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RESEARCH ARTICLE

EFFECT OF STRESS - INDUCED MALONDIALDEHYDE LEVEL IN DIFFERENT BRAIN TISSUES IN SELECTIVE SUBCORTICAL LESIONED WISTAR RATS

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Abstract

Brain is the target for different stressors because of its high sensitivity to stress induced degenerative conditions. A number of pathological conditions of the brain have been implicated due to free radical formation. Subcortcial structures are known to influence stress responses. Among them, the amygdala nucleus and the paraventricular nucleus (PVN) of the hypothalamus has been linked in the regulation of stress responses. The present study was aimed to elucidate the comparative analysis of amygdala and paraventricular nucleus in regulating the acute and chronic stress-induced malondialdehyde level in different brain tissues. Wistar albino rats were divided into amygdala lesioned and PVN lesioned groups. Each group was further subdivided into lesioned control group and lesioned stress group. The lesioned stress group animals were subjected to acute and chronic types of swimming and immobilization stress with bilateral lesions of nucleus of amygdala and PVN. Each subgroup contained ten animals. MDA levels of cerebral cortex , hypothalamus and cerebellum were estimated. Exposure to acute and chronic swimming stress in amygdala lesioned groups showed a significant ($p < 0.05$; $p < 0.001$) increase in the hypothalamus lipid peroxidation level when compared to the PVN lesioned swimming stress groups. Exposure to acute stressors significantly increased ($p < 0.001$) the cerebellar lipid peroxidation level in the amygdala lesioned groups when compared to the PVN lesioned groups. Based on the present study, it appears that the amygdala nucleus has a prominent role in decreasing the stress induced free radical formation in brain tissues. It is our attempt to put forth the evidence for greater involvement of the amygdala in the prevention of stress- induced free radicals formation

Introduction

Oxidative stress induced by excessive formation of free radicals is considered to be crucial in cell injury in a variety of diseases. Brain is considered abnormally sensitive to oxidative damage. Free radicals are produced in the human body both in physiological and pathological conditions (Hemnani T and Parihar MS, 1998). In the central nervous system, the membrane lipids of neurons have a high content of polyunsaturated fatty acids which is the main substrate for lipid peroxidation (Abuja PM and Albertoni R, 2001). The products of lipid peroxidation are themselves reactive species and lead to extensive membrane, organellar and cellular damage (Cotran RS et al., 1999). Elevated levels of

malondialdehyde (MDA), one of the by-products of lipid peroxidation has been reported in the cardiovascular, neurological and in other diseases (Mehrotra A et al., 1996).

Forebrain stress systems have come under considerable scrutiny in recent years. Several forebrain structures are known to influence stress responses (Herman JP et al., 1996). Among them, the amygdala has attracted continued interest because of its central role in processing emotional information (Joseph E and Le Doux, 2000). Numerous lesion studies have shown the importance of the amygdala and its involvement in autonomic responses to stress (Cardinal RN et al., 2002; Williams LM et al., 2007). The paraventricular nucleus (PVN) of the hypothalamus is another important area which plays a key role in the regulation of the HPA activation and it is considered as a focal point in the complex interacting systems regulating stress responses (Pacak K , 2000). Extensive research has provided evidence for a crucial role of the paraventricular nucleus as an integrator of endocrine and autonomic functions (Benarroch EE, 2005).

There is a lack of information available in literature on the precise influence of subcortical structures on acute and chronic stress induced formation of free radicals in different brain tissues . There are not enough evidences to show the effects of damage to the subcortical brain regions on stress and lipid peroxidation. Therefore, the present study was designed to address the issue of the role of subcortical structures on the stress induced formation of free radicals. Lipid peroxidation was estimated in different brain tissues following electrolytic lesions in two separate subcortical areas i.e. amygdala nucleus and the paraventricular nucleus of hypothalamus. The present study was an attempt to elucidate the regulatory role of the amygdala and paraventricular nuclei on acute and chronic stress- induced formation of free radicals.

Materials and methods

All procedures in this study were performed in accordance with the guidelines established by the Institutional Animal Ethics Committee and of the Society for Neuroscience Policy on the Use of Animals in Research. Adult albino rats (150 to 250 g) of Wistar strain were used in the present study. The rats were procured from the central animal breeding center at our university. Animals were housed individually in polypropylene cages (29cms x 22cms x 14cms) during the experimental period at 28±2° C temperature and 50±5% humidity. The rats were maintained under standard laboratory conditions with 12h light: 12h dark cycle. Animals were fed on laboratory chow (Gold Mohur; Lipton India, Ltd) and tap water in drinking bottles were made available *ad libitum.* After 1 week acclimation to vivarium conditions, during which time they were handled extensively, rats were subjected to bilateral and sham bilateral lesions of the amygdala and PVN and then allowed to recover for 1 week before undergoing the stress procedures. Rats were anesthetized (Pentobarbitone sodium, 40 mg/kg, intraperitonially) and then sacrificed by giving the lethal dose of Pentobarbitone sodium. The amygdala and the PVN lesion were performed according to the stereotaxic coordinates prescribed in the Paxinos and Watson rat stereotaxic atlas (Paxinos G and Watson C, 1986) . The coordinates of amygdala were as follows: anteroposterior (AP) = -2.8 mm posterior to bregma, lateral (L) = 4.8 mm from midline, vertical (V) = 7.8 mm from the surface of the skull. The PVN lesion coordinates were as follows: anteroposterior (AP) = -1.3 mm posterior to bregma, lateral (L) = 0.3 mm from midline, vertical (V) = 8.0 mm from the surface of the skull. The lesion was produced using stainless steel electrode (gauge 22) which was insulated except for 0.5 mm at the tip. An anodal DC (direct) current of 2 mA was passed for 20 sec to produce the lesion. The cathode was connected to the tail. The procedure was repeated on either side to produce bilateral lesions. The animals were divided into two major groups as lesioned control group and lesioned stress group. The lesioned stress groups were again subdivided into experimental acute and chronic stress groups. Each subgroup had ten animals.

Lesioned control groups:

Amygdala lesioned control (AL-C)

This subgroup of amygdala lesioned rats was not subjected to experimental stress.

PVN lesioned control (PVNL-C)

This subgroup of PVN lesioned rats was not subjected to experimental stress.

Lesioned stress groups:

Amygdala lesioned acute swimming stress (AL-ASS)

This subgroup of rats was lesioned in amygdala nucleus and subjected to one day acute swimming stress until the animals were exhausted.

PVN lesioned acute swimming stress (PVNL-ASS)

This subgroup of rats was lesioned in the PVN and subjected to one day acute swimming stress until the animals were exhausted.

Amygdala lesioned acute immobilization stress (AL-AIS)

This subgroup of rats was lesioned at amygdala nucleus and was subjected to acute immobilization stress for one hour.

PVN lesioned acute immobilization stress (PVNL-AIS)

This subgroup of rats was lesioned at paraventricular nucleus and was subjected to acute immobilization stress for one hour.

Amygdala lesioned chronic swimming stress (AL-CSS)
This subgroup of rats was lesioned at amygdala nucleus and subjected to chronic swimming stress for seven days.

PVN lesioned chronic swimming stress (PVNL-CSS)
This subgroup of rats was lesioned at PVN nucleus and subjected to chronic swimming stress for seven days.

Amygdala lesioned chronic immobilization stress (AL-CIS)
This subgroup of rats were lesioned at amygdala nucleus were subjected to chronic immobilization stress one hour per day for seven days.

PVN lesioned chronic immobilization stress (PVNL-CIS)
This subgroup of rats was lesioned at PVN nucleus was subjected to chronic immobilization stress one hour per day for seven days.

STRESS PROCEDURE (Nayanatara AK et al., 2011):

a) ACUTE STRESS

Acute immobilization stress:
The immobilization chambers used in this study were plastic tubes of varying sizes to accommodate all sizes of rats (15cms long and 4cms diameter, 16cms long and 5cms diameter, 17cms long with 6cms diameter). The tubes had a conical head at one end. The conical head area contained numerous perforations which served as breathing holes. The rat was placed inside the tube with head in the conical end. The rats were totally restrained by packing the rear end of the tube and closing it firmly with a stopper. This immobilization procedure minimized the space around the rat and prevented it from turning and moving and thus provided a rather strong stressful condition without causing any injury to the animal. Rats were immobilized for a period of one hour daily.

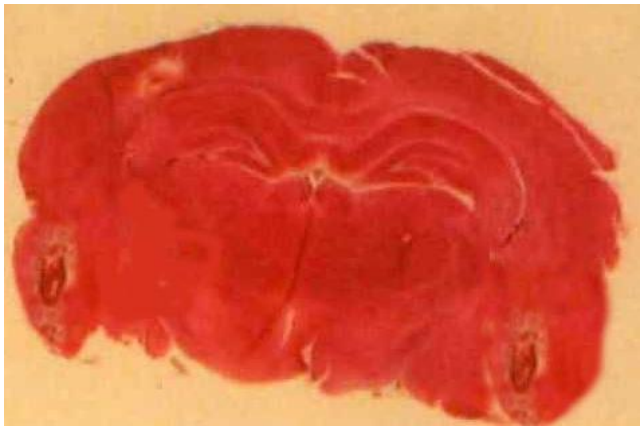
Acute swimming stress: The rats were allowed to swim in the plastic tubs containing tap water maintained at room temperature. The water level in the plastic tub was always kept at 30cms from the bottom. Rats were forced to swim in this tub until exhaustion. The point at which the animals became unable to stay at surface and showed signs of sinking was considered to be the point of exhaustion. All the experiments were done between 10AM to 12 Noon to minimize circadian variability. After the stress session, the rats were towel dried and then placed back in their respective cages where water and food were available *ad libitum.*

b) CHRONIC STRESS

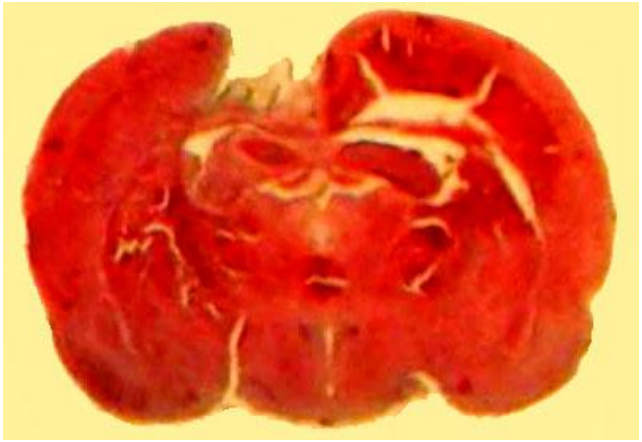
Chronic immobilization stress: The animals were exposed to chronic stress in the form of immobilization for 1 hour per day for a period of 7 days.

Chronic swimming stress: Animals were subjected to forced swimming daily for 7 days.

(Fig 1). AMYGDALA LESION



(Fig 2). PVN LESION



DISSECTION OF DISTINCT BRAIN REGIONS

All the sample collections were done between 8AM – 9AM in order to avoid circadian rhythm induced variation. After sacrificing the rats, the brain was removed quickly. To expose the brain the tip of the curved scissors was inserted into the foramen magnum and a single lateral cut was made into the skull extending forward on the left and right side. With the help of a bone cutter, the dorsal portion of the cranium was peeled off and by means of blunt forceps brain was placed on an ice-cold glass plate

leaving behind the olfactory bulbs. Further dissections were made on an ice-cold glass plate as per the methods followed by (Culling CFA et al., 1985). The dissected portions of the brain were weighed immediately.

STUDY OF BIOCHEMICAL PARAMETERS

Preparation of the tissue homogenate:

Brain tissues were washed with cold saline and dried. Each of these tissues was separately transferred to a glass homogenizer containing 10ml of 10mM cold phosphate buffer saline (PBS - pH 7.4). The tissues were homogenized using an electrical homogenizer (Remi 8000 RPM). The unbroken cells and cell debris were removed by centrifugation at 3000 RPM for 10 minutes by using Remi C 24 refrigerated centrifuge (-4°C). The obtained supernatant was used for estimation of lipid peroxidation:

Estimation of Lipid Peroxidation:

Lipid peroxidation was estimated according to the method of Kartha and Krishnamurthy (Kartha R and Krishnamurthy S, 1978). This assay is based upon the reaction of TBA with malondialdehyde (MDA) which is one of the aldehyde products of lipid peroxidation. Five ml of the homogenate (freshly prepared) was incubated in 50 ml conical flask at 38-39°C for 30 minutes along with the blank in a water bath. After incubation, 1ml of aliquot was added to the tube containing 1.5ml of 20% cold trichloroacetic acid (TCA) and then centrifuged for 10 minutes. After centrifugation, 2ml of supernatant fluid was taken in a test tube and 2ml of 0.7% thiobarbituric acid (TBA) was added to it and kept in the boiling water bath for 10 minutes. The development of pink color was measured at 535nm by using Spectronic D-20 Spectrophotometer. TBA reactive material was expressed in terms of nanomoles of malondialdehyde (MDA)/gram wet tissue, taking molar extinction coefficient of malondialdehyde (MDA) as 1.56 X 105.

STATISTICAL ANALYSIS

The datas were summarized using mean ± SEM or median and interquartile range depending on the skewness. For normally distributed data one way ANOVA was used and Kruskal Wallis test was used for skewed data. This was followed by multiple comparison tests for significant F value in ANOVA. The data of pre and post lesion group was analyzed using two way ANOVA followed by post hoc tests in case of significant F value of ANOVA. $p<0.05$ were considered as statistically significant.

TABLE 1. Comparison between the amygdala and the PVN lesioned groups on MDA level of the cerebral cortex, hypothalamus, cerebellum following swimming stress and immobilization stress.

GROUPS	CEREBRAL CORTEX	HYPOTHALAMUS	CEREBELLUM
AL-C (n=10)	16.040±1.262[NS]	106.927±0.521 [NS]	110.386±4.964 [NS]
PVNL-C (n=10)	15.274±0.460	104.400±5.130	130.500±3.850
AL-ASS (n=10)	28.169±1.365	204.877±6.400 [‡]	205.035±3.061[‡‡]
PVNL-ASS (n=10)	44.187±0.76	188.978±4.167	104.924±1.644
AL-CSS (n=10)	41.635± 3.945	276.217±9.459[¶¶]	124.428±2.336
PVNL-CSS (n=10)	19.031±0.837	119.76±5.417	126.431±1.277
AL-AIS (n=10)	87.017±1.830	110.581± 2.575	227.417±3.304[§§]

PVN-AIS (n=10)	53.921±1.4094	109.51± 0.664	142.76±0.555
AL-CIS (n=10)	36.989±0.368	149.285 ± 2.258	184.770±5.144
PVN-CIS (n=10)	38.171±3.029	100.864 ± 3.024	150.251±7.116

n= number of rats
Values are expressed as mean±SEM; nanomoles of MDA/gram wet tissue.
AL-C versus PVN-LC – Non significant (NS)
‡ p < 0.05; AL-ASS versus PVNL-ASS
¶¶ p < 0.001; AL-CSS versus PVNL-CSS
§§ p < 0.001; AL-AIS versus PVNL-AIS

RESULTS:

It is evident from the table 1 that the MDA level of the cortex, hypothalamus and cerebellum did not show any significant changes in between the AL-C and PVNL-C groups. The MDA level of the cerebral cortex did not show significant changes between the amygdala lesioned groups (AL-ASS, AL-CSS, AL-AIS, AL-CIS) and PVN lesioned groups (PVNL-ASS, PVNL-CSS, PVNL-AIS, PVNL-CIS). Further, swimming stress in the amygdala lesioned groups (AL-ASS, $P < 0.05$; AL-CSS; $P < 0.001$) showed a significant increase in the hypothalamus lipid peroxidation level when compared to the PVN lesioned swimming stress groups. Exposure to acute stress significantly increased ($P < 0.001$) the cerebellar lipid peroxidation level in the amygdala lesioned groups (AL-ASS, AL-AIS) when compared to the PVN lesioned groups (PVNL-ASS, PVNL-AIS).

DISCUSSION:

The central nervous system has anatomic and metabolic features that make it sensitive to oxidative stress (Gupta YK et al., 2003). The brain, spinal cord and peripheral nerves are rich in both unsaturated fatty acids and iron (Skaper S D , 1999). . The high lipid content of the nervous tissue coupled with its high aerobic metabolic activity make it particularly susceptible to oxidative damage (Skaper S D , 1999). Some selective areas of the brain have high iron content (Gilgun Sherki Y , 2002). A number of pathological conditions of the brain have been implicated due to free radical formation (Subir Kumar Das 2007). Lipid peroxidation was estimated in different brain tissues in this study , in order to evaluate their relative role in stress-induced regulatory mechanisms.

In the present study, the MDA level of the tissues was estimated by TBARS (thiobarbituric acid reactive species) assay to evaluate lipid peroxidation level in, cerebellum, cerebral cortex and hypothalamus following exposure to two different models of acute and chronic stress. This assay is the easiest method used to study the effects of different treatments on the lipid peroxidation and can be applied to crude biological extracts (Torres RL, 2004). Although its specificity has been questioned (Janero DR , 1990), this particular assay is widely used for in vivo and in vitro measurements and is accepted as an empirical window for the examination of the complex process of lipid peroxidation (Janero DR , 1990) .

The extensive neural connections between the amygdala and the hypothalamus are already well documented (Storozhuk VM , 2005). The centromedial amygdala projects to stria terminalis primarily to the hypothalamus where it can influence behavior and emotion (Michael Davis , 1997). In the present study, exposure to swimming stress increased the hypothalamus MDA level in the amygdala lesioned rats. This finding suggests the greater involvement of the amygdala nucleus in regulating the hypothalamus free radical formation during the prolonged physical stress. Further, acute immobilization stress also increased the hypothalamus MDA level in the amygdala lesioned rats suggesting the additional role played by the amygdala nucleus in minimizing the hypothalamus free radical formation during sudden exposure to psychological stress.

The Cerebellum is an essential modulator and co-coordinator for integrating motor, visceral and behavioral responses (Zhu JN , 2006). A Recent study showed the involvement of the cerebellum in emotions (Zhu JN , 2006) . In the present study, acute stress mediated increase in the MDA level in the

cerebellum was greater in the amygdala lesioned animals when compared to the PVN lesioned animals. These observations suggest the dominant role played by the amygdala in regulating the cerebellar lipid peroxidation level during the acute stress exposure.

Based on the present study, it appears that the amygdala nucleus has a prominent role in stress induced free radical formation in selective brain tissues. There might be the presence of an intricate web of reciprocal independent connections of amygdala nucleus and the paraventricular nucleus of the hypothalamus to the brain areas regulating the free radical formation responses designed for the psychological and physical stress. However, the exact role of these nuclei and their interactions among themselves and other brain areas in regulating stress- induced formation of free radicals needs further in- depth study.

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