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Depression, anxiety-like behavior and memory impairment are associated with increased oxidative stress and inflammation in a rat model of social stress

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Abstract

In the present study, we have examined the behavioral and biochemical effect of induction of psychological stress using a modified version of the resident-intruder model for social stress (social defeat). At the end of the social defeat protocol, body weights, food and water intake were recorded, depression and anxiety-like behaviors as well as learning-memory function was examined. Biochemical analysis including oxidative stress measurement, inflammatory markers and other molecular parameters, critical to behavioral effects were examined. We observed a significant decrease in the body weight in the socially defeated rats as compared to the controls. Furthermore, social defeat increased anxiety-like behavior and caused memory impairment in rats ($P<0.05$). Socially defeated rats made significantly more errors in long term memory tests ($P<0.05$) as compared to control rats. Furthermore, brain extracellular signal-regulated kinase-1/2 (ERK1/2), and an inflammatory marker, interleukin (IL)-6 were activated ($P<0.05$), while the protein levels of glyoxalase (GLO)-1, glutathione reductase (GSR)-1, calcium/calmodulin-dependent protein kinase type (CAMK)-IV, cAMP-response-element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) were significantly less ($P<0.05$) in the hippocampus, but not in the prefrontal cortex and amygdala of socially defeated rats, when compared to control rats. We suggest that social defeat stress alters ERK1/2, IL-6, GLO1, GSR1, CAMKIV, CREB, and BDNF levels in specific brain areas, leading to oxidative stress-induced anxiety-depression-like behaviors and as well as memory impairment in rats.

1. Introduction

Stressful life events are believed to contribute to development of human psychopathologies including anxiety and depression (Kessler, 1997; Post, 1992), as well as cognitive

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impairment (Arnsten, 2009; Arnsten and Rubia, 2012; Burri et al., 2013; Ronnlund et al., 2013; Shansky and Lipps, 2013). Although there exists large body of evidence demonstrating the negative impact of stress on emotional symptoms including depression, anxiety (Millan et al., 2012) and cognitive impairment (Alzoubi et al., 2013a; Devilbiss et al., 2012; Jonsdottir et al., 2013; Ohman et al., 2007; Ronnlund et al., 2013; Schwabe et al., 2012), however, studies investigating role of stress in comorbid prevalence of anxiety, depression and cognitive impairment in humans (Andreotti et al., 2013; Millan et al., 2012), or co-occurrence of anxiety and depression-like behaviors as well as learning-memory impairment, in animals are limited (Gomez et al., 2013; Haridas et al., 2013). Although impressive mechanistic insights have been offered by several groups, with regards to co-occurrence of anxiety-and depression-like behaviors in animal models (Mineur et al., 2013; Roth et al., 2012; Venzala et al., 2012), studies addressing the underlying biology of stress-induced co-occurrence of depression, anxiety and cognitive impairment are scarce.

In the last few years, using variety of animal models, we have focused on examining the mechanistic basis for co-occurrence of anxiety, cognitive impairment and hypertension (Chugh et al., 2012; Salim et al., 2010a; Salim et al., 2010b; Salim et al., 2011; Vollert et al., 2011), More recently, we have reported that direct pharmacological induction of oxidative stress in rats caused anxiety-like behavior and learning and memory impairment while antioxidant treatment prevented these behaviors, suggesting causal role of oxidative stress in this co-occurrence (Allam et al., 2013). While these observations are interesting, an important question has emerged-whether induction of psychological stress is associated with the pathogenesis of depression. These behavioral and biochemical consequences are similar to those produced upon pharmacological induction of oxidative stress? Therefore, in the present study, we have examined behavioral and biochemical outcome of application of chronic stress in rats using the social defeat model. Social stress in rats is known to induce long-lasting, adverse physiological, behavioral and neuronal deficits, which seem to resemble certain human psychopathologies of depression and anxiety (Bartolomucci and Leopardi, 2009). An ethologically relevant animal model of social stress used for studying the link between stress and psychopathologies is the resident-intruder paradigm (Wood et al., 2010). This model involves intimidations and aggressive encounters by a large, aggressive male rat (resident) toward a smaller male rat (intruder) (Wood et al., 2010), and is regarded as one of the most robust models of post-traumatic stress disorder (PTSD), depression, and other stress-related illnesses (Berton et al., 2006; Krishnan et al., 2007), and hence is considered to be of translational relevance. Socially defeated animals reportedly demonstrate social avoidance for weeks after the last social defeat session, and also exhibit depression-and anxiety-like behavioral abnormalities (Berton et al., 2006; Krishnan et al., 2007) as well as cognitive impairment (Yu et al., 2011). Simultaneous occurrence of these behaviors in this model has never been examined.

Therefore, in the present study, using this animal model we have studied the effect of social defeat induced stress, on depression-like behavior, anxiety-like behavior and cognitive impairment as well as the status of oxidative stress in rats. Oxidative stress has been reported to modulate several behaviors including learning and memory function (Alzoubi et al., 2012; Alzoubi et al., 2013a; Alzoubi et al., 2013b), anxiety- (Allam et al., 2013; Hovatta et al., 2005; Masood et al., 2009; Salim et al., 2010a; Salim et al., 2011), depression-

(Brocardo et al., 2012; Leonard and Maes, 2012; Pedreanez et al., 2011), mania- (Macedo et al., 2013), nociceptive- (Arcan et al., 2012) and schizophrenia- (Rao et al., 2012) like behaviors. We also measured the effect of social defeat-induced stress on oxidative stress within three critical brain areas, considered vital for depression, anxiety and cognition, namely, hippocampus, amygdala and prefrontal cortex (McEwen et al., 2012). Oxidative stress which is defined as the imbalance between production of reactive oxygen and nitrogen species (RONS) and their inefficient decomposition by the anti-oxidant system (Lau et al., 2011; Patki et al., 2009; Sies, 1997), has been implicated in the pathophysiology of depression, anxiety and other psychiatric disorders (Frey et al., 2006; Gibson et al., 2012; Kiecolt-Glaser et al., 2013; Maurer et al., 2001; Rezin et al., 2009). Actually, large consumption of oxygen, high amount of polyunsaturated fatty acids and iron content with diminished antioxidant enzymatic activity make brain a vulnerable target for oxidative stress (Evans, 1993) increasing its vulnerability to disease. Relevant to this, recently we have reported that hippocampus was most susceptible to oxidative stress-induced damage and also seemed to regulate the antioxidant pathway (Allam et al., 2013). Herein, we offer new mechanistic insights into the behavioral deficits observed in the social defeat model.

Results

1.1. General parameters

Food intake during 7-day social defeat protocol was not different between control or socially defeated rats [Control vs SS (g/rat/day): 25.4±1.5 vs 25.9±1.9, t=0.172, df=18] (Fig. 1A). However, daily water intake increased with social defeat [Control vs SS (ml/rat/day): 31.4 ± 2.4 vs 61.8± 3.2, t=6.122, df=18] rats (Fig. 1B). The socially defeated animals gained less weight during the 7-day social defeat protocol [Control vs SS (gain in body weight in g/ 7days): 18.0 ± 3.9 vs 4.5 ± 3.3, t=3.752, df=18] (Fig 1C).

1.2. Anxiety-like and depression-like behavior tests

Light-dark (LD), open-field (OF) and elevated-plus maze (EPM) are tests used to examine anxiety-like behavior in rodents (Salim et al. 2010b). Herein, we examined anxiety-like behavior in rats conducting these tests on the same set of animals. First, OF test was conducted followed by LD and EPM test, as previously published by us (Salim et al., 2010b; Vollert et al., 2011). In light-dark test, a rat is exposed to a novel environment with protected (dark) and unprotected (light) areas. More time spent in the dark during a 5-min test session is indicative of high anxiety-like behaviors. Control rats spent more time (99.3 ± 14.2 seconds, t=2.28, df=18) in the light compartment, when compared to socially defeated rats (53.5 ± 13.1 seconds) (Fig. 2A). Elevated-plus maze model is based on rat's aversion for open spaces. This aversion leads to the behavior termed as thigmotaxis, which means avoidance to open areas by restricting movements to enclosed spaces or to the edges of a confined space. Increased amount of time spent in the closed arms during a 5-min session is indicative of high anxiety-like behavior. The amount of time the control rats spent in the open arms (58.6 ± 14.7 seconds, t=2.31, df=18) was significantly higher than the socially defeated (16.8 ± 7.8 seconds) rats (Fig 2B).

Furthermore, socially defeated rats had lower total activity (Control [counts]: 4772 ± 359, SS [counts]: 3465 ± 330, t=2.7, df=18) (Fig. 3A) and ambulatory activity (Control [counts]: 4072 ± 371, SS [counts]: 2904 ± 288, t=2.51, df=18) (Fig. 3B) and covered lesser distance (Control [cm]: 3824 ± 369, SS[cm]: 2789 ± 315, t=2.38, df=18) than the control rats (Fig. 3C). Also, the percent time spent in the center bylast paragraph the control (45.5 ± 2.1, t=7.32, df=18) rats was significantly greater than the socially defeated (26.8 ± 1.8) rats. The sucrose consumption assay consists of a two-bottle choice, one consisting of drinking water and the other a 1% sucrose solution. The rats choose between consuming water versus sucrose solution. This test is used to assess motivational state in rodents, including stress-induced anhedonia, which is regarded as a sign of depression-like behavior (Yu et al., 2011). The preference for sucrose over water is considered as a measure for rodents' sensitivity to a natural reward. Social defeat exposure influenced sucrose intake, a measure of natural reward, 24 h after the last stress session. As a percent of the total amount of fluid intake the socially defeated rats consumed (22.8 ± 5.1, t=3.12, df=18) percent of sucrose as compared to the control (51.6 ± 5.3) rats, symbolizing decreased preference for sucrose as compared to control (Fig 3D).

1.3. Memory function

Twenty-four hour after the conclusion of anxiety-like behavior tests, radial arm water maze (RAWM) test was conducted. This test is used to assess memory impairment in rats (Aleisa et al., 2011; Alhaider et al., 2010a; Alhaider et al., 2010b). For Short-term memory test (STM), the rats were subjected to 12 learning trials and tested for short-term memory 30 min after the end of 12^{th} trial and the rats were returned to the home cage. For Long-term memory test (LTM), the rats were tested 24 h after the STM. Both control and social defeat rats made comparable errors in the STM test, with control rats making (1 ± 0.24, t=0.972, df=18) error while social defeat rats making (0.8 ± 0.16) errors on an average. On the other hand, in the LTM, social defeat significantly increased the number of errors (1.6 ± 0.18, t=2.39, df=18) as compared to the control (0.5 ± 0.1) rats. Thus, social defeat did not significantly affect STM but the longterm memory consolidation was affected in these rats (Fig. 4 A, B). The rats were sacrificed 24h after completion of LTM tests, their brains harvested and processed for biochemical analysis.

1.4. Analysis of indices of oxidative stress, levels of corticosterone and enzymes of antioxidant defense

Protein carbonylation assay allows detection of carbonyl groups introduced into proteins by oxidative reactions (Allam et al. 2013). Protein carbonylation was measured in oxidative stress susceptible brain areas, previously reported to be important for blood pressure regulation, anxiety and learning-memory function (Salim et al., 2010a; Salim et al., 2011; Vollert et al., 2011). Protein carbonylation significantly increased in the hippocampus (t=2.44, df=6) (Fig. 5C) of the social defeat rats, while the levels were not altered in the prefrontal cortex and amygdala (Fig. 5A, B). Plasma 8-isoprostane (Fig. 5D) significantly increased in social defeat rats as compared to control rats (control: 28.29 ± 1.7 ng/ml, SS: 41.0 ± 4.2 ng/ml, t=2.87, df=18). Thus two different markers of oxidative stress indicate that, socially defeated rats have higher oxidative stress than control rats. Plasma

corticosterone levels significantly increased in response to social defeat when compared to the control rats (Control: 31.8 ± 3.1 ng/ml, SS: 66.9 ± 6.4 ng/ml, t=4.1, df=18) (Fig. 5E).

Protein expression levels of glyoxalase (GLO)-1(t=2.41, df=6) (Fig. 6A–C), glutathione reductase (GSR)-1 (t=2.22, df=6) (Fig. 6D–F), Cu-Zn SOD (t=2.35, df=6) (Fig. 6G–I) and Mn-superoxide dismutase (SOD) (t=2.41, df=6) (Fig. 7J–L), were examined in the hippocampus, amygdala and prefrontal cortex. While Mn-SOD, Cu-Zn SOD and GSR-1 protein expression levels decreased only in the hippocampus of the social defeat rats, no change was observed in the cortex and amygdala between the social defeat and control groups. GLO-1 protein expression levels decreased in the amygdala (Fig. 6B) and hippocampus (Fig. 6C), but not in the cortex (Fig. 6A) of the social defeat rats. The β-actin (loading control) protein levels remained unchanged.

1.5. Assessment of ERK-1/2, IL-6, CAMK-IV, CREB, and BDNF protein expression

Induction of social defeat stress caused ERK-1/2 activation (phospho ERK-1/2 normalized to total ERK-1/2 protein) in the hippocampus (t=5.47,df=6) (Fig. 7A–C). Moreover, ERK-1/2 activation was associated with upregulation in the level of an inflammatory cytokine IL-6, in the cortex and the hippocampus of social defeat rats when compared to the control rats (t=4.46, df=6) (Fig, 7D–F). Furthermore, social defeat significantly decreased the protein levels of CAMKIV (t=2.32, df=6) (Fig. 8A–C), p-CREB/total CREB (t=3.13, df=6) (Fig. 8D–F) and BDNF (t=2.37, df=6) (Fig. 8G–I) in the hippocampus when compared to the control rats. The levels of these proteins remained unchanged in the prefrontal cortex and amygdala. The β-actin (loading control) protein levels remained unchanged.

2.6. Correlation analysis between behavioral assays and indices of oxidative stress

Pearson's correlation analysis was conducted and a negative correlation was observed between LD, OF, EPM tests and oxidative stress parameters as provided in Table 1, which indicates less anxiety-like behavior corresponds to less oxidative stress. On the other hand, a positive correlation depicts when one factor increases the other increases as well. When the number of errors the rats performed in the radial arm water maze (for the long term memory measurement) increased (memory impairment), the level of 8-isoprostane increased too (more oxidative stress), suggesting that higher oxidative stress corresponds to greater memory impairment. Similarly, more sucrose consumed (less depression-like behavior) corresponded with less protein carbonylation (less oxidative stress). And, more sucrose consumed (less depression-like behavior) corresponded to more GLO-1, Mn SOD and Cu/Zn SOD levels (greater antioxidant defense and lesser oxidative stress).

2. Discussion

In this study, we demonstrate that social defeat stress of 7 days in rats produces significant behavioral and biochemical alterations. Socially defeated rats exhibited heightened anxiety-like behavior, as indicated by reduced time spent in the light compartment of the LD box, decreased total and ambulatory activity and distance traveled as well as reduced time spent in the open arms of the EPM test. Moreover, socially defeated rats exhibited decreased

sucrose preference, indicating anhedonia and depression-like behavior. It is well known that rodents are born with an interest in sweet foods or sweetened solutions. Reduced preference for sweet solution (1% sucrose) represents anhedonia, while this reduction can be reversed by treatment with antidepressants (Warren et al. 2013). Therefore, decreased sucrose preference is likely due to the influence of stress on the brain's reward circuitry. The assessment of cognitive functions in socially defeated rats revealed impairments in long-term but not short-term learning and memory function. These results suggest that social defeat of 7 days, generally used to model depressionlike behaviors, can also be used as a model for co-occurrence of depression, anxiety and cognitive impairment.

Furthermore, our data shows that socially defeated rats do not attain normal rodent weight gain profile (Wood et al., 2010) even though they consume comparable amount of diet. Moreover, socially defeated rats consumed more water indicative of increased dehydration during stress. Although, dehydration is only one potential cause of increased drinking, changes in the hypothalamus or changes in vasopressin or angiotensin could both contribute to the change in drinking behavior (Bhatnagar et al., 2006; Fitzsimons, 1978). Importantly, thirst and water drinking are associated with the experience of stress (Bhatnagar et al., 2006; Greenleaf, 1992). Elevated stress is also indicated by increased plasma corticosterone levels in socially defeated rats. Moreover, oxidative stress parameters (plasma 8-isoprostane, and protein carbonylation in brain tissues) showed a significant increase with social defeat. Furthermore, increased level of oxidative stress was observed in the hippocampus but not in the prefrontal cortex or amygdala. These observations are quite interesting and in agreement with our previous report in which we found this brain region to be most affected by pharmacological application of oxidative stress (Allam et al., 2013). Considering present and previous data, it seems reasonable to suggest that hippocampus is a brain area that seems most susceptible to both pharmacological and physiological induction of oxidative stress. Relevant to this, elevated oxidative stress levels in socially defeated rats correspond to reduced antioxidant enzyme levels of GSR-1, Mn SOD and Cu/Zn SOD in the hippocampus but not in the amygdala or prefrontal cortex. GLO-1 levels, on the other hand were reduced in the hippocampus as well as in the amygdala upon social defeat stress. Earlier, both GLO-1 and GSR-1 levels were reported to be reduced in rats that exhibited anxiety-like behavior and cognitive impairment (Allam et al., 2013). The difference between the previous and present study is that the former utilized application of drug-induced oxidative stress whereas the present study involves application of a psychological stressor which is a more physiological scenario. Pro-oxidant mediated oxidative stress is likely to engage many more antioxidant enzymes than a more indirect method of inducing oxidative stress. Interestingly, Pearson's correlation analysis revealed a negative correlation between LD, OF, EPM tests and oxidative stress parameters (Table 1), suggesting less anxiety-like behavior corresponds to less oxidative stress. On the other hand a positive correlation was observed between RAWM and oxidative stress parameters. Higher oxidative stress corresponded to greater memory impairment. Similarly, more sucrose consumed (less depression-like behavior) corresponded with less oxidative stress and more GLO-1, Mn SOD and Cu/Zn SOD levels i.e, greater antioxidant defense and lesser oxidative stress.

Furthermore, social defeat-induced stress significantly decreased the levels of CAMKIV, p-CREB/total CREB and BDNF protein levels in the hippocampus but not in the amygdala or

the prefrontal cortex. Earlier, Fanous et al (2010) in a different model of social defeat (exposed rats to social defeat once every 72 h over 10 days) had reported increased BDNF mRNA and protein expression (Fanous et al., 2010). This is in contrast to our data although it must be noted that Fanous et al. used a different time of sacrifice after the social defeat (2h or 28 days after last social defeat session). It is likely that BDNF expression is highly susceptible to different times and types of stress where regional differences within the amygdala exist that cannot be detected in gross sections of this structure. Increased BDNF levels in other brain areas including nucleus accumbens upon social defeat stress also have been reported (Covington et al., 2011; Krishnan et al., 2007). This is interesting considering involvement of BDNF in regulation of learning and memory function (Duman et al., 1997; Schaaf et al., 2001). BDNF expression is known to be regulated via activation of CREB dependent CAMKIV signaling (Einat et al., 2003; Pandey, 2003). GLO-1 and GSR-1 already have been implicated in anxiety-like behavior (Allam et al., 2013; Ditzen et al., 2006; Hovatta et al., 2005; Kromer et al., 2005; Landgraf et al., 2007; Salim et al., 2011; Thornalley, 2006; Vollert et al., 2011), while CAMKIV and CREB mediated regulation of BDNF is proposed to be involved in cognitive impairment and synaptic plasticity (Abel and Kandel, 1998; Duman et al., 1997; Einat et al., 2003; Markesbery, 1997; Pandey, 2003). We conclude that social defeat mediated stress causes depression-like and anxiety-like behavior as well as LTM impairment with a concomitant rise in oxidative stress (Scheme 1). Increased oxidative stress is a result of reduced antioxidant response which most likely occurs due to diminished GLO-1, GSR-1, Mn SOD and Cu/Zn SOD protein expression. Failing antioxidant response perhaps leads to a further buildup of RONS, leading to inflammation and cytotoxicity. Pertinent to this, we found increased expression of IL-6 and mitogen-activated protein kinase ERK-1/2 in the hippocampus and prefrontal cortex. Increased ERK1/2 activation has been observed in socially defeated animals (Krishnan et al., 2007). ERK-1/2–mediated increase in inflammatory markers is well known (Shin et al., 2012). Moreover, several studies including our own suggest activation of ERK-1/2 upon induction of oxidative stress (Allam et al., 2013; Klann and Thiels, 1999; Serrano and Klann, 2004), and role of ERK-1/2 in anxiety, stress, memory, plasticity, and depression (Ailing et al., 2008; Mazzucchelli and Brambilla, 2000; Qi et al., 2006; Reul and Chandramohan, 2007) is also well established. These observations are quite significant considering that psychological stress is known to trigger comorbid prevalence of anxiety, depression and cognitive impairment (Cohen et al., 2007; McEwen, 2008). This is not surprising as chronic psychological stress is well known to negatively impact an individual's physical and social performance and overall quality of life (Cohen and Wills, 1985). Thus, it is critical to investigate the cause of this highly prevalent comorbidity. Identifying the causality will open new avenues for prevention and treatment. Pertinent to this, mechanistic insights obtained from the present study, are likely to be important for psychological stress-induced comorbid occurrence of anxiety-depression-cognitive impairment.

Finally, hippocampus seems to represent a common brain area that potentially mediates anxiety-depression-like behaviors and cognitive dysfunction induced by social defeat. Studies directly examining causality of oxidative stress in anxiety-like behavior and cognitive impairment in an animal model of depression are currently ongoing.

3. Experimental Procedures

3.1. Animals

Male Sprague Dawley (SD) rats (275–300 g) were used as controls or intruders, and male Long-Evans (LE) retired breeders (400–500 g) served as residents (Charles River, Wilmington, MA). Rats were singly housed with a 12-h light, 12-h dark cycle (lights on at 0600 h) in a climate-controlled room with *ad libitum* food and water. All experiments were conducted in accordance with the NIH guidelines using approved protocols from the University of Houston Animal Care Committee.

3.2. Social defeat

3.2.1. Screening of aggressor Long-Evans rats—Successful application of chronic social defeat stress to SD rats was dependent on appropriate selection of LE rats with consistent levels of aggressive behaviors, as determined from the 3-d screening process mentioned below. It is critical to note that although many male retired breeders showed aggression, the degree, quantity and quality of aggressive behavior varied across LE rats. Almost half of all screened LE rats did not reach the criterion for inclusion in the study, a fact that should be considered when designing experiments. Those aggressors that did meet the inclusion criteria (residents performing a defeat, characterized by the intruder surrendering or acquiring a supine position for approximately 3 sec), were used in multiple social defeat experiments for up to 3 months following their initial screening. Since there is a possibility for the aggressors to habituate to the presence of SD rats over time, thus leading to decrease in their antagonistic interactions, all aggressors were rescreened in a single screening session prior to be used in consecutive social defeat experiments (Golden et al., 2011).

3.2.2. Experimental design—The social defeat model used in the present study was modified from the resident-intruder model originally developed by Miczek (Miczek, 1979). Rats were randomly assigned to either a social defeat or control group for a consecutive 7 days (Bhatnagar and Vining, 2003; Bhatnagar et al., 2006; Wood et al., 2010). This paradigm consisted of 7 encounters, carried out for 7 consecutive days, with an aggressive male Long Evans (LE) rat. Each intruder (Sprague Dawley) was defeated by six different resident LE rats. (Bhatnagar et al., 2006; Golden et al., 2011). A typical social defeat was observed by intruder defeat, indicated by the intruder surrendering or acquiring a supine position for approximately 3 sec. After defeat, a plexiglass partition with holes was placed in the cage to avoid direct physical contact between the LE and intruder. The plexiglass partition with holes allowed intense visual, auditory, and olfactory interactions for the remainder of the 30-min session. If a resident struggled to defeat the intruder for 10 min, rats were separated with the plexiglass partition for the remainder of the 30-min session. Controls were placed behind a plexiglass partition in a fresh cage for 30 min daily. Rats were returned to their home cage after each social defeat session, and body weight was recorded on days 1 and 8. All Sprague-Dawley rats were used for behavioral assessment and sacrificed thereafter for collection of brains.

3.3. Anxiety and depression-like behavior tests

First, open-field test was conducted followed by light-dark (LD) and elevated-plus maze (EPM) tests as previously published by us (Salim et al., 2010b; Vollert et al., 2011).

3.3.1. Open Field (OF) activity—Rats were placed in the center of the OF (60×40 cm) and left free to explore the arena for 15 min and movement quantified using Opto-Varimex Micro Activity Meter v2.00 system (Optomax, Columbus Instruments; OH) as previously published by us (Salim et al., 2010b; Vollert et al., 2011). The light intensity was adjusted at 300 lux. Percent time spent in the center of the arena, rearings, total activity, ambulatory activity, distance covered and fecal boli were examined.

3.3.2. Light-Dark (LD) exploration—Time spent in light is considered as a measure of anxiety-like behavior. The light-dark box consisted of a light and a dark compartment separated with a single opening for passage from one compartment to the other and total time spent in the lit area was recorded (Salim et al., 2010b; Vollert et al., 2011).

3.3.3. Elevated plus-maze—A standard rat elevated plus-maze with 43 cm arms extending from a 10 cm central area was obtained from Med Associates Inc., (St. Albans, VT). The arms of the maze were approximately 90 cm above the floor. The rat's movements were tracked manually. The observer was blinded to the group classification to avoid bias. Each session was started by placing the rat in the central area facing the open arms of the maze and lasted 5 min. In between each test animal, the maze was wiped down with alcohol. The amount of time the rat spent in the open arms was noted (Bert et al., 2002).

3.3.4. Sucrose Consumption—The sucrose consumption test has been used extensively to assess motivational state in rodents, including stress-induced anhedonia (Barrot et al., 2002; Iniguez et al., 2010; Warren et al., 2013), which is regarded as a sign of depressive behavior (Yu et al., 2011). This test consists of a two-bottle choice paradigm in which rats are given the choice between consuming water and a 1% solution of sucrose. The preference for sucrose over water is considered as a measure for rodents' sensitivity to a natural reward. Thus, anhedonia and depression-like behavior is revealed by a reduction in sucrose preference (Papp et al., 1991; Willner et al., 1992). The sucrose preference test was carried out during the 7-day social defeat protocol and 4 days after the last social defeat session.

3.4. Memory Function Test

3.4.1. Radial Arm Water Maze (*RAWM*)—The RAWM procedures were done as previously published by us (Aleisa et al., 2011; Alhaider et al., 2010a; Alhaider et al., 2010b). Basically the apparatus consisted of a black circular pool filled with water at 25°C containing six swim paths in a dimly lit room. Each rat was randomly assigned a goal arm which contains a hidden black platform near the end of the arm. The rats were randomly released at an arm different from the goal arm, allowed to swim and locate the platform which is submerged 1 cm under water. The rats were allowed 1 minute for each learning trial or memory test. An error was counted when the rat entered more than halfway into an arm other than the goal arm or if the rat entered more than half of the goal arm but failed to approach the platform. Number of errors ranged from 1–7, as the rat can only swim into 7

arms within 1 minute. If the rat failed to locate the platform within 1 minute, the rat was manually guided to the platform and was scored with 7 errors. Upon reaching the platform, the rat was allowed 15 seconds rest before the next trial began.

3.4.2. Short-term learning and long-term memory tests—The rats were subjected to the first set of six learning trials (trials # 1–6) followed by a five min rest period and then another set of six learning trials (trials # 7–12) and tested for short-term memory 30 min after the end of 12th trial. The rats were subjected to learning trials (trials #1–12) as above. At the end of the 12th trial, the rats were returned to their home cages and 24 h later subjected to long-term memory test.

3.5. Brain Dissections and Preparation of Homogenates

Rats were anesthetized (Isoflurane, #57319-479-06, Phoenix Pharmaceuticals) immediately after behavior tests (4 days after the last social defeat session). The brains were quickly removed and rapidly frozen and stored at −80°C until analysis. Blood was collected and plasma was separated immediately and stored at −80°C. Hippocampus, amygdala and cerebral prefrontal cortex were identified according to Paxinos and Watson (Paxinos, 1986) and grossly dissected out, homogenized and prepared as published (Vollert et al., 2011).

3.6. Western Blot Analysis

Equal amounts of brain tissue homogenate proteins diluted with 4× laemmli sample buffer were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting as previously published by us (Vollert et al., 2011). Primary antibody dilutions used were as follows; GLO-1 (1:200 dilution; Abcam, Cambridge, MA - Cat No: ab81461), GSR-1 (1:100 dilution), Cu/Zn SOD (1:1000 dilution; Cat No: 07-403) and Mn SOD (1:1000 dilution; Cat No: 06-984) were purchased from Millipore, Temecula, CA. CAMKIV (1:1000 dilution; Cat No: 4032S), p-p44/42 MAPK (1:200 dilution; Cat No: 9106S) and p44/42 MAPK (1:1000 dilution; 9107S) were purchased from Cell Signaling technology, Danvers, MA. BDNF (1:1000 dilution; Cat No: sc-546), p-CREB (1:200 dilution; Cat No: sc-7978), total-CREB (1:1000 dilution; Cat No: sc-58), IL-6 (1:1000 dilution; Cat No: ARC0062) was purchased from Life Technologies, Grand Island, NY and β-actin (1:1000 dilution; Cat No: sc-47778) were from Santacruz biotechnology, Santacruz, CA. The membranes were incubated with respective antibody for 1 h, followed by incubation with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000), anti-rat horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000) or anti-mouse HRP-linked secondary antibody (1:1000) at room temperature for 1 h. The images of immunoblots were captured by a Fluorchem 8900 imaging system with intensity of each immunoreactive band determined using Alpha Ease FC 4.0 (Alpha Innotech Corp., San Leandro, CA) that were normalized to β-actin protein loading control.

3.7. Protein oxidation and corticosterone levels

The OxyBlot™ Protein Oxidation Detection Kit (EMD Millipore Corp. #S7150) was used for immunoblot detection of carbonyl groups introduced into proteins by oxidative reactions. Equal amount (20 μg) of protein homogenate from different brain regions (prepared as indicated above) were subjected to this kit based reaction following manufacturer's

instructions, which allows detection of carbonylation of proteins in the homogenates using western blotting method. Plasma corticosterone levels, released in response to stress and anxiety (Arranz et al., 2007) were measured using an EIA based kit (cat#500655, Cayman Chem. Co., Ann Arbor, MI) per manufacturer's instructions.

3.8. Statistical Methods and Data Analysis

Data are expressed as mean ± SEM. Statistical significance was done by using student's t-test (GraphPad Software, Inc. San Diego, CA). A value of $P < 0.05$ was considered significant.

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Abbreviations used

SD	Sprague Dawley
SS	Social stressed
LE	Long-Evans
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF	Polyvinyledene difluoride
ECL	enhanced chemiluminescence
RAWM	radial arm water maze
ERK1/2	extracellular signal-regulated kinase-1/2
P-CREB	phospho-cAMP response element–binding protein
T-CREB	total-cAMP response element–binding protein
CAMKIV	calcium/calmodulin-dependent protein kinase type IV
BDNF	brain derived neurotrophic factor
SOD	superoxide dismutase
GLO-1	glyoxalase-1
GSR	glutathione reductase-1

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Research Highlights

- Social defeat stress leads to anxiety-like and depression-like behaviors along with memory deficits in rats.
- Co-occurrence of anxiety-depression like behaviors and memory deficits in rats correlates with elevated oxidative stress.
- Potential cross-talk between oxidative stress and inflammation might regulate affected behaviors during social stress in rats.

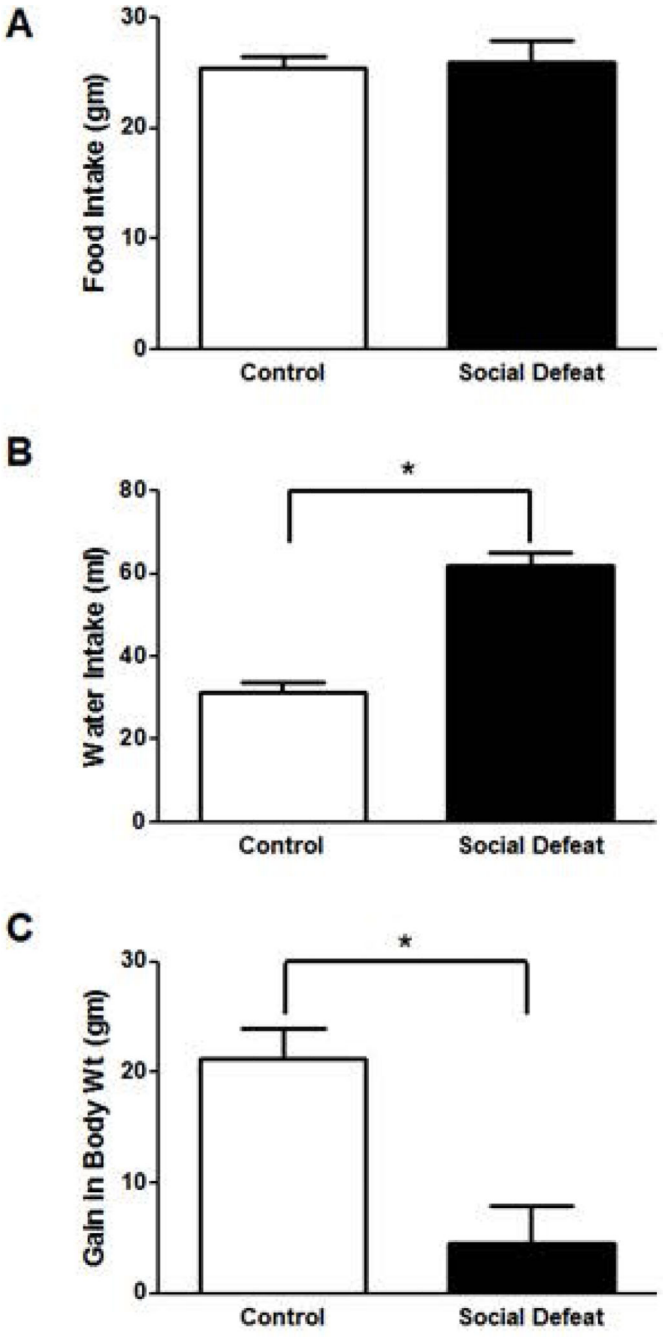


Fig.1.

Examination of general parameters including gain in body weight and food and water intake was measured. Food intake (A), water intake (B) and gain in body weight (C) were measured. (*) significantly different from control, $P<0.05$. Bars represent means ± SEM, n = 10 rats/group.

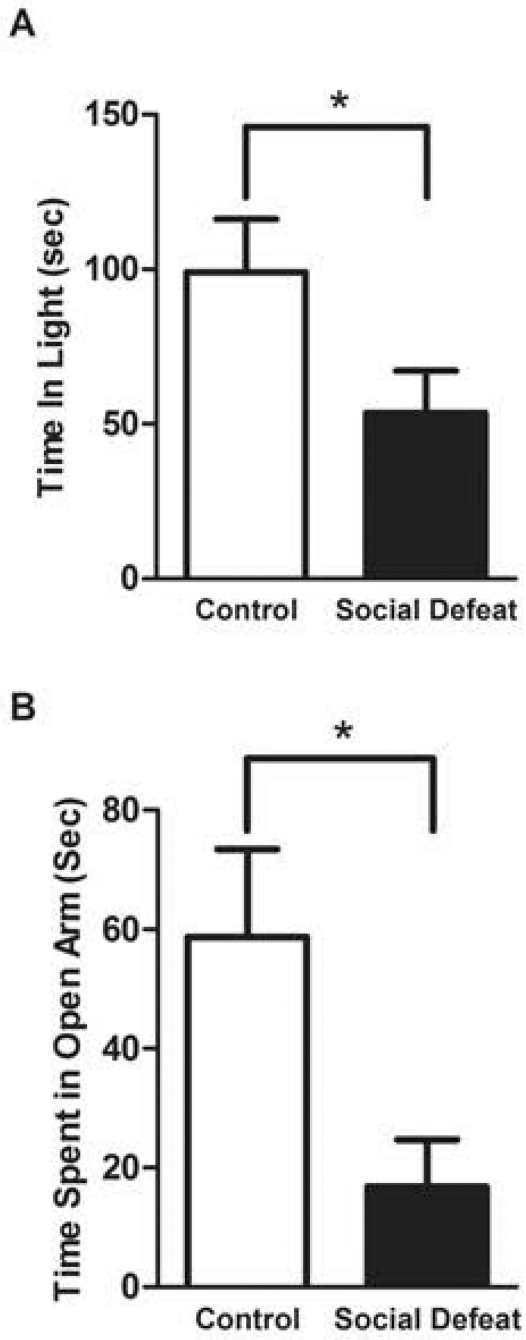


Fig.2.

Examination of anxiety-like behavior tests including light dark (A) and elevated plus maze (B) tests in rats subjected to social defeat. (*) significantly different from control, $P<0.05$. Bars represent means ± SEM, n =10 rats/group.

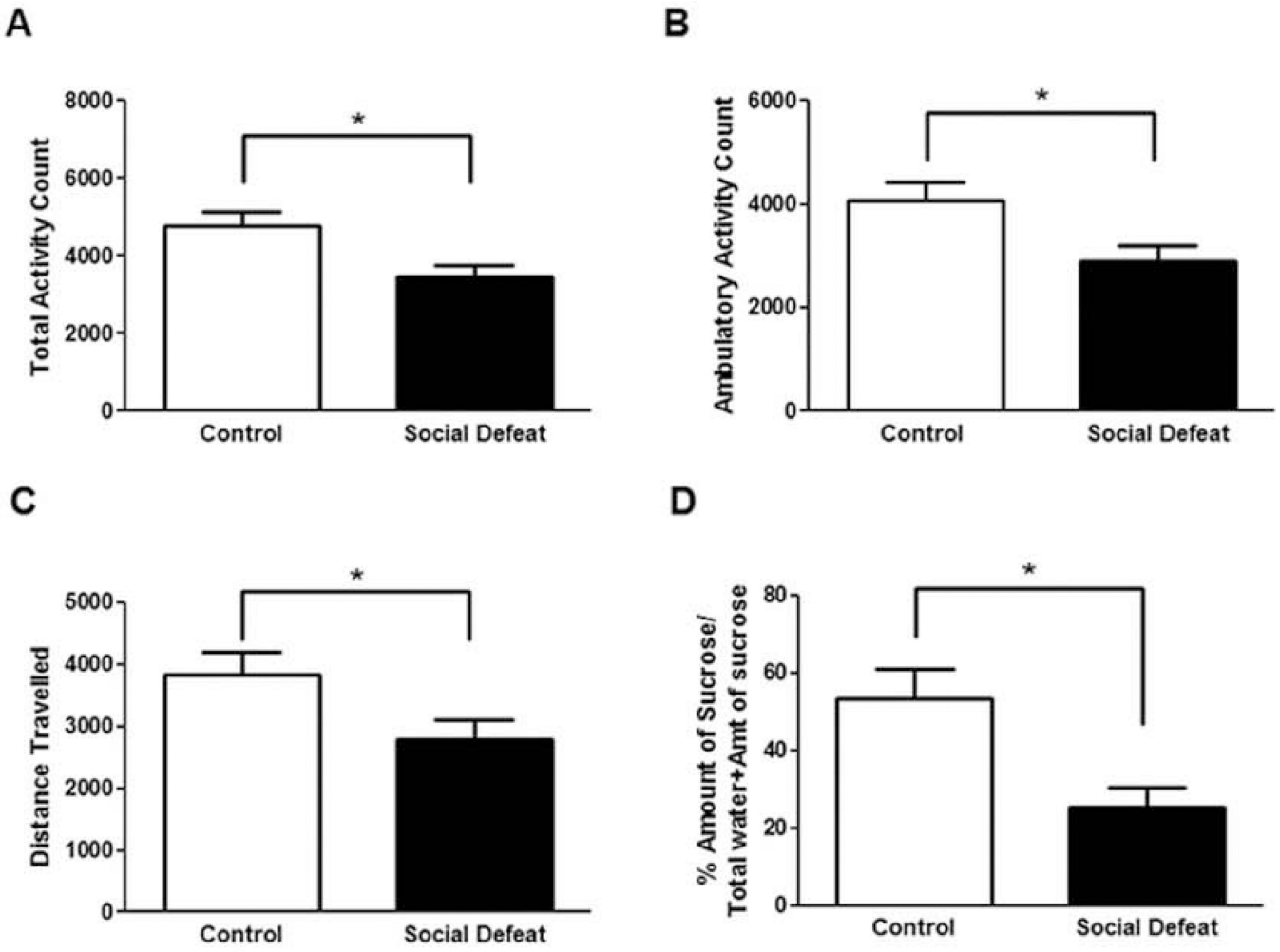


Fig.3.

Examination of anxiety- and depression-like behaviors using open-field and sucrose preference test. The open-field test determined total (A), ambulatory (B) activities and distance traveled (C). Depression-like behavior in rats subjected to social defeat was examined using sucrose preference test (D). Social defeat exposure influenced sucrose preference, a measure of natural reward, 24 h after the last stress session. (*) significantly different from control, $P<0.05$. Bars represent means ± SEM, n =10 rats/group.

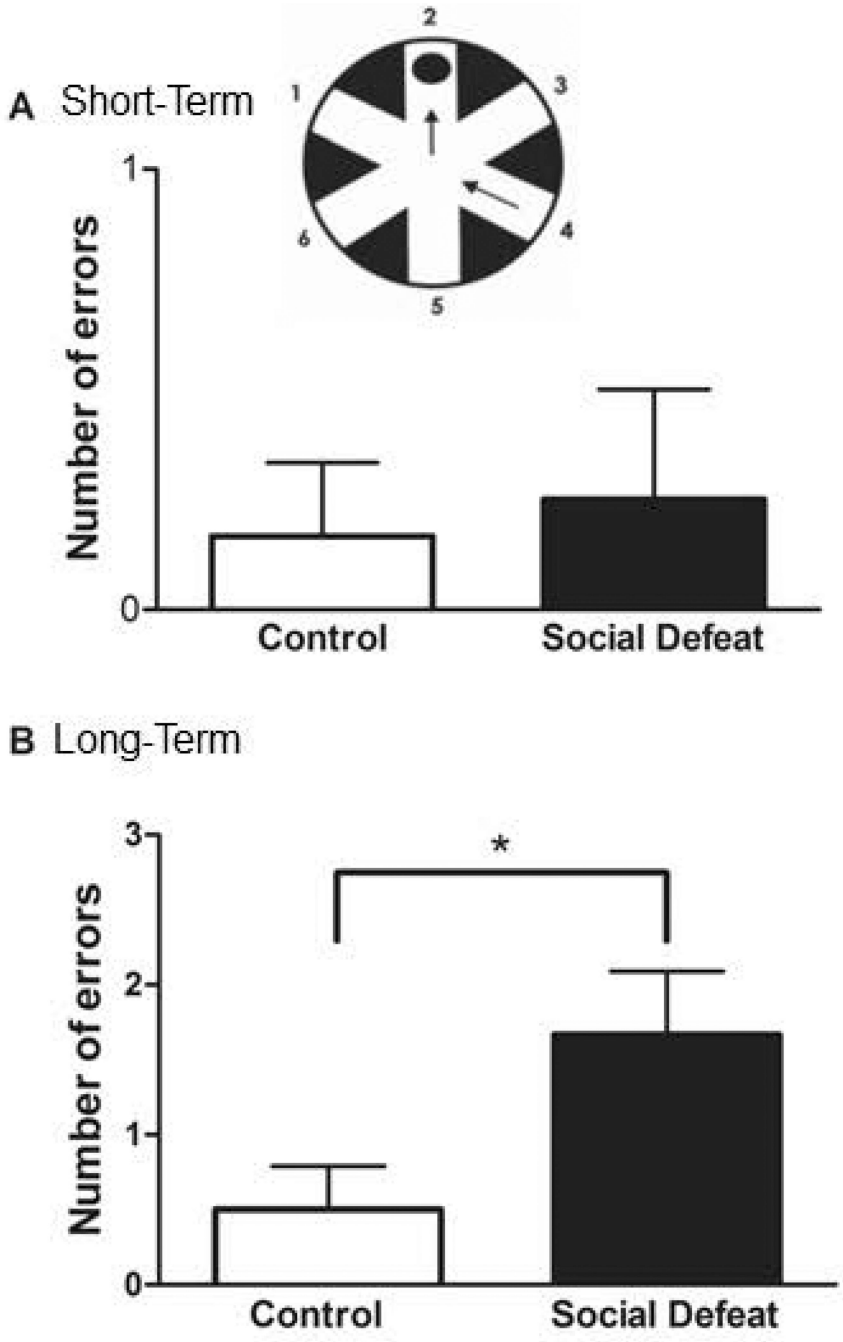


Fig.4.

Examination of radial arm water maze (RAWM) memory tests in rats subjected to social defeat. Short-term (A) and long-term (B) memory was assessed by using a series of 12 RAWM trials. The RAWM apparatus is shown as an insert containing a circular water pool with 6 swim paths. (*) significantly different from control, $P<0.05$. Bars represent means ± SEM, n =10 rats/group.

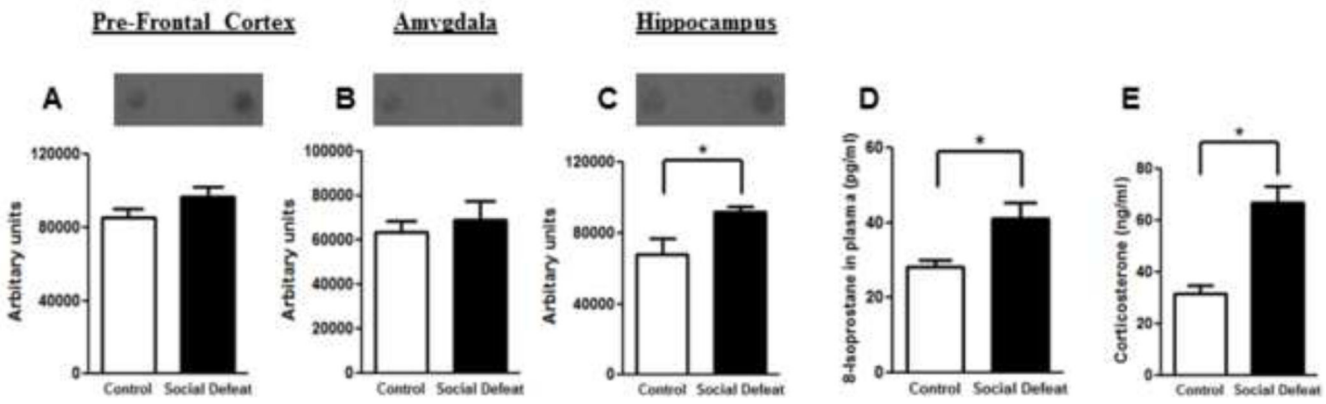


Fig.5.

Analysis of markers of oxidative stress, protein oxidation, 8-isoprostane and corticosterone in rats subjected to social defeat. Protein carbonylation was measured using the OxyBlot™ Protein Oxidation Detection Kit following manufacturer's instructions (A–C). Dot blots in panels (A–C) are 10 μl homogenate-oxyblot reaction mixture. (*) significantly different from control, $P<0.05$. Bars represent means ±SEM, n = 6 rats/group. The 8-isoprostane concentrations in plasma (D) and plasma corticosterone (E) were measured by using an Enzyme Immuno Assay kit. (*) significantly different from control, $P<0.05$. Bars represent means ± SEM, n = 10 rats/group.

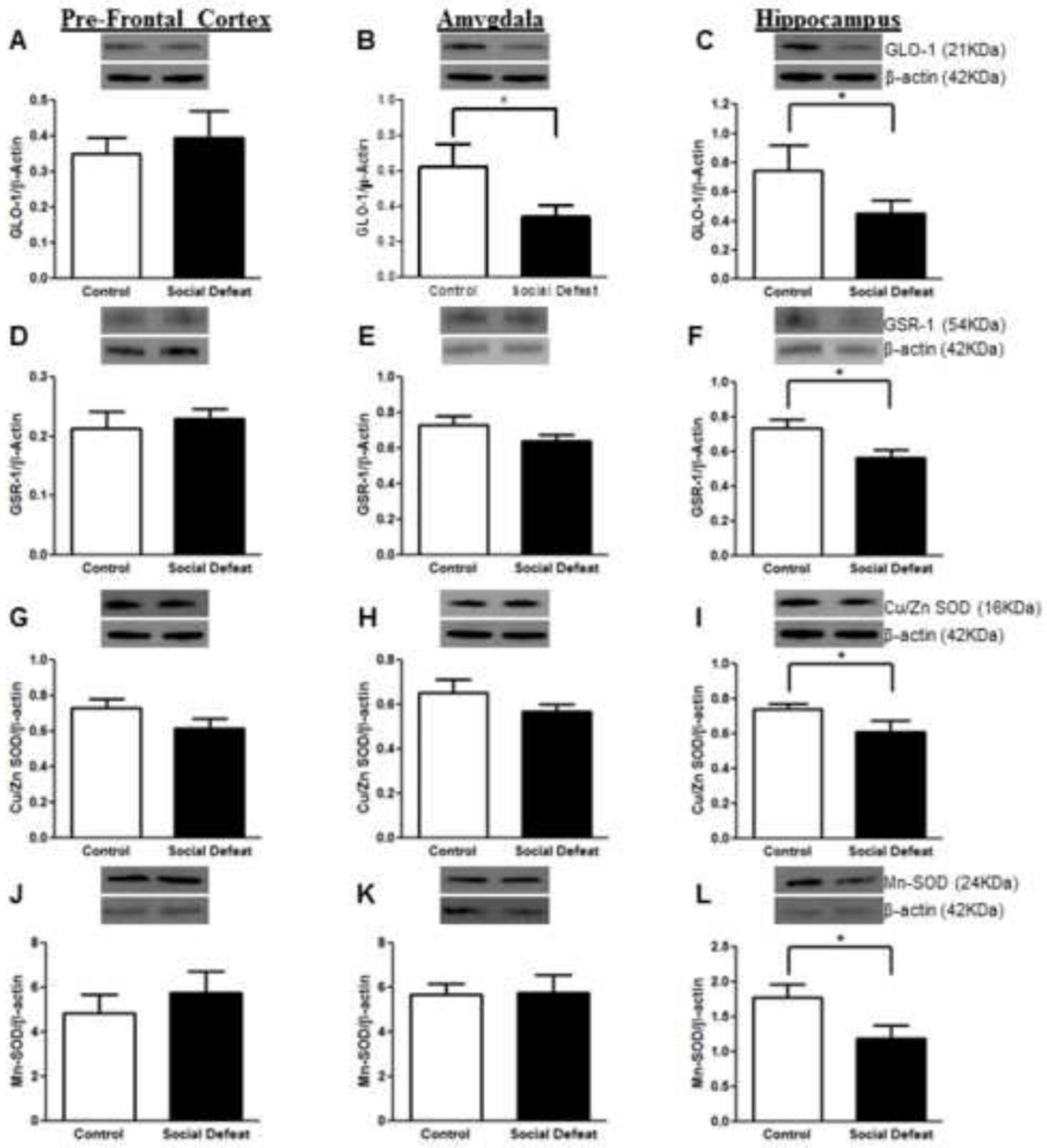


Fig.6.

Examination of GLO-1, GSR-1, Cu-Zn SOD and Mn SOD protein levels in the hippocampus, amygdala, and prefrontal cortex of rats subjected to social defeat stress. Protein levels of GLO-1 (A–C), GSR-1 (D–F), Cu-Zn SOD (G–I) and Mn-SOD (J–L) were determined by Western blotting. The upper panels are representative blots for GLO-1, GSR-1, Cu-Zn SOD, Mn-SOD and the lower panels are protein loading control β-actin. Bar graphs are ratios of GLO-1, GSR-1, Cu-Zn SOD and Mn-SOD to β-actin, respectively. (*) significantly different from control, $P<0.05$. Bars represent means ± SEM, n =4 rats/group.

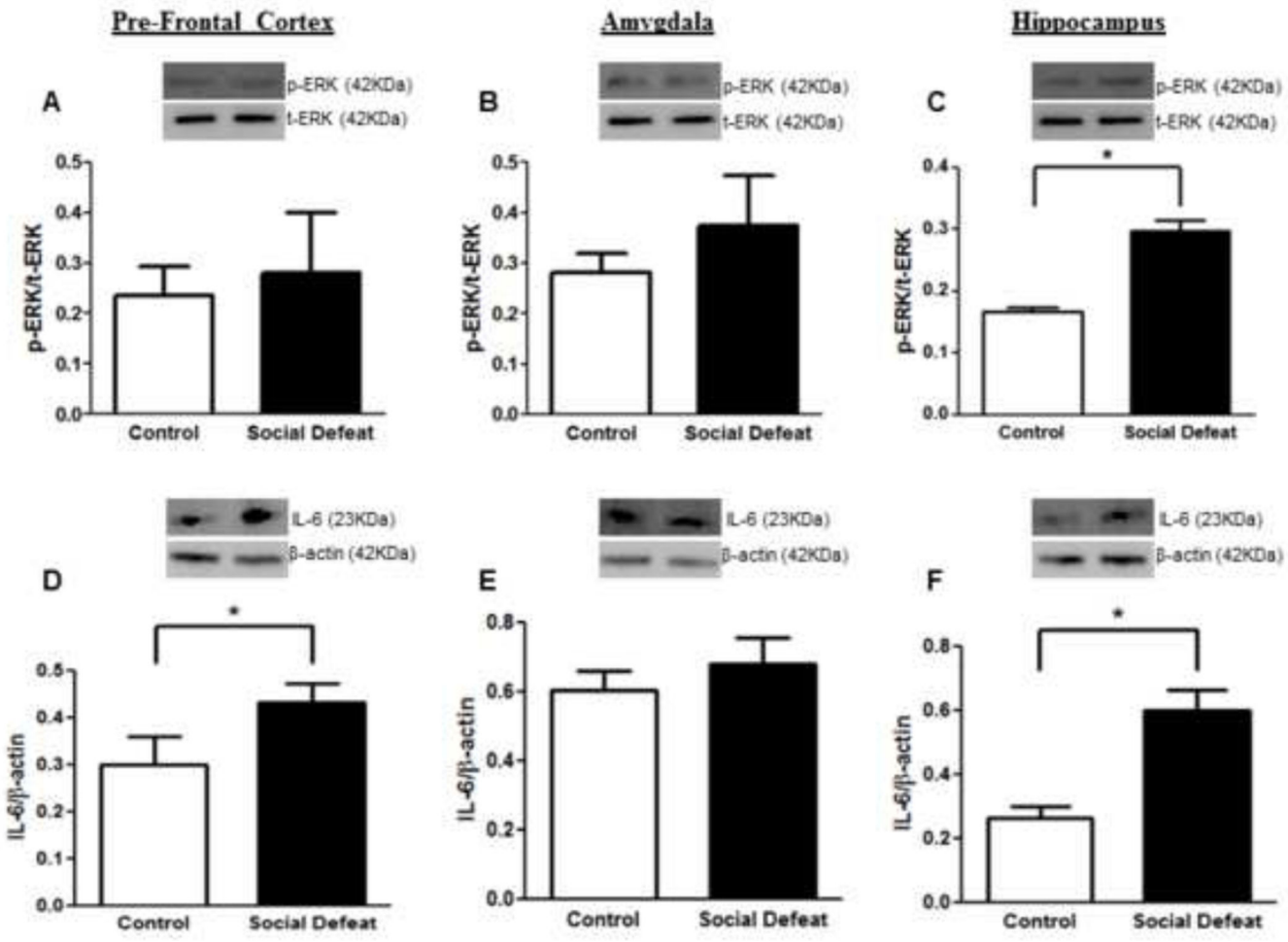


Fig.7.

Analysis of ERK-1/2 activation and IL-6 in the prefrontal cortex, amygdala and hippocampus of rats subjected to social defeat stress. Protein expression levels of ERK-1/2[phospho(P)/total] (A–C) and IL-6 (D–F) in the prefrontal cortex, amygdala and hippocampus were determined by western blotting. Upper panels are representative blots for phospho-ERK(1/2)/total ERK(1/2) and IL-6/β-actin. Bar graphs are ratios of phospho to total ERK-1/2, and IL-6 to β-actin, respectively. (*) significantly different from control, $P<0.05$. Bars represent means ± SEM, n =4 rats/group.

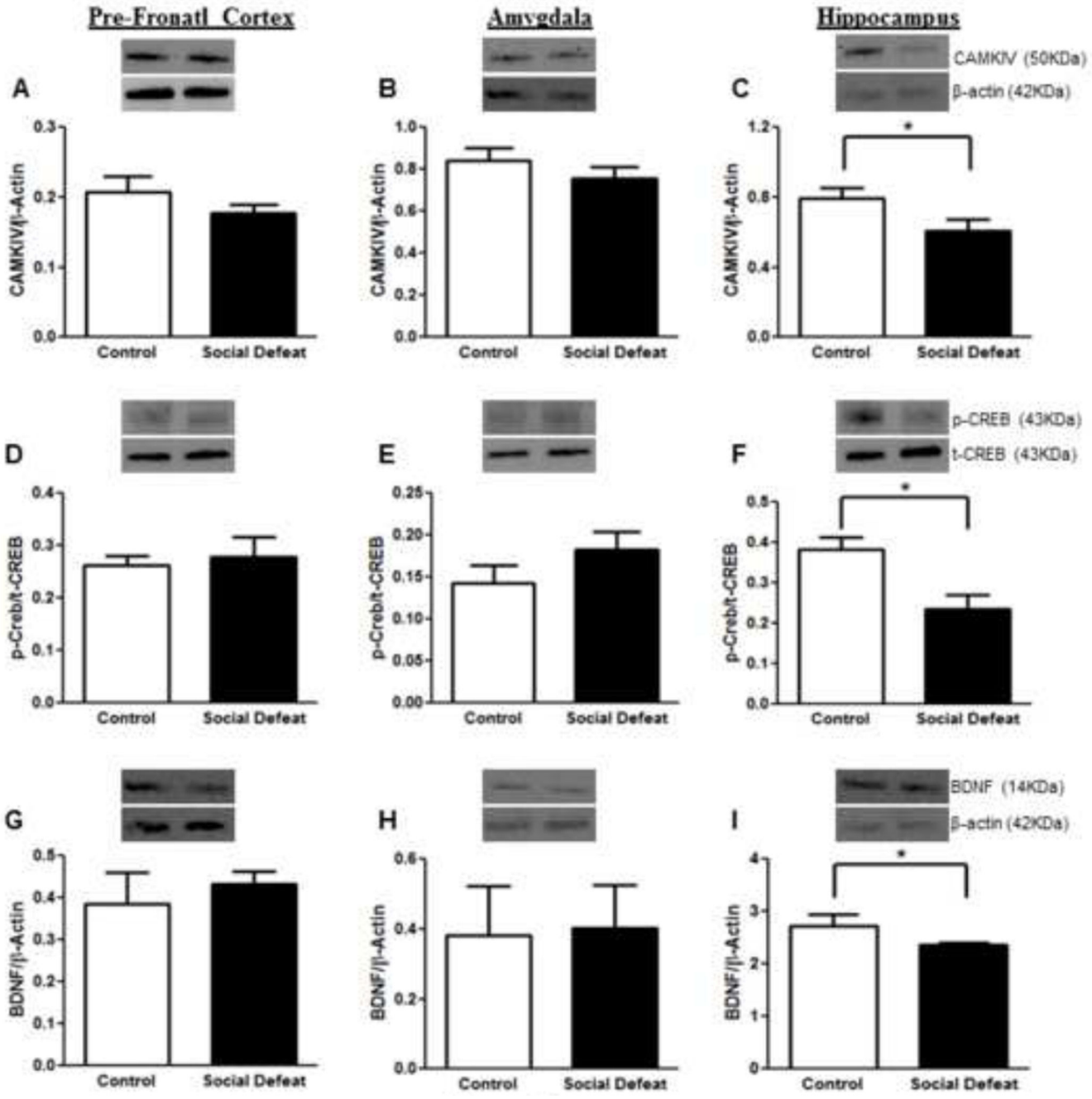
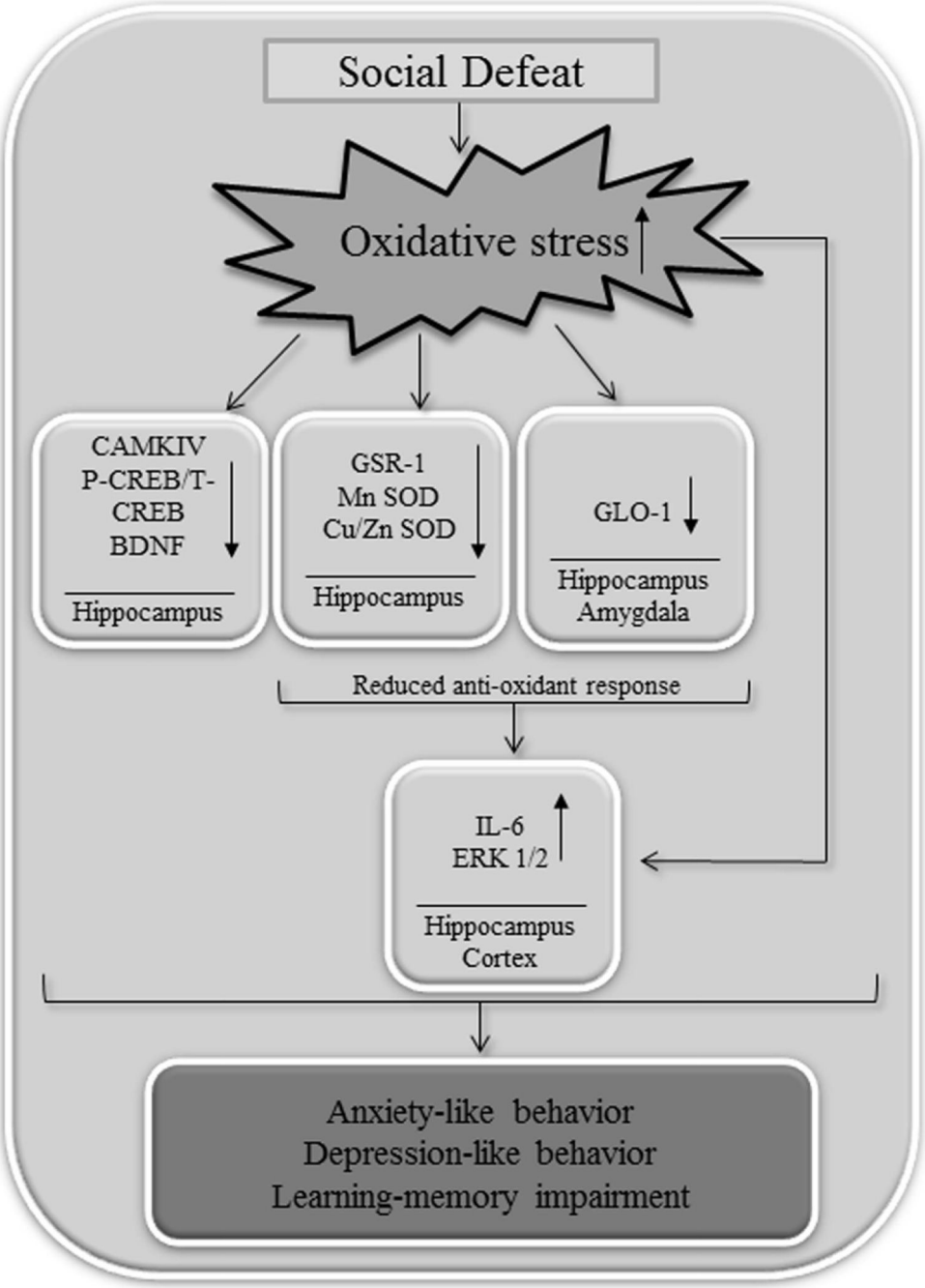


Fig.8.

Examination of CAMK-IV, CREB, and BDNF protein levels in the prefrontal cortex, amygdala and hippocampus of rats subjected to social defeat stress. Protein expression levels of CAMK-IV (A–C), CREB [phospho (P)/total] (D–F), and BDNF (G–I) in the prefrontal cortex, amygdala and hippocampus were determined by using western blotting. Upper panels are representative blots for CAMK-IV/β-actin, phospho-CREB/total CREB, and BDNF/β-actin. Bar graphs are ratios of CAMK-IV to β-actin, phospho to total CREB, and BDNF to β-actin, respectively. (*) significantly different from control, $P<0.05$. Bars represent means ± SEM, n =4 rats/group.



Scheme 1.

A schematic representation of different pathways modulated by activation of oxidative stress that collectively affect anxiety-, depression-like behaviors and cognitive impairment, upon induction of social defeat in rats.

Table 1

Examination of correlation between behavioral and molecular changes in socially defeated and control rats. The correlation was determined using Pearson's correlation coefficient.

Groups	Pearson's Correlation coefficient (r)
Light Dark (n=10) Vs 8-Isoprostane (n=10)	−0.53*
Light dark (n=10) Vs Protein carbonylation (n=6)	−0.79*
Elevated plus maze (n=10) Vs 8-Isoprostane (n=10)	−0.84*
Elevated plus maze (n=10) Vs Protein carbonylation (n=10)	−0.63*
Ambulatory activity (n=10) Vs 8-Isoprostane (n=10)	−0.42*
Ambulatory activity (n=10) Vs Protein carbonylation (n=10)	−0.71*
Total activity (n=10) Vs 8-Isoprostane (n=10)	−0.42*
Total activity (n=10) Vs Protein carbonylation (n=6)	−0.69*
Distance travelled (n=10) Vs 8-Isoprostane (n=10)	−0.47*
Distance travelled (n=10) Vs Protein carbonylation (n=6)	−0.73*
Sucrose consumed (n=10) Vs 8-Isoprostane (n=10)	−0.54*
Sucrose consumed (n=10) Vs Protein carbonylation (n=6)	−0.32
LTM (RAWM, n=10) Vs 8-Isoprostane (n=10)	0.39*
LTM (RAWM, n=10) Vs Protein carbonylation (n=6)	0.35
GLO-1 (n=4) Vs Sucrose consumption (n=10)	0.82*
Mn-SOD (n=4) and Sucrose consumption (n=10)	0.23
Cu/Zn-SOD (n=4) and Sucrose consumption (n=10)	0.42*

(*) significantly different from control, $P<0.05$.