2).

Changes in serum level of nitrite/nitrate

As shown in Figure 3, serum levels of nitrate/-nitrate were significantly different between the groups ($F_{(4, 15)}$=1.43; P=0.0001). In comparison with the IR group, serum levels of nitrite/nitrate were reduced in the BIC+IR group (261.43±13.42 vs 142.69±14.33; P<0.05) and increased in the ASD+IR group (261.43±13.42 vs 598.68±37.14; P<0.001). Injection of bicuculline prior to ASD reduced nitrite/-nitrate levels in the BIC+ASD+IR group as compared to the ASD+IR group (292.77±40.27 vs 261.43±13.42, P<0.001). No statistically significant difference was observed between the MLP+IR and IR groups.

eNOS expression

The gene expression of eNOS was evaluated in the infarcted and non-infarcted regions of the left ventricle.

eNOS expression in the infarcted tissue

One-way ANOVA analysis showed a significant difference between the groups in terms of mRNA expression of eNOS in the infarcted region ($F_{(4, 15)}$ = 16.92; P=0.0001) (Figure 4). ASD increased eNOS gene expression in the ASD+IR group when compared to the IR group (19.09±4.9 vs 1±0, P<0.001). Injection of bicuculline in the BIC+IR group attenuated eNOS expression in comparison with the IR group (0.77±0.19 vs 1±0, P<0.05). Administration of bicuculline prior to ASD reduced eNOS expression (2.73 ± 0.70) as compared to the ASD+IR group (2.73± 0.70 vs 19.09±4.9, P<0.001). There was no significant difference between the MLP+IR and IR groups.

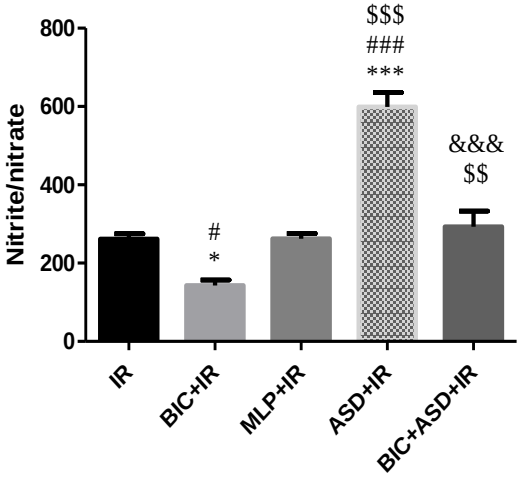


Figure 3. Serum levels of Nitrite/nitrate. All samples were analyzed in duplicate. Data are presented as mean± SEM (animals/group n=6). ASD: Acute sleep deprivation, BIC: bicuculline, IR: ischemia-reperfusion, MLP: Multiple large platforms. $^{*}P$<0.05 and $^{***}P$<0.001 vs IR, $^{\#}P$<0.05 and $^{\#\#\#}P$<0.001 vs MLP, $^{\$\$}P$<0.01 and $^{\$\$\$}P$<0.001 vs BIC, $^{\&\&\&}P$<0.001 vs ASD

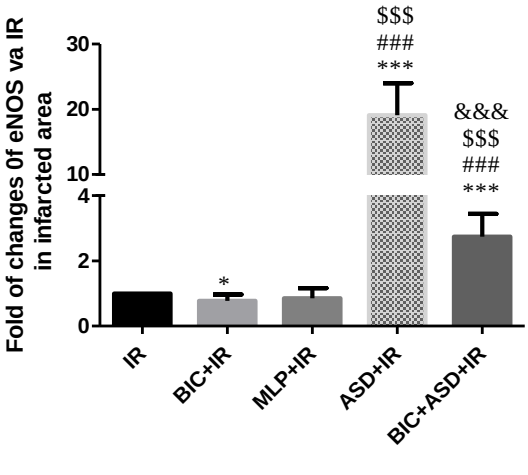


Figure. 4 Fold changes of eNOS mRNA expression vs IR in infarcted area. All samples were analyzed in duplicates. Data are presented as mean± SEM (animals/group n=5). ASD: Acute sleep deprivation, BIC: bicuculline, IR: ischemia-reperfusion, MLP: Multiple large platforms. $^{*}P$<0.05 and $^{***}P$<0.001 vs IR, $^{\#\#\#}P$<0.01 vs MLP, $^{\$\$\$}P$<0.001 vs BIC, $^{\&\&\&}P$<0.001 vs ASD

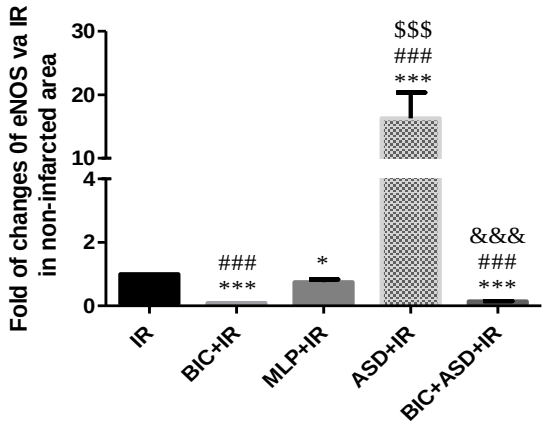


Figure 5. Fold changes of eNOS mRNA expression vs IR in non-infarcted area. All samples were analyzed in duplicates. Data are presented as mean±SEM (animals/group n=5) ASD: Acute sleep deprivation, BIC: bicuculline, IR: ischemia-reperfusion, MLP: Multiple large platforms.* $P<0.05$ and *** $P<0.001$ vs IR, ### $P<0.01$ vs MLP, $$$ $P<0.001$ vs BIC, &&& $P<0.001$ vs ASD

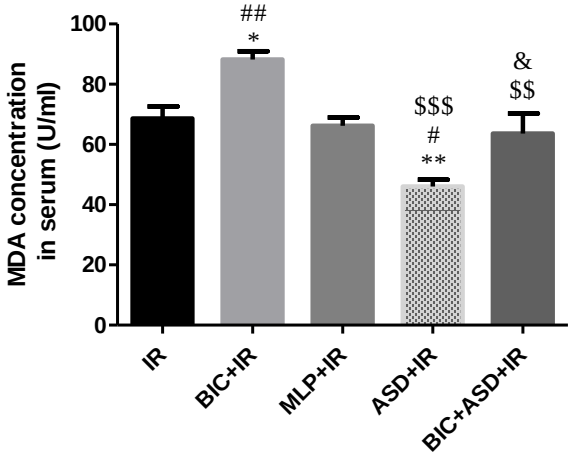


Figure 6. Serum levels of malondialdehyde (MDA). All samples were analyzed in duplicates. Data are presented as mean ± SEM (animals/group n=6). ASD: Acute sleep deprivation, BIC: bicuculline, IR: ischemia-reperfusion, MLP: Multiple large platforms. * $P<0.05$ and ** $P<0.01$ vs IR, # $P<0.05$ and ## $P<0.01$ vs MLP, $$ $P<0.01$ and $$$ $P<0.001$vs BIC, & $P<.05$ vs ASD

eNOS expression in the non-infarcted tissue

We observed a significant difference between the groups in terms of mRNA expression of eNOS in the non-infarcted region ($F_{(4, 15)}=12.49$; $P=0.0001$) (Figure 5). In comparison with IR group, bicuculline administration in the BIC+IR group reduced eNOS expression (1±0 vs 0.09±0.001, $P<0.001$), and induction of sleep deprivation in the ASD+IR group increased eNOS expression (1±0 vs 19.28±4.07, $P<0.001$). Injection of bicuculline prior to ASD reduced mRNA expression of eNOS when compared to the ASD+IR group (0.14±0.008 vs 19.28±4.07, $P<0.001$). The mRNA expression of eNOS was significantly reduced in the MLP+IR group as compared to the IR group (0.74± 0.1 vs 1±0, $P<0.05$).

Serum level of MDA

The serum MDA levels were significantly different between the groups ($F_{(4, 20)}=14.12$; $P=0.0001$). The *post hoc* test confirmed that intra-CeA injection of bicuculline increased MDA levels when compared to the IR group (88.2±2.72 vs 68.6±4.01, $P<0.05$). Furthermore, MDA levels in the ASD+IR group were reduced as compared to the IR group (46±2.3 vs 68.6±4.01, $P<0.05$). Administration of bicuculline prior to ASD led to an increase in MDA levels in comparison with the ASD+IR group (63.6±6.72 vs 46±2.3, $P<0.05$). There was no significant difference in MDA levels between the MLP+IR and IR groups (Figure 6).

Changes of corticosterone level

Serum corticosterone levels differed significantly between the groups ($F_{(4, 18)}=136.12$; $P=0.000$). In comparison with the IR group, corticosterone levels were elevated in the ASD+IR group (101.84±3.41 vs 205.57±1.96, $P<0.001$), BIC+IR group (101.84±3.41 vs 245.93±2.27, $P<0.001$), BIC+ASD+IR group (101.84 ±3.41 vs 212.13±2.19, $P<0.001$) and MLP+IR group (101.84± 3.41 vs 180±10, $P<0.001$). Induction of ASD in the ASD+IR group markedly reduced corticosterone levels when compared to the BIC+IR group (205.57± 1.96 vs 245.93±2.27, $P<0.001$) (Figure 7).

Neural degeneration

There was a statistically significant difference in the number of Nissl stained neurons between the groups ($F_{(2, 27)} = 17.82$; $P<0.001$) (Figure. 8. a, b). Pair-wise comparisons showed that neural degeneration was increased in the bicuculline administered group as compared to the IR group (19.66 ± 3.7 vs 1.33 ± 0.33, $P<0.001$). The number of degenerated neurons in the ASD+IR and BIC+ASD+IR groups were reduced in comparison with the BIC+IR group (2 ± 0.57 vs 19.66 ± 3.7, $P<0.001$ and 3.66 ± 1.2 vs 19.66 ± 3.7, $P<0.01$, respectively).

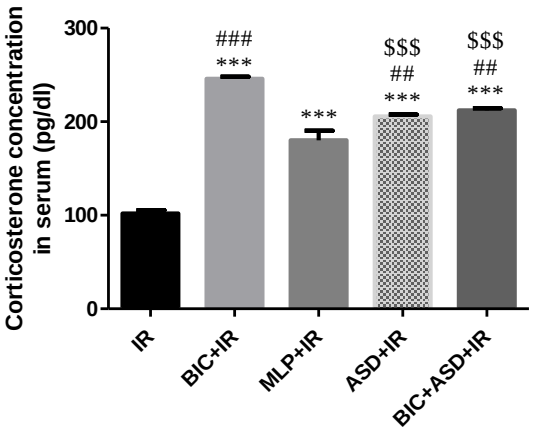
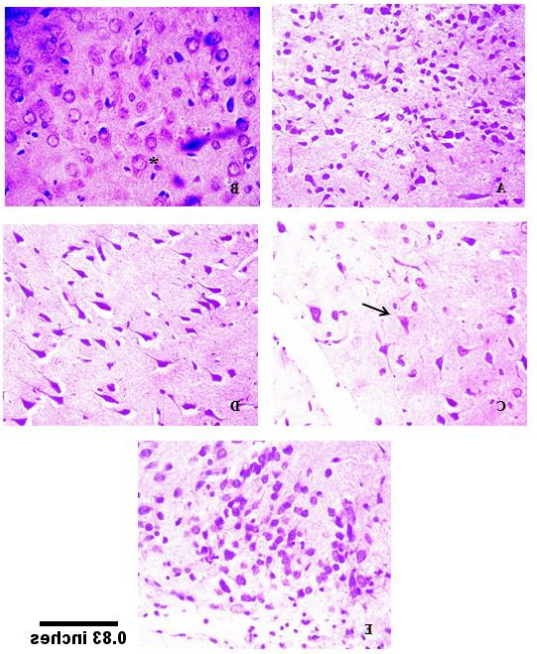


Figure 7. Serum levels of corticosterone. All samples were analyzed in duplicates. Data are presented as mean± SEM (animals/group n=6). ASD: Acute sleep deprivation, BIC: bicuculline, IR: ischemia-reperfusion, MLP: Multiple large platforms. *** $P<0.001$ vs IR, ## $P<0.01$ and ### $P<0.001$ vs MLP, $$$ $P<0.001$ vs BIC

8a)



8b)

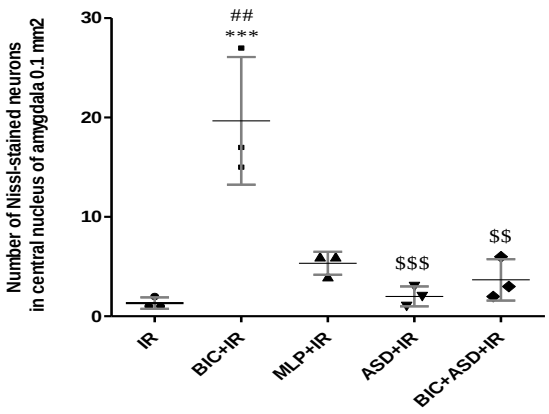


Figure 8. a: Nissl–stained neurons in the amygdala. Data are presented as mean± SEM (animals/group n=3). A) IR, B) Bicuculline+IR, C) MLP+IR, D) ASD, E) Bicuculline+ASD+IR. Arrow: natural neuron
Star: Degenerated neuron
b: Number of Nissl–stained neurons in the amygdala. ASD: Acute sleep deprivation, BIC: bicuculline, IR: ischemia-reperfusion, MLP: Multiple large platform. $^{***}P<0.001$ vs IR, $^{\#\#}P<0.01$ vs MLP, $^{\$\$}P<0.01$ and $^{\$\$\$}P<0.001$ vs BIC

Discussion

Sleep is a restorative process that plays an important role in the balance of psychological and physical health. It is associated with various physiological processes like growth, tissue regeneration, wound healing and memory consolidation (34). There are numerous studies suggesting how the lack of sleep can result in dramatic health consequences. Extended hours of wakefulness can increase motor activity and elevate stress (12). In this regard, the current study demonstrates the beneficial effect of ASD against IRI and elucidates the role of GABA-A receptors in CeA in mediating ASD-induced cardioprotection. In the present study, intra-CeA injection of bicuculline prior to induction of IR aggravated the myocardial IRI. Based on these results, it can be speculated that the endogenous activation of GABA-A receptors in CeA during IR prevents myocardial injury. Interference with GABA neurotransmission evokes various cardiovascular and sympathetic responses (35). Da Silva *et al.* demonstrated that blockade of GABAergic inputs in the hypothalamic paraventricular nucleus (PVN) increases sympathetic outflow, whereas activation of GABA- A receptors in the PVN reduces sympathetic activity and blood pressure (36). Muscimol, a GABA-A agonist, has been shown to induce anti-apoptotic and anti-inflammatory effects in amygdala and brain stem in hypertensive rats (7). Furthermore, reduced GABA availability during chronic heart failure contributes to exaggerated sympathoexcitation in both, human patients and animal models (37). Vaz *et al.* demonstrated that microinjection of Muscimol in the lateral ventricle reduces MAP, HR and sympathetic activity in spontaneously hypertensive rats (9). Conversely, intracerebroventricular injection of bicuculline has been shown to increase BP, HR and sympathetic outflow (38). In the current study, we observed that ASD reduces HR and bicuculline administration lowered this ASD-induced reduction in HR. This may suggest that the reduction in HR observed in the ASD group can be at least partly attributed to the activation of GABA-A receptors. Moreover, it may further indicate the favorable effect of ASD as decreased HR can contribute to the reduction in myocardial oxygen consumption and improvement in oxygen supply to oxygen demand ratio Moreover, ASD induced decrease in heart rate may indicate reduced myocardial oxygen demand following ASD and therefore, improved in oxygen supply to oxygen demand ratio (39).

GABA is a well-known inhibitory neurotransmitter that has been shown to mediate the sympatho-inhibitory effects of NO (40, 41). Moreover, numerous studies have reported the beneficial effect of eNOS-derived NO against myocardial IRI (42). On the other hand, IRI to myocardium is considered to be a consequence of the imbalance between the formation of ROS and endogenous availability of antioxidants (43). Oxidative stress induces intercellular Ca^{+2} overload, contractile dysfunction, inflammation and cell death (44). Lipids are prone to oxidation and therefore, lipid peroxidation products like MDA are considered important biomarkers of oxidative stress (45). Chronic sleep deprivation (CSD) has been shown to exert devastating effects on cardiac health. Using biochemical and histological analyses, Periasamy *et al.* observed a CSD-induced rise in markers of

cardiac injury and MDA levels (21). Furthermore, metabolism-induced free radicals accumulate during wakefulness, and they are responsible for many of the adverse effects caused by sleep deprivation (21). For the first time, the current study indicates an ASD-induced increase in eNOS expression in the left ventricle. Interestingly, this effect was not only limited to infarcted area but was also observed in non-infarcted regions of the heart. The aim of measuring the eNOS expression in the two different areas (infarcted and non-infarcted) of the left ventricle was to determine whether or not the effects of ASD or bicuculline administration are limited to the infarcted area or they may exert the protective effects through activation of non-infarcted area. Our findings showed that these manipulations (ASD or bicuculline administration) affected both ventricular areas, thereby conferring cardioprotection. Furthermore, we observed a significant increase of NO metabolites in the ASD+IR group when compared to the IR group. Moreover, ASD lowered MDA levels in comparison with the IR group. Blockade of central GABA-A receptors markedly attenuated the ASD-induced rise in eNOS expression and serum NO metabolites, and increased MDA levels. These novel findings suggest that the ASD-induced rise in GABA confers cardioprotection by potentially targeting NO and oxidative stress. Interestingly, in comparison with the IR group, bicuculline administration before IR reduced eNOS expression and NO metabolites, while increasing MDA levels in serum. These results indicate the protective role of central endogenous GABA against myocardial injury (30).

Although using aquarium for induction of sleep deprivation is common in some studies, elevated levels of corticosterone in the MLP+IR group (with normal sleep) is suggestive of protocol-induced stress. Moreover, exposure to novel environment may have also induced stress in ASD animals (46). In addition, the fact that ASD itself appears to be a stressor, cannot be neglected (47).-It is believed that stress-induced rise in corticosterone enhances ones' ability to cope with stressors. Previous findings from our laboratory have demonstrated that acute stress improves cardiac hemodynamics, and reduces infarct size, incidence of VT ventricular tachycardia (VT), and markers of myocardial injury during IR. In addition, our behavioral study using elevated plus maze demonstrated that blocking GABA-A receptors is associated with increased anxiety behavior (48). Our results are in line with Keim *et al.* who demonstrated that blockade of GABA-A receptors in the dorsomedial hypothalamic nucleus (DMH), a region known to elicit cardiovascular and anxiety responses, results in augmented corticosterone secretion in addition to the increase in HR and BP (49). It seems that the neural connections between infralimbic cortex, CeA, PVN and NTS, and the autonomic effectors can modulate the sympatho-adrenimedullary system and promote glucocorticoid release (50). This pattern of findings suggests that ASD may confer cardioprotection against myocardial IRI via development of acute stress. Furthermore, elevated corticosteroids observed during chronic stress are implicated in neurodegenerative processes. We observed significantly increased neurodegene-ration in the bicuculline group as compared to the ASD groups, which may suggest the protective role of GABA-A receptors in mediating ASD-induced neuroprotection as a potential role for GABA-A receptors against ASD (potential stressor)-induced neurodegeneration (31).

There is growing evidence that neuroactive steroids modulate neuronal function and play an important role in behavioral processes. Altered neurosteroid levels can modify the sensitivity of GABA-A receptors and modulate the synthesis of neurosteroids. In this regard, it has been demonstrated that 3α, 5α-tetrahydrodeoxycorticosterone attenuates the stress-induced elevation of adrenocorticotropic hormone and corticosterone in rats (3).

Conclusion

ASD reduces myocardial IRI at least partly by targeting oxidative stress and NO. Since injection of bicuculline prior to IR increased cardiac injury and intra-CeA bicuculline administration attenuated the cardioprotective effects of ASD, it can be suggested that GABA-A receptors mediate ASD-induced cardioprotection against IR.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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