Brain Region Specific Monoamine and Oxidative Changes During Restraint Stress

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ABSTRACT: Background and Purpose: Stress-induced central effects are regulated by brain neurotransmitters, glucocorticoids and oxidative processes. Therefore, we aimed to evaluate the simultaneous alterations in the monoamine and antioxidant systems in selected brain regions (frontal cortex, striatum and hippocampus) at 1 hour (h) and 24h following the exposure of restraint stress (RS), to understand their initial response and possible crosstalk. Methods and Results: RS (150 min immobilization) significantly increased the dopamine levels in the frontal cortex and decreased them in the striatum and hippocampus, with selective increase of dopamine metabolites both in the 1h and 24h RS groups compared to control values. The serotonin and its metabolite levels were significantly increased in both time intervals, while noradrenaline levels were decreased in the frontal cortex and striatum only. The activities of superoxide dismutase, glutathione peroxidase and the levels of lipid peroxidation were significantly increased with significant decrease of glutathione levels in the frontal cortex and striatum both in the 1h and 24h RS groups. There was no significant change in the catalase activity in any group. In the hippocampus, the glutathione levels were significantly decreased only in the 1h RS group. Conclusions: Our study implies that the frontal cortex and striatum are more sensitive to oxidative burden which could be related to the parallel monoamine perturbations. This provides a rational look into the simultaneous compensatory central mechanisms operating during acute stress responses which are particular to precise brain regions and may have long lasting effects on various neuropathological alterations.

RÉSUMÉ: Changements au niveau des monoamines et changements oxydatifs spécifiques à certaines régions du cerveau pendant le stress de contention. Contexte et objectif : Les effets centraux induits par le stress sont régulés par les neurotransmetteurs cérébraux, les glucocorticoides et les processus oxydatifs. Notre but était d’évaluer les changements simultanés dans les systèmes des monoamines et des antioxydants dans des régions particulières du cerveau (cortex frontal, striatum et hippocampe) 1 heure (h) et 24 h après une exposition à un stress de contention (SC) afin de comprendre la réponse initiale et l’intermodulation s’il y a lieu. Méthode et résultats : Le SC (immobilisation de 150 minutes) augmentait les niveaux de dopamine de façon significative dans le cortex frontal et le striatum et les diminuait dans le striatum et l’hippocampe, et il augmentait de façon sélective certains métabolites de la dopamine tant après 1 h qu’après 24 h par rapport aux valeurs témoins. Les niveaux de sérotonine et de ses métabolites étaient augmentés de façon importante après 1 h et 24 h et les niveaux de noradrénaline étaient diminués dans le cortex frontal et le striatum seulement. L’activité de la superoxyde dismutase, de la glutathion peroxydase et les niveaux de peroxydation lipidique étaient augmentés de façon importante, tandis que les niveaux de glutathion étaient diminués de façon importante dans le cortex frontal et le striatum après 1 h et après 24 h. Aucun changement significatif de l’activité de la catalase n’a été observé. Dans l’hippocampe, les niveaux de glutathion étaient diminués de façon significative seulement après 1 h. Conclusions : Selon ces observations, le cortex frontal et le striatum seraient plus sensibles au fardeau oxydatif ce qui pourrait être relié aux perturbations parallèles des monoamines. Notre étude donne un aperçu rationnel des mécanismes compensatoires centraux simultanés, qui existent pendant les réponses au stress aigu, et qui sont particuliers à des régions précises du cerveau. Ils pourraient avoir des effets à long terme sur différents changements neuropathologiques.

simulate human-related clinical conditions. However, examining the effect of single stress exposure on the central nervous system at different time intervals is also crucial. This may help in determining which of the central systems (monoamines or oxidative processes) is more sensitive to stress; which brain regions show pronounced changes; and how long these changes exist.

The brain is more vulnerable to oxidative damage compared to other organs as it has a higher rate of oxygen consumption per unit mass of tissue, contains high levels of peroxidizable lipids, excitotoxic amino acids and low levels of antioxidants. Some studies indicate a link between the alterations in the central monoaminergic systems and increased oxidative load during physiologically adverse conditions. However, the extent to which oxidation products contribute to the perturbed redox state during acute stressful conditions in the brain regions with monoaminergic innervation is still unclear. Furthermore, a study in vitro indicating the pro-oxidant effect of phenol-containing neurotransmitters, animal studies that address the simultaneous changes in the brain monoamine levels and antioxidant defense system are lacking.

The frontal cortex and striatum have a high dopamine (DA) content, while the hippocampus has a high concentration of glucocorticoids (GC) receptors. These brain regions are connected with each other through different neurotransmitter systems and have been proposed to play an important modulatory role in stress responses. In the present study, we hypothesized that these brain regions could be particularly vulnerable to stress exposure, and that changes in central monoamine and antioxidant systems could be interrelated. It was also hypothesized that the source of oxidative damage during stress may vary in a region and time specific manner depending on the microenvironment of the brain region.

Therefore, the aim of the present investigation was to evaluate the response of monoamines and their metabolite concentrations with simultaneous changes in the enzymatic and non-enzymatic antioxidant defense systems and lipid peroxidation in the selected brain regions at 1 hour (h) and 24 h following the exposure of restraint stress (RS) in rats. In order to evaluate oxidative alterations during RS, we measured the activities of Cu-Zn superoxide dismutase (SOD), catalase (CAT), selenium dependent glutathione peroxidase (GSH-Px), extent of lipid peroxidation (Malondialdehyde concentrations) and reduced glutathione (GSH) concentrations in the selected brain regions.

METHODS

Animals

Experimental protocols were approved by the Institutional Ethical and Usage Committee of Central Drug Research Institute (CDRI), Lucknow, following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Adult male Sprague Dawley rats, weighing 180-220g procured from National Laboratory Animal Centre, CDRI, were used in the study. For both control and stressed groups three rats were housed per cage, in a room with temperature regulated at 22 ± 2°C, with a 12h/12h light/dark cycle (lights on 07:00 h, lights off 19:00 h). Standard chow pellets and water were given ad libitum, except during the period when food or water deprivation was applied.

Chemicals

Dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindole acetic acid (5-HIAA), dihydroxybenzylamine (DHBA), norepinephrine (NE), epinephrine, t-butyl hydroperoxide, reduced glutathione (GSH), glutathione reductase, thiobarbituric acid, 1,3,3 -tetroxypropane and 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used in the study were of analytical or high performance liquid chromatography (HPLC) grade and were purchased locally.

Experimental protocol

All the rats were acclimatized to laboratory conditions prior to the experiment. The rats were randomly divided into two groups i.e. a control non-stress (NS) and restraint stress (RS) group.

**NS group:** Rats were kept undisturbed in their home cages. Standard chow pellets and water were given ad libitum.

**RS groups:** In RS model, after 12 hr fasting (food deprivation) of rats, one stress session consisting of a 2.5 hr immobilization period inside the cylindrical steel tube (7cm diameter, 17.5cm long, with holes for ventilation) at room temperature was performed during the early phase (7:00 a.m. to 9:30 a.m.) of the light cycle. The RS group was further divided into two groups, based upon the time of killing the rats after the stress regimen. In one group rats were killed 1h after the stressor (1h RS group), the other group was killed 24h after the stressor (24h RS group).

Sample preparation

The rats were killed by conscious cervical dislocation followed by decapitation, and the brains were immediately removed. The discrete brain regions (frontal cortex, striatum and hippocampus) were dissected on an ice-cold glass plate, homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1000g for 5 min at 4°C, the supernatants were passed through a 0.22 µm membrane filter and were used for analysis. For the estimations of antioxidant parameters and lipid peroxidation, the dissected brain regions were homogenized in ice cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1000g for 15 min at 4°C, the supernatants thus obtained were used for the analysis.

Estimation of monoamines and their metabolites

The endogenous levels of NE, DA, 5-HT and their non-conjugated metabolites DOPAC, HVA, and 5-HIAA were determined by reverse phase HPLC with electrochemical detection. The sample (20 µL) was injected via an HPLC pump.
(Model 1525, Binary Gradient Pump, Waters, Milford, MA, U.S.A.) into a column (Spherisorb, RP C18, 5 µm particle size, 4.6 mm i.d x 250 mm at 30°C) connected to an electrochemical detector (Model 2465, Waters, Milford, MA, U.S.A.). Oxidation potential was fixed at 0.80 V using a glass carbon working electrode versus an Ag/AgCl reference electrode. The mobile phase consisted of 32 mM citric acid, 12.5 mM disodium hydrogen orthophosphate, 1.4 mM sodium octanyl sulfonate, 0.05 mM EDTA and 16% (v/v) methanol. The pH of the mobile phase was adjusted to 4.05. Separation was carried out at a flow rate of 1.2 ml/min. The neurotransmitters were quantified using Breeze version 3.2. The levels were expressed in nanograms of neurotransmitter per gram of wet weight of the brain tissue. Quantification was made by comparing peak heights of the samples to the corresponding standard curve.

**Cu-Zn, superoxide dismutase (SOD, EC 1.15.1.1) activity**

SOD activity was measured based on its ability to inhibit the autoxidation of epinephrine to adrenochrome at alkaline pH. The absorbance of reaction mixture was followed for 4 min at 480 nm in a spectrophotometer (Model 1201, Shimadzu). Enzymatic activity was expressed as U/mg protein at 30°C. The amount of enzyme that caused 50% inhibition of epinephrine autoxidation was defined as one unit (U).

**Catalase (CAT, EC 1.11.1.6) activity**

CAT activity was measured using H$_2$O$_2$ as substrate. A molar absorption of 43.6 M/cm was used to determine CAT activity. Enzymatic activity was expressed as U/mg protein at 25°C, one unit (U) of which was equal to 1 mole of H$_2$O$_2$ degraded/min/mg of protein.

**Glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity**

GSH-Px activity was measured using t-butyl hydroperoxide as substrate. The absorbance of reaction mixture was measured at 340 nm for 180 s in a spectrophotometer. A molar absorption of 6.22x10$^3$ M/cm was used to determine enzyme activity, expressed as U/mg protein at 37°C. One unit (U) of activity was equal to mM of NADPH oxidized/min/ mg protein.

**Reduced glutathione (GSH) levels**

GSH was determined by its reaction with DTNB (Ellman’s reagent) yielding a yellow chromophore, the absorbance of which was measured at 412 nm within 15 min spectrophotometrically. GSH levels were determined from a standard curve and expressed as µM/mg protein.

**Lipid peroxidation assay**

MDA (a thiobarbituric acid reactive species: TBARS) an indicator of lipid peroxidation was measured using 1,1,3,3 - tetraethoxypropane as standard. The absorbance of the reaction mixture was measured at 532 nm and the values were expressed as nM MDA /mg protein.

**Protein assay**

The Protein content of the samples was determined using Folin phenol reagent.

**Statistical analysis**

Data were evaluated by one-way analysis of variance (ANOVA) with post-hoc analysis by Tukey Kramer multiple comparison test. Significance was set at P<0.05. All data are presented as means ± standard error (S.E.) of the means. Comparisons were done between the stress groups (RS; 1h or 24h after the stressor) and NS control group, and also between the 1h and 24h RS groups.

**RESULTS**

Effect of RS on the levels of monoamine and their metabolites in the selected brain regions:

**Frontal cortex**

As shown in Figure 1A and 1B, there was a significant increase in the cortical DA [P<0.05-0.01], HVA [P<0.01-0.001] and 5-HT [P<0.01] levels in both the 1h and 24h RS groups as compared to the NS control group. However, the levels of NE [P<0.01] and 5-HIAA [P<0.05] were significantly altered only in the 1h RS groups as compared to the NS control group. The post
hoc tests (Tukey Kramer) revealed no significant differences [P>0.05] in the levels of NE and 5-HIAA between the 1h and 24h RS groups. Further, the post hoc analysis showed a significant decrease in the levels of DA [P<0.05], HVA [P<0.01] and 5-HT [P<0.05] in the 24h RS group when compared with 1h RS group.

**Striatum**

Figure 2A and 2B depict that there was a significant decrease in the levels of striatal DA [P<0.05-0.001], with significantly increased DOPAC [P<0.05], HVA [P<0.05-0.001], 5-HT [P<0.05-0.01] and 5-HIAA [P<0.05] levels in both the 1h and 24h RS groups when compared with the NS control group. Post hoc tests (Tukey Kramer) revealed a significant increase of the DA levels in the 24h RS group when compared to the 1h RS group. However, the levels of DOPAC, HVA, 5-HT and 5-HIAA were significantly [P<0.05] decreased in the 24h RS group when compared with the 1h RS group. The levels of NE altered insignificantly [P>0.05] in all the groups.

**Hippocampus**

As shown in Figure 3A and 3B, there was a significant decrease in the DA [P<0.01-0.001] and NE [P<0.01-0.001] levels in both the 1h and 24h RS groups compared with the NS control group. The 5-HT and 5-HIAA levels were significantly [P<0.05] increased in the 1h RS group only. Post hoc tests (Tukey Kramer) revealed significant differences in the NE [P<0.05] and 5-HT [P<0.05] levels when compared between the 1h and 24h RS groups.
Effect of RS on the antioxidant enzyme activities in selected brain regions:

Alterations in the activities of SOD, CAT and GSH-Px are depicted in the Table.

SOD: SOD activity was significantly increased in both the 1h and 24h RS groups in the frontal cortex [P<0.05-0.01], striatum [P<0.05-0.01], with no significant [P>0.05] change in the hippocampus compared to the NS control group. Further, post hoc tests (Tukey Kramer) revealed no significant differences [P>0.05] in SOD activities between the 1h and 24h RS groups in any of the brain regions (Table).

CAT: There was no significant change in the CAT activity in any of the groups studied. Further, there were no significant differences [P>0.05] in CAT activities between the 1h and 24h RS groups in any of the brain regions (Table).

GSH-Px: There was a significant increase in the GSH-Px activity in the frontal cortex [P<0.05-0.01] and striatum [P<0.05-0.01] in both the 1h and 24h RS groups, with no significant differences in the hippocampus [P>0.05] compared to the NS control group. Further, post hoc tests (Tukey Kramer) revealed no significant differences [P>0.05] in GSH-Px activity between the 1h and 24h RS groups in any of the brain regions (Table).

Effect of RS on the GSH levels in the selected brain regions:

As shown in the Table, the non-enzymatic antioxidant defense marker GSH was significantly decreased in the frontal cortex [P<0.05-0.01] and striatum [P<0.05-0.001] both in the 1h and 24h RS groups, whereas in the hippocampus the significant [P<0.05] decrease was observed only in the 1h RS group compared to the NS control group. Further, the post hoc tests (Tukey Kramer) revealed no significant differences [P>0.05] in the GSH levels between the 1h and 24h RS groups in any of the brain regions.

Effect of RS on lipid peroxidation in the selected brain regions:

The Table shows that there was a significant increase in the lipid peroxidation expressed as increased MDA levels in both the 1h and 24h RS groups in the striatum [P<0.01-0.001], while in the frontal cortex [P<0.01] the increase was observed only in the 1h RS group with no significant change in the hippocampus [P>0.05] compared to the NS control group. Further, the post hoc tests (Tukey Kramer) revealed no significant differences [P>0.05] in the MDA levels between the 1h and 24h RS groups in any of the brain regions.

Discussion

The stress-induced effects are an outcome of altered activity of different mechanisms such as central neurotransmitters, neurohormonal factors, particularly those linked with the HPA-axis, and free radical generation2,3. The development of stress-induced disorders might be associated with a temporal prolongation of initial stress response in discrete brain regions. These responses are believed to be developed during the first exposure of stress and/or novel environment. Thus, in the present investigation we aimed to evaluate the initial response of monoaminergic and oxidative systems in various important brain regions in RS condition after different time intervals. We have
selected the RS model to investigate changes induced by a short acting severe stressors. It represents the reaction to an immediate threat. Among the various stress models, RS has been used extensively and accepted widely as it produces both physical and inescapable psychological stress.

The present study demonstrates that the exposure of a single immobilization stress episode caused a differential initial response in the monoaminergic and anti-oxidant systems in a brain region specific manner, and most of these changes last for at least 24 h after the stress exposure, indicating their long lasting effects. RS led to a significant decrease of NE levels in the frontal cortex and hippocampus. The NE system is known to be activated by stress; although only for a short duration, which could explain the decreased levels of NE by RS, as the stressor applied were of relatively longer duration (2.5 hr). Also, increased corticosterone level during RS can reduce the efficacy of NE to enhance firing frequency with a delay of one to two hours, through a GC-dependent mechanism.

Results also demonstrated a differential and brain region specific DA response. RS significantly increased DA levels in the frontal cortex while the levels decreased in the striatum and hippocampus both in the 1h and 24h RS groups. The increased DOPAC and HVA levels in the striatum and increased HVA level in the frontal cortex probably reflect increased synthesis and/or metabolism of DA. The increased DA metabolism may be due to either the increased intraneuronal monoamine oxidase (MAO) activity to DOPAC and/or by catechol-O-methyl transferase to HVA. Our observations concur with reports suggesting that acute stressful conditions activate monoaminergic systems leading to an increase in the extracellular levels of NE, DA and 5-HT in different regions of brain. Generally, it has been thought that exposure to acute stress led to a transient activation of monoamine systems to restore homeostasis. However, our finding of decreased DA level in the striatum and hippocampus in response to RS warrants special attention. In the striatum the decreased DA level was accompanied by subsequent increases in DOPAC and HVA levels, suggesting increased conversion of DA to its metabolite in response to RS. On the other hand in the hippocampus, the DA level was decreased with no significant changes in its metabolite levels. The hippocampus is rich in cholinergic innervations and a reciprocal relation exists between acetylcholine and DA responses in brain. The decrease of DA in the hippocampus could be attributed to decreased cholinesterase activity and excess acetylcholine turnover during acute stress conditions. These differential monoamine responses could also be due to variations in their level of distribution, synthesis and degradation.

Increased 5-HT and 5-HIAA levels during RS, in all three brain regions, both in the 1h and 24h RS group, could be attributed to an overall activation of 5-HT systems and subsequent increase in the firing rate of 5-HT neurons. The turnover and/or synthesis of 5-HT in the brain is reported to increase in response to physical and psychological stressors.

The increased 5-HT and 5-HIAA levels could also be explained on the basis that an acute stress exposure activates tryptophan hydroxylase activity in the cortex and midbrain. Stressful stimuli adversely affect different brain regions and cause changes in the normal homeostasis of the anti-oxidant defense system. However, the anti-oxidant system may respond diversely depending upon the different stress protocols used in various studies. In order to neutralize reactive oxygen species (ROS), the body uses enzymatic (SOD, CAT and GSH-Px) and non-enzymatic (GSH) antioxidant systems. In our study, RS significantly enhanced the activity of defensive anti-oxidant enzymes SOD and GSH-Px in the frontal cortex and striatum, and these changes remain altered even after 24 h of the stress exposure. Interestingly, in the hippocampus there were no significant changes in the activity of these enzymes. The CAT activity was increased in all three brain regions but did not reach statistical significance. This differential elevation of oxidative markers in the selected brain regions may arise from the differences in the antioxidant buffering capacities or differential susceptibilities to oxidative stress. The increased activity of SOD is an indicator of a relative increase in super oxide radical production and suggests that the brain’s antioxidant machinery is activated in response to excessive generation of free radicals. Enhanced SOD activity catalyzes the conversion of superoxide anions to H2O2 which in turn could stimulate the second line of defence which includes GSH-Px and CAT. These enzymes convert H2O2 into water and molecular oxygen, rationalizing the cause for the elevation of these two during RS. However, a lesser change in CAT activity as compared to GSH-Px could be attributed to the fact that in brain CAT activity has been found to be low and confined to peroxisomes. GSH-Px, therefore, appears to be primarily responsible for the destruction of excess H2O2 formed in nervous tissue. The increased GSH-Px activity also indicates the increase in cellular peroxides. Furthermore, such an increase in the activities of enzymes during acute stress exposure can be related to long term compensatory mechanisms, including activity modulation of enzymes related to ROS catabolism. Decreased GSH levels could be explained on the basis that loss of GSH may occur during an oxidative stress event due to its increased rate of utilization. The significant elevation of lipid peroxidation marker (MDA) in the frontal cortex and striatum is in agreement with previous reports. However, the unaffected lipid peroxidation in the hippocampus might reflect the difference in the microenvironment (concentration of monoamines and anti-oxidants) of various brain regions.

We found simultaneous alterations in both monoamines and oxidative processes, pointing towards the possibility of interconnection between the two systems during RS. These observations are strengthened by other reports too, showing some direct and indirect involvement of the production of free radicals in brain with catecholamine metabolism. Elevated catecholamine levels may undergo auto-oxidation, in which electrons are generated that in turn can produce ROS. DA forms ROS through its metabolism by MAO and by auto-oxidation. Molecular oxygen can react with DA forming quinones and semiquinones, which can further deplete GSH with simultaneous generation of ROS. Thus, increased monoamine metabolite concentrations and turnover could be one of the contributing factors for increased oxidative load and altered antioxidant enzyme activities during RS. These results support the hypothesis of Siraki and O’Brien that biogenic amines form pro-oxidant radicals. The increase in DA turnover and metabolism might produce H2O2 through MAO activity, which could be responsible for neuronal membrane lipid peroxidation observed in our study.
Since increased activity of antioxidant enzymes is observed only in the frontal cortex and striatum and not in the hippocampus, it could be hypothesized that monoamine-associated oxidation might be the primary source of ROS during RS conditions. Considering the fact that both these brain regions have extensive amounts of DA, its oxidation could contribute more oxidative burden in these brain regions than in the hippocampus. These oxidative changes in the frontal cortex and striatum, although decreased with time, (evidenced in the 24 h RS group), still remained significantly altered compared to the control values. Thus we cannot rule out the possibility that RS-induced alteration of monoamine metabolism in these brain regions contributes to the increased oxidative load.

Altered 5-HT transmission might also contribute to oxidative load during RS in different brain regions as other reports also highlighted the potential neurotoxic role for 5-HT metabolites arising from superoxide mediated oxidation. Thus, the perturbed central monoamine response and metabolism could amplify the oxidative load during RS, in a region specific manner. Thus, the different levels of free radicals in various brain regions during acute stress exposure may determine modulation of neurotransmitters either through the changes in neurotransmitter levels and/or by effects on receptor functioning. Thus, both the monoaminergic and oxidative systems may influence each other’s functioning.

CONCLUSIONS

The present study clearly suggested that the frontal cortex and striatum are more sensitive to oxidative burden than the hippocampus, which could be related to the parallel monoamine perturbations during RS. The pattern of RS-induced monoamine and oxidative changes after 1 h and 24 h later were similar when compared with control group. Although, in a few instances the changes in these parameters decreased significantly when compared between the 1 h and 24 h RS groups. Nevertheless, the changes induced by RS remained significantly altered even after 24 h of stress regime compared to control values, indicating that the observed changes may have long lasting effects. The present findings support the hypothesis that neurotransmitter intervention in oxidative damage should be seriously considered, however understanding its precise mechanisms warrants further studies. Our study contributes to the understanding of the neurochemical and antioxidant defense system interactions that are possibly involved in the biological basis of central stress-induced disorders such as depression, anxiety and other neurological conditions.

Our study provides a rational look into the possible interactions between the two systems and stipulates the compensatory mechanisms operating during acute stress responses which are particular to precise brain regions. Studies suggest implications for pharmacological interventions targeting both central monoamines and cellular antioxidants as a potential stress management strategy for protecting against stress-induced brain disorders.

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