CENTRAL IMMUNE ALTERATIONS IN PASSIVE STRATEGY FOLLOWING CHRONIC DEFEAT STRESS

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ABSTRACT

The relationship between stress, mood disorders and immune disorders is known, but what remains to be resolved is why certain individuals are more susceptible than others to suffer different disorders, along with the biological mechanisms that underlie these differences. The objective of this study was to analyze the changes in the expression patterns of proinflammatory cytokines in the hypothalamus, hippocampus, amygdala and prefrontal cortex after chronic defeat, depending on the coping strategy used. The expression levels of $\alpha 1_b$ and $\alpha 2_a$ adrenergic receptors and cytokine-inducible nitric oxide synthase (iNOS) in the prefrontal cortex were also measured. The results indicated that subjects with a passive coping strategy showed high levels of interleukin-6 (IL-6) and interleukin-1β (IL-1β) expression in several cerebral structures in resting conditions after 21 days of chronic stress and increases in these cytokine levels in the hippocampus following an additional stress. Low expression levels of tumour necrosis factor-alpha (TNF-α) in the prefrontal cortex in active subjects at rest and in passive subjects after an additional defeat were detected. The iNOS expression levels were lower in the prefrontal cortex of the active group at rest. With respect to adrenergic receptor expression, there were no changes as a function of stress, but there were changes as a function of coping strategy. These results indicate differences in the variables studied in terms of the coping strategy adopted, with passive subjects having a biological profile that could be considered more vulnerable to the development of stress-related disorders.

KEYWORDS

chronic social stress, stress-related disorders, cytokines, adrenergic receptors, coping, iNOS

1. Introduction

In recent years, a strong relationship has been established between depression and an increase in proinflammatory cytokine levels [1,2]. We also know that psychosocial stressors are triggers of depressive episodes [3] and that these stressors may also activate the inflammatory response at a peripheral level, resulting in an increased production of proinflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α), in both humans and animals [2,4,5]. However, there are few studies about the effects of stress on the immune system at the central level, and not all point in the same direction. For instance, studies of chronic stress in animals report increases of interleukin-1 (IL-1) in the hippocampus [6], IL-1β in the hypothalamus, and TNF-α in the pituitary and hypothalamus [7]. However, there are also data showing decreases in both IL-1β and TNF-α in the stria terminalis and hippocampus [8]. It has also been observed that exposure to chronic stress along with the administration of lipopolysaccharide enhances IL-1β, IL-6 and TNF-α expression in both the prefrontal cortex and the hippocampus [9,10], although a less pronounced response of IL-6 was observed.

On the other hand, not all individuals respond in the same manner to a stressful situation. This differential individual response gives rise to different behavioural, neuroendocrine, and immune alterations in each organism, which can constitute the basis of vulnerability to developing different types of pathologies [11,12]. Previous work in our laboratory found that subjects who showed a passive strategy after chronic defeat stress, characterized by behaviours of immobility and low exploration, had low corticosterone levels at rest, a high glucocorticoid responsiveness, and higher peripheral levels of IL-6 and TNF-α than subjects with an active strategy [4,13]. Studies examining cytokine levels at the central level have reported increased IL-1β in the hippocampus and low levels of TNF-α in the prefrontal cortices of submissive mice that were high corticosterone responders after exposure to social stress for three days [14]. However, a stress challenge after seven days of defeat resulted in increased TNF-α, IL-1β, and IL-6 expression in all stressed subjects [9], although a smaller increase in IL-6 was observed among mice that had previously experienced a single or repeated social defeat.

Despite the evidence for an association between depression, stress and proinflammatory cytokines, the mechanism by which cytokines can alter behaviour is still unknown. Several possible mechanisms exist, one of which is the activation of the cytokine-inducible nitric oxide synthase (iNOS) enzyme. This enzyme, which requires tetrahydrobiopterin (BH_4) as a cofactor, converts arginine to nitric oxide (NO) and induces oxidative stress, thereby contributing to neuropsychiatric pathogenesis [15,16]. Furthermore, iNOS activation by proinflammatory cytokines can also lead to decreased availability of BH_4, a critical factor for the rate-limiting

enzymes involved in the synthesis of the catecholamine neurotransmitters, and can thus reduce noradrenaline synthesis [17], the deficit of which has been linked to depression. The anxiolytic-like and antidepressant-like effect observed after inhibition of the iNOS enzyme in a variety of animal paradigms supports this idea [18,19].

There is evidence, from studies in both animals and humans, of noradrenergic receptor alterations in different stress-related psychopathologies [20]. Studies in humans suggest that α1b adrenergic receptors are responsible for modulating cerebral catecholaminergic systems and play an important role in depressive disorders [20]. Furthermore, studies with animal models of depression have reported elevated levels of α2 adrenergic receptors in the brain [21] and that treatment with antagonists of these receptors produces antidepressant-like effects [22]. In patients with depressive disorders, changes have been observed in the level of α2 adrenergic receptors in the prefrontal cortex [23,24], which is one of the fundamental structures involved in stress-related disorders [25]. However, the data on changes in central adrenergic receptors as a function of the coping strategies adopted by subjects in situations of social stress are limited.

Taking into account the above data, the objective of this study was to analyze the effects generated by stress induced by chronic defeat, as well as the mechanisms through which individual differences in coping with stress may have repercussions on vulnerability to the development of stress-related psychopathologies. For this purpose, changes were measured in the expression levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α as a result of chronic defeat and the coping strategy adopted by subjects in this situation, in different cerebral structures that have been implicated in stress-related psychopathologies, such as the amygdala, hippocampus, hypothalamus and prefrontal cortex. We also studied changes in α1b and α2a adrenergic receptor and iNOS enzyme expression levels in the prefrontal cortex, since this structure appears to be one of those that are particularly sensitive to the effect of stress-induced catecholamines.

2. Material and methods

2.1. Animals and husbandry

Six-week-old male OF1 mice (Charles River, Oncins, France) were individually housed for 7 days in transparent plastic cages measuring 24.5×24.5×15 cm for adaptation to the laboratory conditions. Food and water were available *ad libitum*. The holding room was maintained at a constant temperature of 20ºC with a 12 h light/dark cycle (white lights on from 20:00 to 08:00 h). The light cycle was reversed to facilitate behaviour assessment during the animal's active phase (dark). All experimental procedures were conducted under dim red light conditions in a room adjacent to the holding facility. European regulations for the care and treatment of

experimental animals were followed, and the procedures were controlled and approved by the Diputación Foral de Gipuzkoa, Spain, in compliance with the European Directive (2010/63/EU) on the protection of animals used for scientific purposes (22 September 2010). The procedures were approved by the Ethical Committee for animal welfare of the Basque Country University (CEBA).

2.2. Experimental procedure

The experiments were initiated after the adaptation period. A control group (manipulated control) and a group of socially stressed mice were established. Male adult mice were exposed to repeated defeat experiences for 21 days (at 11:00 am). On day 21, the behaviour in the confrontation was recorded for subsequent assessment. The stressed mice were divided into two groups according to their behavioural profile during defeat: active and passive (see below). Two days later, on day 23 (at 12:00 pm), half of the animals were sacrificed by cervical dislocation. On day 24, the remaining animals were subjected to another social defeat and 50 minutes after (at 12:00 pm), the animals were sacrificed by cervical dislocation. The brains from all of the animals were then quickly removed and the whole hypothalamus, hippocampus, prefrontal cortex and amygdala of both hemispheres were dissected [26] and flash-frozen in liquid nitrogen for subsequent measurement of proinflammatory cytokine (IL-6, IL-1β and TNF-α), iNOS and adrenergic receptor (α1b and α2a) mRNA expression in each animal (see Fig. 1). All dissections were performed under stereomicroscopic observation with reference to mouse brain atlas [27].

2.2.1. *Socially stressed mice*

The mice experienced repeated social defeat using the sensorial contact model [28]. Eighty-five pairs of mice matched by weight were allowed a 10 min confrontation, with half of the subjects being placed in their opponent's cage (i.e. in the cage of a resident mouse) for 3 successive days in order to select defeated subjects. After the third day, only those defeated subjects which had clearly shown defensive/submissive behaviour during the agonistic confrontations, and their aggressive counterparts, continued with the experiment (eighty pairs of mice). Between the fourth and last day of chronic stress, the defeated mice were exposed daily to 5 min of agonistic interactions with a different aggressive resident mouse. Daily transfer of males after the fighting precludes habituation of males to each other and consolidates the submissiveness of the defeated animal placed in an alien territory [29].

As a result, the mice were repeatedly defeated by a different aggressive resident mouse every day. After each daily confrontation, the mice were separated by transparent partitions with holes, and remained in the same cage in which the confrontation took place until the next

confrontation. These partitions permitted the mice to see, hear and smell each other, but prevented physical contact. Although the defeated mice received some bites during the direct interaction period, most of the mice did not have evident wounds. In the case of visible wounds, the mice were removed from the experimental procedure. The final number of defeated mice was seventy-nine. The behaviours manifested by the defeated mice on day 21 during the 5 min of social interaction were recorded using video cameras (JVC, GZ-MG77E). The behavioural assessment was carried out using an ethogram for the mouse [30], in which 51 behaviours are described. The assessment was conducted by two independent evaluators (kappa index=0.81). The behaviours observed were divided into the following behavioural categories: avoidance/flee, defence/submission, digging/self-grooming, exploration at a distance, immobility, non-social exploration and social exploration. The behavioural evaluation was carried out using The Observer 3.0 (Noldus, ITC, Wageningen, The Netherlands). To classify the subjects according to behaviour, two cluster analyses were performed on all of the defeated mice based on the behavioural characteristics they displayed in the social confrontation on day 21, using the mean percentage of time spent on each assessed behavioural element [4].

2.2.2. *Manipulated controls*

The manipulated control group (30 subjects) was treated identically to the stressed group but was not exposed to agonistic interactions or sensorial contact with other mice. These mice were housed individually in cages containing a transparent barrier to subject them to the same space restrictions as the experimental mice. The manipulated controls were moved daily to an experimentation room where the barrier was removed for the same period of time as that allotted to the confrontations in the defeated mice.

2.3. Physiological determinations

Tissue from the hypothalamus, hippocampus, amygdala and prefrontal cortex were homogenized using the Trizol reagent (Invitrogen, Madrid, Spain), and total RNA was isolated using the standard phenol chloroform extraction method [31]. A UV spectrophotometric analysis of nucleic acids was performed at 260 nm to determine the RNA concentrations, and the 260:280 absorbance ratio was used to assess the nucleic acid purity. All samples had absorbance ratios of over 1.81. The samples were DNAse treated (DNAse I, Invitrogen, Madrid, Spain) to remove contaminating DNA prior to cDNA synthesis, and the total RNA was reverse transcribed using PrimeScript reverse transcriptase (Conda, Madrid, Spain). The resulting cDNA underwent SYBRGreen-based (QuantiTect SYBR Green PCR, Conda, Madrid, Spain) real-time PCR, and the amplification was monitored using the Applied Biosystems 7500 Real Time PCR System. The cDNA sequences were obtained from Genbank at the National Center for Biotechnology Information (NCBI: www.ncbi.nlm.nih.gov), and glyceraldehyde-6-

phosphate dehydrogenase (GAPDH) was used as a reference gene, with an ANOVA being conducted to check that there were no differences in its expression between groups. The primer sequences were designed using the Primer Express Software v3.0 (Applied BioSystems, Madrid, Spain) (Table 1). The primers were obtained from Applied Biosystems (Madrid, Spain), and the specificity of the primers was verified by a melting curve analysis. The relative gene expression was determined using the 2ΔΔCt method [32].

2.4. Statistical analysis

All statistical analyses were carried out using SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA) with the level of significance set at <0.05. The social behavioural variables were analyzed using hierarchical cluster and multivariate discriminant analyses (see socially stressed mice). The physiological variables were analyzed using multivariate analyses of variance (MANOVAs), with compliance with the criteria of normality and homogeneity being taken into consideration. Outliers (2 subjects) were eliminated from all analyses in accordance with the boxplot outlier labelling rule. When appropriate, specific comparisons were made using the Bonferroni correction. Finally, the relationships between mRNA measures in each brain structure were examined using Pearson's correlation coefficient and the Bonferroni correction.

3. Results

3.1. Analysis of behavioural profiles during chronic defeat

A cluster analysis using the mean percentage of time spent on each assessed behavioural element was performed on all defeated mice on day 21 to separate the mice into groups based on the behavioural characteristics they demonstrated during the social confrontation. This analysis resulted in two final clusters. The multivariate discriminant analysis confirmed the statistical validity of the established groups and accounted for 94.5% of the cases obtained by the cluster solution, thus confirming the behavioural descriptions. The variables that best discriminated between the two clusters were non-social and social exploration. Cluster 1 (n=39), which was designated the "active group", was characterized by a higher exploratory behaviour. Cluster 2 (n=40) was characterized by lower levels of social and non-social exploration. The group of mice that belonged to this second cluster was termed the "passive group". The passive subjects had higher levels of immobility ($F[1,77]=9.568$; $p<0.001$), avoidance/flee ($F[1,77]=13.813$; $p<0.001$) and defence/submission ($F[1,77]=21.603$; $p<0.001$) and lower levels of non-social exploration ($F[1,77]=85.268$; $p<0.001$), social exploration ($F[1,77]=10.965$; $p<0.001$), exploration at a distance $F[1,77]=4.060$; $p<0.05$), and digging/self-grooming ($F[1,77]=10.597$; $p<0.05$) than their more active counterparts (Figure 2).

3.2. Analysis of physiological parameters

3.2.1. *Proinflammatory cytokine mRNA expression in the hypothalamus, hippocampus, amygdala and prefrontal cortex*

When the expression levels of hypothalamic cytokines were analyzed, the analysis of variance of the data obtained on day 23 revealed that there was a significant effect on the expression levels of IL-6 ($F(2, 51) = 3.201$; $p < 0.05$) and IL-1β ($F(2, 51) = 3.373$; $p < 0.05$), but not TNF-α mRNA levels. The post hoc analysis indicated that the passive subjects had higher IL-6 expression levels than the active subjects ($p < 0.05$) and higher IL-1β expression levels than the manipulated controls ($p < 0.05$) (Figure 3). The analysis of the data obtained on day 24, one hour after the defeat, revealed no significant differences in the expression levels of the hypothalamic cytokines studied (Figure 3).

In the case of the values obtained in the amygdala on day 23, the analysis of variance showed a significant effect on IL-6 expression levels ($F(2, 51) = 3.920$; $p < 0.05$), although no significant differences were observed for TNF-α and IL-1β expression levels. The passive subjects had higher proinflammatory cytokine expression levels than animals that exhibited an active strategy ($p < 0.01$). However, on the 24th day after the defeat, no significant differences were found in the expression levels of the three proinflammatory cytokines in the amygdala (Figure 3).

In the prefrontal cortex, no significant differences in the IL-6 and IL-1β expression levels were detected on day 23. However, in the case of TNF-α expression, the data revealed a significant effect ($F(2, 51) = 3.440$; $p < 0.05$). Specifically, the active subjects had lower expression levels of the proinflammatory cytokine than the manipulated controls ($p < 0.05$) (Figure 3). With respect to the values obtained on day 24 after the social defeat, the analysis revealed a significant effect in the case of TNF-α expression in the prefrontal cortex ($F(2, 52) = 4.860$; $p < 0.05$). The passive subjects had lower TNF-α expression levels than the manipulated controls ($p < 0.05$) (Figure 3). No significant differences in the IL-6 and IL-1β expression levels were detected on the 24th day after the defeat.

When analyzing the data obtained for the expression levels of cytokines in the hippocampus on day 23, a significant effect was observed only in the case of IL-1β expression ($F(2, 51) = 4.390$; $p < 0.05$). The post hoc analysis revealed that passive subjects had higher IL-1β expression levels than both subjects with an active strategy ($p < 0.05$) and the manipulated controls ($p <0.05$) (Figure 3). No significant differences were detected between the control, passive and active groups as a function of the expression levels of the other two cytokines. With respect to the data obtained on day 24, the analysis of variance revealed significant differences in the expression levels of IL-1β ($F(2, 52) = 3.644$; $p < 0.05$) and IL-6 ($F(2, 52) = 4.939$; $p < 0.05$).

Specifically, subjects with a passive strategy exhibited higher expression levels of these two cytokines with respect to the manipulated controls ($p < 0.05$ for both cytokines) (Figure 3). No significant differences in TNF-α expression were detected on the 24th day after the defeat.

3.2.2. *Adrenergic receptor mRNA expression in the prefrontal cortex*

When analyzing the expression levels of $\alpha 1_b$ and $\alpha 2_a$ adrenergic receptors in the prefrontal cortex of subjects sacrificed on day 23, a significant effect was observed only in the case of the $\alpha 2_a$ receptor ($F(2, 51) = 3.676$; $p < 0.05$). The post hoc analysis revealed that active subjects have lower receptor $\alpha 2_a$ expression levels than individuals with a passive strategy ($p < 0.05$) (Figure 4). With respect to the data obtained on day 24 after the social defeat, significant effects on the expression levels of both adrenergic receptors, $\alpha 1_b$ ($F(2, 52) = 7.194$; $p < 0.01$) and $\alpha 2_a$ ($F(2, 52) = 3.548$; $p < 0.05$), were detected. Specifically, subjects with a passive strategy had lower expression levels of both receptors than the active subjects ($\alpha 1_b$: $p < 0.05$; $\alpha 2_a$: $p < 0.01$) (Figure 4).

3.2.3. *iNOS mRNA expression in the prefrontal cortex*

The analysis revealed significant differences in iNOS expression in the prefrontal cortex on day 23, two days after the last social confrontation ($F(2, 51) = 3.278$; $p < 0.05$). Active subjects had lower iNOS expression levels than the manipulated controls ($p < 0.05$) (Figure 5). With respect to the values obtained on day 24 after the social defeat, the analysis revealed no significant differences in the iNOS expression levels in the prefrontal cortex of any of the study groups.

3.3. Correlations between physiological variables studied in the prefrontal cortex

With respect to day 23, after applying the Bonferroni correction, the study of the relationships of the adrenergic receptors in the prefrontal cortex showed that expression levels of both adrenergic receptors are positively correlated with each other ($r = 0.603$, $p < 0.0001$). Positive correlations were also identified between the expression levels of IL-1β and TNF-α ($r = 0.690$, $p < 0.0001$), and IL-6 and TNF-α ($r = 0.467$, $p < 0.001$). Additionally, the study of the correlations between adrenergic receptor and the cytokine expression levels revealed that $\alpha 1_b$ adrenergic receptor expression positively correlated with IL-1β ($r = 0.622$, $p < 0.0001$) and TNF-α ($r = 0.544$, $p < 0.0001$) expression. Positive correlations were identified between the expression levels of the $\alpha 2_a$ adrenergic receptor and IL-1β ($r = 0.633$, $p < 0.0001$) and TNF-α ($r = 0.699$, $p < 0.0001$). Positive relationships were also detected between both $\alpha 1_b$ adrenergic receptor and iNOS expression levels ($r = 0.447$, $p < 0.001$) and $\alpha 2_a$ adrenergic receptor and iNOS expression levels ($r = 0.635$, $p < 0.0001$). Furthermore, some cytokine expression levels studied correlated positively with iNOS expression (IL1β: $r = 0.508$, $p < 0.0001$; TNF-α: $r = 0.700$, $p < 0.0001$) (Table 2).

With respect to the expression levels measured on day 24 after the defeat, after applying the Bonferroni correction, positive correlations between the adrenergic receptors ($r = 0.614$, $p = 0.0001$) were observed. Furthermore, adrenergic receptor expression levels correlated positively with iNOS expression ($\alpha 1_b$: $r = 0.399$, $p < 0.001$; $\alpha 2_a$: $r = 0.475$, $p < 0.0001$) (Table 3).

4. Discussion

The results obtained in this study reveal for the first time that chronic social stress triggers different dynamic responses in the immune system, in the central noradrenergic system, and in iNOS expression levels at the central level as a function of the type of coping strategy adopted by the individual. In this study, two behavioural profiles were differentiated in response to repeated defeat experiences for 21 days. Subjects with passive behavioural profiles were characterized by lower social and non-social exploration than active subjects. Passive subjects also expressed greater immobility, avoidance/escape and defence/submission.

Previous work in our laboratory using the same paradigm showed that chronic social stress resulted in increased expression levels of different peripheral proinflammatory cytokines, depending on the coping strategy used [4]. In the present study, social stress was also accompanied by changes in the expression of various cytokines at the central level, with these changes differing in accordance with the coping strategy adopted. Moreover, such changes varied depending on the cytokine and the cerebral structure analyzed. These results agree with those reported by other authors [6,10]. Despite the limitations imposed by the fact that protein levels were not measured, the data seem to indicate that a greater immune disturbance occurred in the case of passive subjects than in the case of active subjects. Passive subjects at rest, after 21 days of repeated defeat, showed higher IL-1β expression levels in the hypothalamus compared to the manipulated controls. Passive subjects also showed higher IL-6 expression levels in the hypothalamus and amygdala than active subjects and also presented increased IL-1β expression levels in the hippocampus compared to the manipulated controls and active subjects. The fact that these changes were observed three days after the last defeat could reflect a loss in regulatory capacity, and therefore a worse adaptive response to the effects of chronic stress, which was more evident in passive subjects. Increased levels of proinflammatory cytokines fulfil an adaptive function in response to one-off stressful events [17,33]; however there is increasing evidence that the persistence of these levels could produce alterations in neural activity, contributing to behaviours associated with different disorders, as measured by immobility in the forced swimming test and tail suspension test, among others [13,34,35,36].

Faced with a new defeat three days after the stress period, passive subjects continued to exhibit a greater alteration in the cytokine expression levels, with increases in IL-6 and IL-1β

expression in the hippocampus compared with the manipulated controls and the active subjects. Thus, the hippocampus was the only structure in which the proinflammatory effect of re-exposure to social stress was observed. Moreover, these findings indicate that the additional stress affects the expression of proinflammatory cytokines to a lesser extent in active subjects. It is not clear how specific cytokine changes in certain structures translate into specific behavioural changes. In this sense, it is interesting to note the increased expression of hippocampal IL-1β, both at rest and after a new defeat in subjects with a passive strategy, as persistently increased expression of this cytokine could affect neuronal plasticity [37] in this structure, producing deteriorations in the learning and memory processes [38], which are often affected in depression [39]. The increase in hippocampal IL-6 expression observed after further exposure to defeat in the passive subjects could contribute to the damage of this structure [40]. Furthermore, some studies suggest that IL-6 in the amygdala and hippocampus is involved in immobility behaviour in the forced swimming test [41].

Although several studies have reported increased TNF-α expression in animal models of depression, as well as increased peripheral levels of this cytokine [4,9,42], there are also studies that, just like this present study, detected a decrease in TNF-α expression in diverse stress situations [8,14]. Interestingly, there are data supporting the vital role of TNF-α, as well as other proinflammatory cytokines, in the development and functioning of the central nervous system [17,43]. Consistent levels of TNF-α are needed for neurogenesis and neurotrophin expression [44], such that low levels can affect behaviour. In our work, we detected reduced levels of TNF-α in the prefrontal cortices of the active subjects at rest (also in the passive subjects, though not reaching a significant level) and in the passive subjects after the application of additional stress (also in the active subjects, though not reaching a significant level), which could favour the development of anxious depressive behaviours [45] in both groups. Recent studies have found a negative correlation between TNF-α levels and symptoms related to chronic stressful experiences in depressed patients [46].

The reason why proinflammatory cytokine expression varies in accordance with cerebral area and the subject's situation (at rest/response to additional stress) is not clear. It is known that the modulation of cytokine expression through signalling pathways activated by stress (the hypothalamic-pituitary-adrenal axis and the catecholaminergic system) is specific to the structure, cell type and gene type upon which they act, and this may be the reason why peripheral cytokine levels and their expression at the central level differ [47,48,49,50]. Nevertheless, other factors, such as differences in the onset of the expression of different cytokines in response to stress, cannot be dismissed [10,49]. In short, the role of proinflammatory cytokines in the response to psychological stress may be more complex than

anticipated and further research is required to understand pro-inflammatory brain effects in response to psychological stress.

No changes were observed in the expression of adrenergic receptors in the prefrontal cortex as a function of chronic defeat stress, but there were differences when subjects with different behavioural strategies were compared. Specifically, the results indicate opposite changes in the expression of adrenergic receptors depending on the behavioural profile of the subjects and the situation considered (rest/response to additional stress). Thus, the active subjects showed lower alfa2 receptor resting expression levels than the passive subjects, which may indicate a down-regulation as a consequence of greater noradrenergic activity after 21 days of stress [51]. However, after a new stress on day 24, these subjects had greater adrenergic receptor $\alpha 1_b$ and $\alpha 2_a$ expression levels in relation to the passive subjects, which might suggest a decreased central noradrenergic response to additional stress in the active subjects. In this regard, Flugge et al. [20] also reported transient changes in the regulation of adrenergic receptors by chronic psychosocial stress, even though in the case of the $\alpha 2_c$, $\beta 1$ and $\beta 2$ receptors. Although the differences between the $\alpha 1_b$ receptors relative to the strategies are only observed on day 24, after the additional stress, there is a high positive correlation in the expression of both types of receptors in both situations (rest/response to additional stress), suggesting that these receptors respond in the same way in both situations. These results could indicate different central noradrenergic activities as a function of strategies adopted, resulting in compensatory changes in the genetic mechanisms of receptor expression that can be generated rapidly following stress.

There is evidence that the expression and function of adrenergic receptors is modulated by TNF-α [52,53] and that this cytokine inhibits the electrically stimulated release of noradrenaline in the hippocampus [52,54]. Based on this hypothesis, the correlations identified in this study could indicate that low TNF-α expression in the active subjects contributes to decreased α receptor expression, perhaps through increased noradrenaline release. However, an inverse relationship could exist, as there are data indicating that noradrenaline reduces TNF-α production through α_2 receptors [55] and that in vitro neuronal TNF-α production in the hippocampus depends on the activation of α2 receptors [56]. The lack of correlation after further stress could be due to the fact that stress may trigger other regulatory mechanisms. Thus, for example, it is known that both serotonin and glucocorticoids regulate the production of proinflammatory cytokines and that both substances are released in response to a stress challenge [57,58,59,60] Moreover, it should be borne in mind that inflammatory changes may also be triggered by beta receptors, since data exist which indicate that repeated defeats alter the inflammatory profile of the microglia through (partly at least) the activation of these receptors [61].

In this study, stress did not increase iNOS expression, in contrast to previous studies using other stress paradigms [62]. Furthermore, we observed a decrease in iNOS expression in the prefrontal cortices of active subjects at rest without changes in its expression either in the group of passive subjects or after the application of additional stress. Because iNOS expression is induced by inflammatory cytokines, including TNF-α [63], one would not expect an increase in its expression, as IL-6 and IL-1β expression levels were not increased in the prefrontal cortices of stressed subjects and as TNF-α expression was reduced in active subjects at rest. Therefore, the data appear to indicate that the observed changes in the passive subjects do not involve the arginine-nitric oxide pathway. However, it would be interesting to investigate the availability of the BH4 cofactor for the synthesis of noradrenaline in stressed subjects, since the correlation data indicate that low iNOS expression levels are accompanied by low adrenergic receptor expression. It should also be noted that there is evidence that a deficiency in cerebral iNOS or decreased NO production can be harmful for cerebral function and can produce changes in behaviour [64,65].

Considering the results, we can conclude that the increase of proinflammatory cytokines in passive subjects, particularly in the hippocampus, appears to indicate that this strategy is more vulnerable to the effects of stress. In addition to these changes, changes in TNF-α and those reported in previous studies in passive subjects [4,13] constitute a biological profile that may be more vulnerable to developing stress-related disorders. The data on adrenergic receptor expression also suggest a different noradrenergic response in accordance with the coping strategy adopted. However, it would be necessary to analyze the turnover of noradrenaline and receptor activity to confirm the existence of such differences. Moreover, the changes in TNF-α and iNOS expression detected in active subjects suggest that an active strategy also entails a cost for the organism. These data can contribute to identifying ways in which cytokines alter behaviour, thus improving our understanding of the mechanisms underlying individual vulnerabilities to the development of specific chronic stress-induced disorders. However, more research is required, particularly into adrenergic receptor and iNOS expression levels in other structures, and the possible alterations in serotonin or other potential pathways involved in the effects of cytokines on behaviour.

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Disclosure Statement

All authors declare that they have no conflicts of interest.

References

[1] Dunjic-Kostic B, Ivkovic M, Radonjic NV, Petronijevic ND, Pantovic M, Damjanovic A, et al. Melancholic and atypical major depression - Connection between cytokines, psychopathology and treatment. ProgNeuropsychopharmacolBiol Psychiatry 2013;43C:1-6.

[2] Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, et al. A meta-analysis of cytokines in major depression. Biol Psychiatry 2010;67(5):446-457.

[3] Kendler KS, Karkowski LM, Prescott CA. The assessment of dependence in the study of stressful life events: validation using a twin design. Psychol Med 1999;29(6):1455-1460.

[4] Gomez-Lazaro E, Arregi A, Beitia G, Vegas O, Azpiroz A, Garmendia L. Individual differences in chronically defeated male mice: behavioral, endocrine, immune, and neurotrophic changes as markers of vulnerability to the effects of stress. Stress 2011;14(5):537-548.

[5] Avitsur R, Powell N, Padgett DA, Sheridan JF.Social interactions, stress, and immunity.Immunol Allergy Clin North Am 2009;29(2):285-293.

[6] Goshen I, Kreisel T, Ben-Menachem-Zidon O, Licht T, Weidenfeld J, Ben-Hur T, et al. Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. Mol Psychiatry 2008;13(7):717-728.

[7] Grippo AJ, Francis J, Beltz TG, Felder RB, Johnson AK. Neuroendocrine and cytokine profile of chronic mild stress-induced anhedonia. PhysiolBehav 2005;84(5):697-706.

[8] Bartolomucci A, Sacerdote P, Panerai AE, Peterzani T, Palanza P, Parmigiani S. Chronic psychosocial stress-induced down-regulation of immunity depends upon individual factors. J Neuroimmunol 2003;141(1–2):58-64.

[9] Audet MC, Jacobson-Pick S, Wann BP, Anisman H. Social defeat promotes specific cytokine variations within the prefrontal cortex upon subsequent aggressive or endotoxin challenges. Brain BehavImmun 2011;25(6):1197-1205.

[10] Gibb J, Hayley S, Poulter MO, Anisman H. Effects of stressors and immune activating agents on peripheral and central cytokines in mouse strains that differ in stressor responsivity. Brain BehavImmun 2011;25(3):468-482.

[11] Teunis MA, Kavelaars A, Voest E, Bakker JM, Ellenbroek BA, Cools AR, et al. Reduced tumor growth, experimental metastasis formation, and angiogenesis in rats with a hyperreactive dopaminergic system. FASEB J 2002;16(11):1465-1467.

[12] Kavelaars A, Heijnen CJ, Ellenbroek B, van Loveren H, Cools A. Apomorphine-susceptible and apomorphine-unsusceptible Wistar rats differ in their susceptibility to inflammatory and infectious diseases: a study on rats with group-specific differences in structure and reactivity of hypothalamic-pituitary-adrenal axis. J Neurosci 1997;17(7):2580-2584.

[13] Perez-Tejada J, Arregi A, Gomez-Lazaro E, Vegas O, Azpiroz A, Garmendia L. Coping with chronic social stress in mice: hypothalamic-pituitary-adrenal/ sympathetic-adrenal-medullary axis activity, behavioral changes and effects of antalarmin treatment: implications for the study of stress-related psychopathologies. Neuroendocrinology 2013;98(1):73-88.

[14] Audet MC, Mangano EN, Anisman H. Behavior and pro-inflammatory cytokine variations among submissive and dominant mice engaged in aggressive encounters: moderation by corticosterone reactivity. Front BehavNeurosci 2010;4:156.

[15] Moylan S, Maes M, Wray NR, Berk M. The neuroprogressive nature of major depressive disorder: pathways to disease evolution and resistance, and therapeutic implications. Mol Psychiatry 2013;18(5):595-606.

[16] Oliveira RM, Guimaraes FS, Deakin JF.Expression of neuronal nitric oxide synthase in the hippocampal formation in affective disorders.Braz J Med Biol Res 2008;41(4):333-341.

[17] Felger JC, Lotric FE. Inflammatory cytokines in depression: neurobiological mechanisms and therapeutic implications. Neuroscience 2013;246:199-229.

[18] Mutlu O, Ulak G, Laugeray A, Belzung C. Effects of neuronal and inducible NOS inhibitor 1-[2-(trifluoromethyl) phenyl] imidazole (TRIM) in unpredictable chronic mild stress procedure in mice. PharmacolBiochemBehav 2009;92(1):82-87.

[19] Spiacci A, Kanamaru F, Guimaraes FS, Oliveira RM. Nitric oxide-mediated anxiolytic-like and antidepressant-like effects in animal models of anxiety and depression. PharmacolBiochemBehav 2008;88(3):247-255.

[20] Stone EA, Lin Y, Rosengarten H, Kramer HK, Quartermain D. Emerging evidence for a central epinephrine-innervated alpha 1-adrenergic system that regulates behavioral activation and is impaired in depression. Neuropsychopharmacology 2003;28(8):1387-1399.

[21] Flugge G, van Kampen M, Meyer H, Fuchs E. Alpha2A and alpha2C-adrenoceptor regulation in the brain: alpha2A changes persist after chronic stress. Eur J Neurosci 2003;17(5):917-928.

[22] Muguruza C, Rodriguez F, Rozas I, Meana JJ, Uriguen L, Callado LF. Antidepressant-like properties of three new alpha2-adrenoceptor antagonists. Neuropharmacology 2013;65:13-19.

[23] Garcia-Sevilla JA, Escriba PV, Ozaita A, La Harpe R, Walzer C, Eytan A, Guimon J. Up-regulation of immunolabeled alpha2A-adrenoceptors, Gi coupling proteins, and regulatory receptor kinases in the prefrontal cortex of depressed suicides. J Neurochem 1999;72(1):282-291.

[24] Gonzalez AM, Pascual J, Meana JJ, Barturen F, del Arco C, Pazos A, Garcia-Sevilla JA.Autoradiographic demonstration of increased alpha 2-adrenoceptor agonist binding sites in the hippocampus and frontal cortex of depressed suicide victims. J Neurochem 1994;63(1):256-265.

[25] Clark DM, Layard R, Smithies R, Richards DA, Suckling R, Wright B. Improving access to psychological therapy: Initial evaluation of two UK demonstration sites. Behav Res Ther 2009;47:910-920.

[26] Spijker, S. Dissection of Rodent Brain Regions.Neuroproteomics 2001;57:13-26.

[27] Paxinos G, Franklin KBJ. The Mouse Brain in Stereotaxic Coordinates. San Diego: Academic Press; 2001.

[28] Kudryavtseva NN, Bakshtanovskaya IV, Koryakina LA. Social model of depression in mice of C57BL/6J strain.PharmacolBiochemBehav 1991;38(2):315-320.

[29] Kudryatseva NN. Sensory contact model: protocol, control, applications. Nature Precedings 2009.

[30] Brain PF, McAllister KH, Walmsley S. Drug effects on social behavior: methods in ethopharmacology. In: Boulton AA, Baker GB, Greenshaw AJ, editors. Neuromethods. Totowa: Humana Press; 1989, p. 687–739.

[31] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidiniumthiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162(1):4.

[32] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):7.

[33] Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 2008;9(1):46-56.

[34] Fu X, Zunich SM, O'Connor JC, Kavelaars A, Dantzer R, Kelley KW. Central administration of lipopolysaccharide induces depressive-like behavior in vivo and

activates brain indoleamine 2,3 dioxygenase in murine organotypic hippocampal slice cultures. J Neuroinflammation 2010;7:43.

[35] Lawson MA, Parrott JM, McCusker RH, Dantzer R, Kelley KW, O´Connor JC. Intracerebroventricular administration of lipopolysaccharide induces indoleamine-2,3-dioxygenase-dependent depression-like behaviors. J Neuroinflammation 2013;10:87-96.

[36] Mello BS, Monte AS, McIntyre RS, Soczynska JK, Custódio CS, Cordeiro RC, Chaves JH, Vasconcelos SM, Nobre HV Jr, Florenço de Sousa FC, Hyphantis TN, Carvalho AF, Macêdo DS. Effects of doxycycline on depressive-like behavior in mice after lipopolysaccharide (LPS) administration. J Psychiatr Res 2013;47(10):1521-1529.

[37] Hayley S, Poulter MO, Merali Z, Anisman H. The pathogenesis of clinical depression: stressor- and cytokine-induced alterations of neuroplasticity. Neuroscience 2005;135(3):659-678.

[38] Barrientos RM, Frank MG, Hein AM, Higgins EA, Watkins LR, Rudy JW, Maier SF. Time course of hippocampal IL-1β and memory consolidation impairments in aging rats following peripheral infection. Brain BehavImmun 2009;23(1):46-54

[39] Song C, Wang H. Cytokines mediated inflammation and decreased neurogenesis in animal models of depression. ProgNeuropsychopharmacolBiol Psychiatry 2011;35(3):760-768.

[40] Allan SM, Rothwell NJ. Cytokines and acute neurodegeneration. Nat Rev Neurosci 2001;2(10);734-744.

[41] Wu TH, Lin CH. IL-6 mediated alterations on immobile behavior of rats in the forced swim test via ERK1/2 activation in specific brain regions. Behav Brain Res. 2008;193(2):183-191.

[42] You Z, Luo C, Zhang W, Chen Y, He J, Zhao Q, et al. Pro- and anti-inflammatory cytokines expression in rat's brain and spleen exposed to chronic mild stress: involvement in depression. Behav Brain Res 2011;225(1):135-141.

[43] Camara ML, Corrigan F, Jaehne EJ, Jawahar MC, Anscomb H, Koerner H, et al. TNF-alpha and its receptors modulate complex behaviours and neurotrophins in transgenic mice. Psychoneuroendocrinology 2013;38(12):3102-3114.

[44] Golan H, Levav T, Mendelsohn A, Huleihel M. Involvement of tumor necrosis factor alpha in hippocampal development and function. Cereb Cortex 2004;14(1):97-105.

[45] Yamada K, Iida R, Miyamoto Y, Saito K, Sekikawa K, Seishima M, et al. Neurobehavioral alterations in mice with a targeted deletion of the tumor necrosis factor-alpha gene: implications for emotional behavior. J Neuroimmunol 2000;111(1-2):131-138.

[46] Bizik G, Bob P, Raboch J, Pavlat J, Uhrova J, Benakova H, et al. Dissociative symptoms reflect levels of tumor necrosis factor alpha in patients with unipolar depression. Neuropsychiatr Dis Treat 2014;10:675-679.

[47] Rohleder N. Variability in stress system regulatory control of inflammation: A critical factor mediating health effects of stress. Expert Rev Endocrinol Met 2011;6(2):269-278.

[48] Silverman MN, Pearce BD, Biron CA, Miller AH. Immune modulation of the hypothalamic-pituitary-adrenal (HPA) axis during viral infection. Viral Immunol 2005;18(1):41-78.

[49] Johnson JD, Campisi J, Sharkey CM, Kennedy SL, Nickerson M, Greenwood BN, Fleshner M. Catecholamines mediate stress-induced increases in peripheral and central inflammatory cytokines. Neuroscience 2005;135(4):1295-1307.

[50] Kolmus K, Tavernier J, Gerlo S. β2-Adrenergic receptors in immunity and inflammation: stressing NF-KB. Brain BehavImmun 2015;45:297-310.

[51] Soares J, Holmes PV, Renner KJ, Edwards GL, Bunnell BN, Dishman RK. Brain noradrenergic responses to footshock after chronic activity-wheel running.BehavNeurosci 1999;113(3):558-566.

[52] Reynolds JL, Ignatowski TA, Sud R, Spengler RN. An antidepressant mechanism of desipramine is to decrease tumor necrosis factor-alpha production culminating in increases in noradrenergic neurotransmission. Neuroscience 2005;133(2):519-531.

[53] Hadri KE, Courtalon A, Gauthereau X, Chambaut-Guerin AM, Pairault J, Feve B. Differential regulation by tumor necrosis factor-alpha of beta1-, beta2-, and beta3-adrenoreceptor gene expression in 3T3-F442A adipocytes. J BiolChem 1997;272(39):24514-24521.

[54] Elenkov IJ, Kovacs K, Duda E, Stark E, Vizi ES. Presynaptic inhibitory effect of TNF-alpha on the release of noradrenaline in isolated median eminence. J Neuroimmunol 1992;41(1):117-120.

[55] Maes M, Lin A, Kenis G, Egyed B, Bosmans E. The effects of noradrenaline and alpha-2 adrenoceptor agents on the production of monocytic products. Psychiatry Res 2000;96(3):245-253.

[56] Renauld AE, Spengler RN. Tumor necrosis factor expressed by primary hippocampal neurons and SH-SY5Y cells is regulated by alpha(2)-adrenergic receptor activation. J Neurosci Res 2002;67(2):264-274.

[57] Tynan RJ, Weidenhofer J, Hinwood M, Cairns MJ, Day TA, Walker FR. A comparative examination of the anti-inflammatory effects of SSRI and SNRI antidepressants on LPS stimulated microglia. Brain Behav Immun. 2012;26:469–479.

[58] Ahern GP. 5-HT and the immune system. Curr Opin Pharmacol 2011;11(1):29-33

[59] Garcia-Bueno B, Caso JR, Leza JC. Stress as a neuroinflammatory condition in brain: damaging and protective mechanisms. Neurosci Biobehav Rev 2008;32(6):1136-1151.

[60] Artz E, Costas M, Finkielman S, Nahmod VE. Serotonin inhibition of tumor-necrosis-factor-alpha synthesis by human monocytes. Life Sci 1991;48(26):2557-2562.

[61] Hanke ML, Powell ND, Stiner LM, Bailey MT, Sheridan JF. Beta adrenergic blockade decreases the immunomodulatory effects of social disruption stress. Brain Behav Immun. 2012;26:1150–1159.

[62] Peng YL, Liu YN, Liu L, Wang X, Jiang CL, Wang YX. Inducible nitric oxide synthase is involved in the modulation of depressive behaviors induced by unpredictable chronic mild stress. J Neuroinflammation 2012;9:75.

[63] Munhoz C, Madrigal JL, Garcia-Bueno B, Pradillo JM, Moro MA, Lizasoain I, et al. TNF-alpha accounts for short-term persistence of oxidative status in rat brain after two weeks of repeated stress. Eur J Neurosci 2004;20(4):1125-1130.

[64] Yermolaieva O, Brot N, Weissbach H, Heinemann SH, Hoshi T. Reactive oxygen species and nitric oxide mediate plasticity of neuronal calcium signaling. ProcNatlAcadSci USA 2000;97(1):448-453.

[65] Nelson RJ, Kriegsfeld LJ, Dawson VL, Dawson TM.Effects of nitric oxide on neuroendocrine function and behavior. Front Neuroendocrinol 1997;18(4):463-491.

Figure 1. Schematic representation of the experimental procedure. (#) The following samples were obtained: hypothalamus, hippocampus, amygdala and prefrontal cortex.

Figure 2. The time (means ± SEM) dedicated to each of the behaviours evaluated during the social defeat on day 21, analyzed in terms of group membership: passive subjects (n = 40) and active subjects (n = 39). *p< 0.05; ***p < 0.001

Figure 3. A) Mean levels (± SEM) of IL-6, IL-1β and TNF-α gene expression in the hypothalami of active subjects, passive subjects and manipulated controls sacrificed on days 23 and 24 following the defeat. B) Mean levels (± SEM) of IL-6, IL-1β and TNF-α gene expression in the amygdala of active subjects, passive subjects and manipulated controls sacrificed on days 23 and 24 following the defeat. C) Mean levels (± SEM) of IL-6, IL-1β and TNF-α gene expression in the hippocampi of active subjects, passive subjects and manipulated controls sacrificed on days 23 and 24 following the defeat. D) Mean levels (± SEM) of IL-6, IL-1β and TNF-α gene expression in the prefrontal cortices of the active subjects, passive subjects and manipulated controls sacrificed on days 23 and 24 following the defeat. *p< 0.05; **p < 0.01

Figure 4. Mean levels (± SEM) of $\alpha 1_b$-adrenoceptor and $\alpha 2_a$-adrenoceptor gene expression in the prefrontal cortices of active subjects, passive subjects and manipulated controls sacrificed on days 23 and 24 after the defeat. *p< 0.05; **p < 0.01

Figure 5. Mean levels (± SEM) of iNOS gene expression in the prefrontal cortices of active subjects, passive subjects and manipulated controls sacrificed on days 23 and 24 following the defeat. *p< 0.05