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Microglial activation, increased TNF and SERT expression in the prefrontal cortex define stress-altered behaviour in mice susceptible to anhedonia

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ABSTRACT

A chronic stress paradigm comprising exposure to predation, tail suspension and restraint induces a depressive syndrome in C57BL/6J mice that occurs in some, but not all, animals. Here, we sought to extend our behavioural studies to investigate how susceptibility (sucrose preference < 65%) or resilience (sucrose preference > 65%) to stress-induced anhedonia affects the 5HT system and the expression of inflammation-related genes. All chronically stressed animals, displayed increased level of anxiety, but susceptible mice exhibited an increased propensity to float in the forced swim test and demonstrate hyperactivity under stressful lighting conditions. These changes were not present in resilient or acutely stressed animals. Compared to resilient animals, susceptible mice showed elevated expression of tumour necrosis factor alpha (TNF) and the 5-HT transporter (SERT) in the pre-frontal area. Enhanced expression of $5HT_{2A}$ and COX-1 in the pre-frontal area was observed in all stressed animals. In turn, indoleamine-2, 3-dioxygenase (IDO) was significantly unregulated in the raphe of susceptible animals. At the cellular level, increased numbers of Iba-1-positive microglial cells were also present in the prefrontal area of susceptible animals compared to resilient animals. Consequently, the susceptible animals display a unique molecular profile when compared to resilient, but anxious, animals. Unexpectedly, this altered profile provides a rationale for exploring anti-inflammatory, and possibly, TNF-targeted therapy for major depression.

1. Introduction

Major depression, a common and recurrent disorder, is associated with considerable morbidity and increased mortality. Indeed, major depression has been projected to become the second leading cause of disability worldwide by 2020. In the human population, it is clear that some individuals are more susceptible to depression than others and this can be explained only in part by genetic factors. However, the neurochemical and cellular changes that accompany such altered susceptibility remain unclear, and any studies seeking to examine alterations in the biochemical profile within the brains of such individuals are difficult to achieve. A recent approach has been to isolate the signs of major depression, highlighted in DSM-IV (APA, 2000; Hamilton, 1967), and study them individually in animal models. Hedonic deficit, the inability to experience pleasure from activities normally thought to be enjoyable, is a key criterion of major depressive syndrome. In rodent models, this can be tested using a preference for sweetened drinking water. Repeated application of mild stressors, such as exposure to predation, restraint, soiled cages, and others over an extended period of time has been shown to reduce this preference (Katz et al., 1981; Strekalova et al., 2004; Willner et al., 1996). While this model shows both face validity and predictive validity in terms of depression, there is remarkable inter-individual variation, even in inbred animals, in vulnerability to develop the characteristics of anhedonia. The approach (Piazza et al., 1991) used in the past to deal with such variation has been to generate a subgroup of individuals as an internal control, which are negative for the induction of a depressive phenotype. There remains a need to characterise the molecular changes which underpin the variation in anhedonia observed in such genetically similar animals.

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Some of the molecular correlates of stress and stress-induced depression are now beginning to be uncovered. Studies in rats undergoing chronic stress in the form of social defeat, where stressed and resilient animals are separated, were able to identify the groups based on both gene expression arrays (principally Gsk3b and Map1b) and on decreased regional metabolism (Kanarik et al., 2011). Another study on out-bred CD1 mice showed that stress susceptible and resilient animals had different corticosterone levels at 5 weeks post-stress (Schmidt et al., 2010). Corticosterone is a key regulator of the immune response, a system which is engendering interest as a regulator of mood (Raison et al., 2006), but from these studies it is unclear whether corticosterone levels might define stress susceptible and resilient animals in inbred mice. Despite these studies, work on the rodent 5-HT system in the chronic stress model is scarce. In humans, mRNA editing of 5-HT receptors in a number of key regions relating to mood regulation, have shown that this process is important in regulating antidepressant activity within the brain (Mombereau et al., 2010). Furthermore, human suicide victims, and depressed patients, frequently show changes within the genes of the 5-HT receptor network of the prefrontal cortex (Anisman et al., 2008; Stanley and Mann, 1983). It is therefore not unreasonable to assume that similar changes occur in rodents, however, these are yet to be investigated.

Interest in the contribution of cytokine and cyclooxygenase activity in the pathogenesis of major depression has also grown over the years. Animals exhibit sickness-related behaviours in response to experimental challenges that induce cytokine expression in the brain; and this has been likened to malaise in humans (Dantzer, 2009; Swain et al., 1998). Parallels clearly exist in humans where interferon therapy is known to induce transient signs of depression or malaise (Dantzer et al., 2011). Perhaps more relevant, cytokines, such as interleukin-1 (IL-1β) and tumour necrosis factor alpha (TNF), have also been shown to contribute to depression-like behaviours after chronic stress in rats (Goshen et al., 2008). Such work has been very important in highlighting the contribution that cytokines might play in depression, but, hitherto, most work has not sought to investigate whether cytokine or cyclooxygenase expression might segregate with resilience or susceptibility in subgroups of animals. On this basis, we have used a new variant of a recently validated mouse model of stress-induced anhedonia (Cline et al., 2012; Katz et al., 1981; Strekalova et al., 2011) to investigate whether 5-HT related gene expression and markers of inflammation can be used to define animals that are resilient or susceptible to the anhedonia induced by chronic stress.

2. Materials and methods

2.1. Animals

Studies were performed using 3.5-month-old male C57BL/6J mice. 3.5-month-old male CD1 mice were used as resident intruders for social stress and 2–5-month-old Wistar rats were used for predator stress. All animals were from the Gulbenkian Institute of Science, Oeiras, Portugal. C57BL/6J mice were housed individually for 10–14 days before the start of experiments; CD1 mice and rats were housed in groups of five before the experiment and then individually. All animals were under a reversed 12-h light–dark cycle (lights on: 21:00 h) with food and water *ad libitum*, under controllable laboratory conditions (22 ± 1 °C, 55% humidity). All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals upon approval by local governmental bodies for animal care and welfare.

2.2. Chronic stress procedure

This study uses a recently validated variant of a 10-day stress protocol (Strekalova et al., 2011) comprising night time rat exposure and day time application of two of three stressors: a social defeat, restraint stress and tail suspension, a combination of which was applied in a semi-random manner (for details see Supplementary Material). Briefly, between the hours of 09:00 and 18:00 two stressors per day were employed in the following sequence: social defeat for 30 min, restraint stress for 2 h and tail suspension for 40 min with an inter-session interval of at least 4 h. This procedure induces anhedonia in a considerably shorter time than previously validated models by increasing the daytime stress load. Details of rat exposure and chronic stressors can be found in supplementary materials.

2.3. Acute stress control

While all assays on chronically stressed mice were carried out at least 12 h after the termination of the last predator (rat) stress session, the effects of acute stress were controlled using the acute stress control group. This group underwent predator stress the night prior to behavioural testing.

2.4. Sucrose preference test

Mice were given 8 h of free choice between two bottles of either 1% sucrose or standard drinking water at day −7 and day 10 (Fig. 1). At the beginning and end of the period the bottles were weighed and consumption calculated. The beginning of the test started with the onset of the dark (active) phase of animals' cycle. To prevent the possible effects of side-preference in drinking behaviour, the position of the bottles in the cage was switched at 4 h, halfway through testing. No previous food or water deprivation was applied before the test. Other conditions of the test were applied as described elsewhere (Strekalova and Steinbusch, 2010). The 1%-sucrose solution is used in tests performed during baseline and chronic stress application. Percentage preference for sucrose is calculated using the following formula: Sucrose preference = Volume (Sucrose solution)/(Volume (Sucrose solution) + Volume (Water)) × 100. No mice from control groups ever exhibited a preference for sucrose of <65% and, accordingly, mice exhibiting a sucrose preference of <65% were defined as susceptible. Mice that had undergone stress but maintained a sucrose preference of >65% were defined as resilient.

2.5. Forced swim test

The Porsolt forced swim test (FST) has been modified to prevent behavioural artifacts caused by stress-induced hyperlocomotion (Strekalova et al., 2011, 2005). Mice were placed into a transparent pool (20 × 35 × 15 cm) lit with red light and filled with warm water (30 °C, to a depth of 9.5 cm) for 2 min. Floating behaviour was defined as the absence of directed movements of animals' head and body, and was measured by visual observation that was validated previously by automated scoring with CleverSys software (CleverSys, VA, US; (Malatynska et al., 2012)). Latency to begin floating was scored as time between introduction of the animal into the pool and the first moment of complete immobility of the entire body for a duration of >3 s. The total time spent floating was scored for the entire duration of the test using post-test video footage.

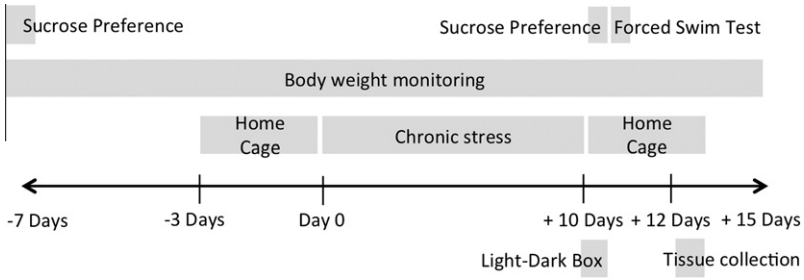

Fig. 1. Schematic timeline of the chronic stress experiments.

2.6. Dark–light activity

Anxiety was measured using a dark–light box on day 11. The dark–light box consisted of two equal Plexiglas compartments; one dark, and one light (5 LUX) connected by a tunnel. Mice were placed into the dark compartment, from where they could visit the lit box. Total duration of time spent in the lit box and number of visits to this compartment were scored over 5 min.

2.7. Monitoring of home-cage activity

Home-cage activity was monitored in animals using the SAMAB (system for automated measurement of behaviour (in-house software). Infrared beams monitored horizontal activity in the home-cage environment, allowing animals' access to food and water *ad libitum* and also allowing normal behaviour to be monitored over a number of days. For specific details of system set-up refer to Supplementary Material.

2.8. Brain dissection and quantitative RT-PCR (qPCR)

Mice were killed by cervical dislocation. RNA extraction was performed as previously described from specifically microdissected snap-frozen brain regions (Blond et al., 2002; Campbell et al., 2005). Standard curves were generated using previously generated samples to enable normalisation to the housekeeping gene glyceraldehyde-3-phosphate (GAPDH) using the Pfaffle method (Pfaffl, 2001). Details of primers appear below (see Table 1). qPCR was run using SYBR green based technology (Primer Design Ltd., Southampton, UK). Results are expressed as relative-fold compared to control animals.

2.9. Immunohistochemistry

Brain tissue was collected as previously described (Couch et al., 2011). Sagittal (40 μm) sections were cut using a Leica vibratome (Leica Microsystems, Nusslock, Germany). Sections were immunostained with anti-Iba-1 (1:500, Abcam ab5076, AbCam, Cambridge, UK) in 2% normal donkey serum in 0.3% triton-X (VectorLabs, UK) at 4 °C for 12 h. visualisation was performed using biotinylated secondary antibodies and a standard avidin–biotin amplification step followed by development in a 0.05% solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB). Immunostaining was examined using a light microscope Olympus IX81, computer-controlled Märzhäuser motorized stage and digital camera Olympus DP72. An area of the prefrontal cortex was specifically delineated and Iba-1 positive cells were counted within this. The periaqueductal grey and striatal areas, which appear on the same sagittal sections, were used as internal controls for this technique. Cell counting was carried out with a software "Cell*" (Olympus Soft Imaging Solution GmbH).

2.10. Statistics

Data were analysed with GraphPad Prism version 5.0 for Windows (San Diego, CA). One-way ANOVA was used followed by a Dunnett's post hoc comparison test. For locomotor activity testing area under curve analysis was performed followed by a Student's *t*-test to compare susceptible vs. resilient animals. The level of confidence was set at 95% ($p < 0.05$) and data are shown as mean ± SEM.

3. Results

3.1. Chronic stress induces a depression-like state in a subgroup of mice

No control animals showed a preference for sucrose of <65%, and, prior to stress induction, mice assigned to the distinct experimental groups displayed similar (>65%) preference for sucrose solutions. At no point did any control animal show a change in total sucrose, total water or total liquid intake (data not shown). Ten days of chronic stress resulted in a significant decrease ($F_{1,38} = 22.81$, $p < 0.001$) in sucrose preference within a specific subgroup of animals (Fig. 2A). These animals displaying anhedonia were defined as 'susceptible'. A further subgroup of animals showed a >65% preference for sucrose, despite expose to 10 days of chronic stress, these animals were defined as 'resilient'. Interestingly, susceptible animals also displayed signs of hyperdipsia (data not shown) in combination with a decrease in sucrose preference. During baseline conditions animals showed a normal distribution of weight gain (data not shown). After 10 days of chronic stress,

Table 1
Primer sequences for qPCR. Primers were custom designed by Primer Design Ltd. (Southampton, UK).

Marker	Forward primer	Reverse primer	Amplicon size (nt)
TNFα	GCTCCCTCTCATCAGTTCTAT	TTTGCTACGADCTGGGCTA	94
IL-1β	CAACCAACAAGTGTATTCTCCAT	GTGTGCCGTCTTTCATTA	127
COX-1	TAGAAAGTTAGAGTTTTTGTGT	CACCCCTACCCTATATATAAGTT	126
IDO	TGCTTACTCTCTTTTCCCTTCC	CATCAGACCTGGTGCTTCA	87
SERT	TGCCTTTTATATCGCCTCCTAC	CAGTTGCCAGTGTTCCAAGA	127
$5HT_{2A}$	CAGGCAAGTCACAGGATAGC	TTAAGCAGAAAGAAAATCCCACAG	93

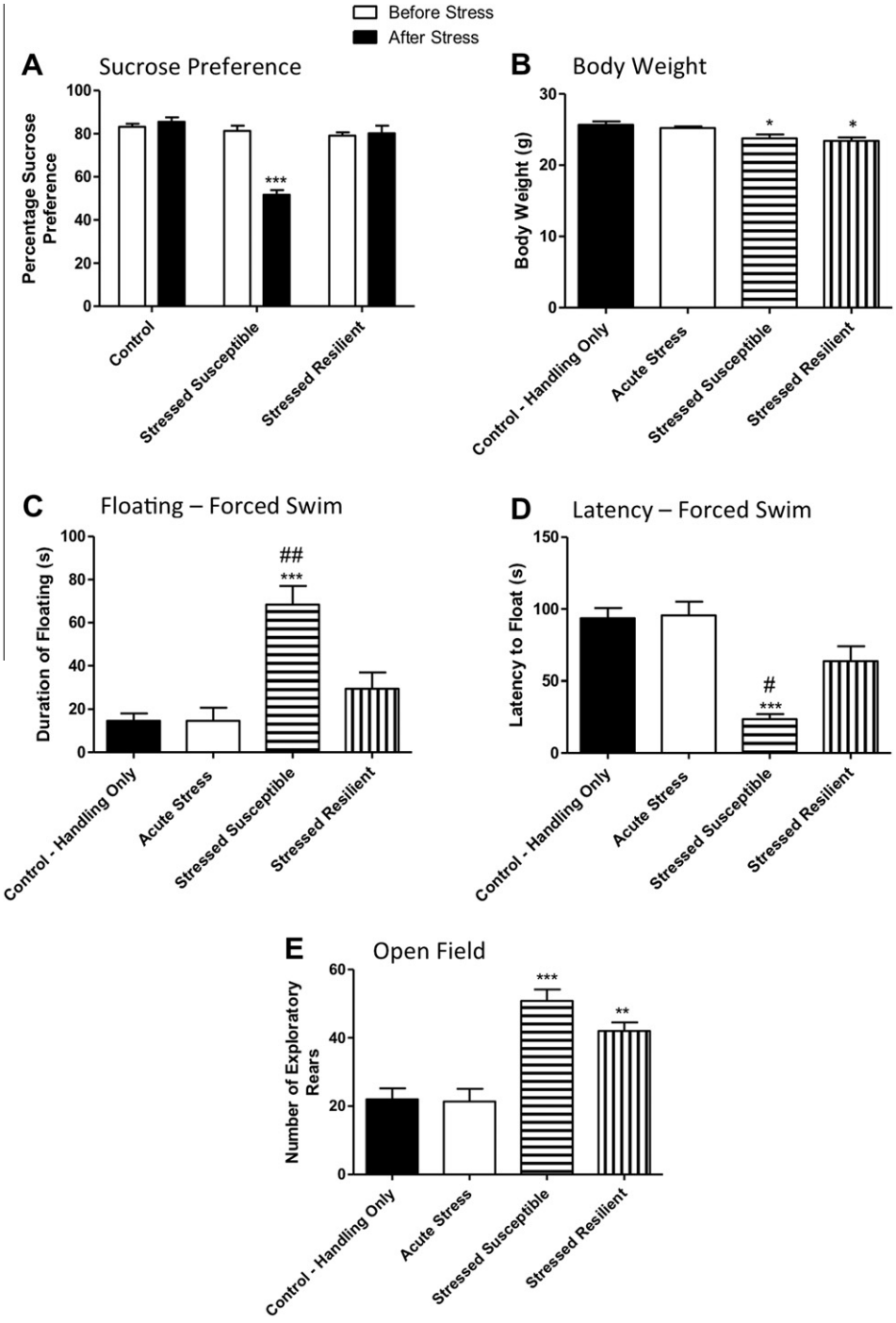

Fig. 2. Chronic stress induces physiological and behavioural changes in a subgroup of anhedonic mice, which are not evident in mice resilient to stress. Mice were divided into control (not stressed; $n = 10$), acute overnight stress (predator exposure; $n = 5$) or susceptible ($n = 6$) and resilient ($n = 6$) to 10 days of chronic stress, depending on sucrose preference levels. (A) After 10 days of chronic stress mice were defined as 'susceptible' with a sucrose preference of <65%. (B) Body weight was significantly reduced in susceptible and resilient groups compared to non-stressed control mice. (C) The duration of floating in the forced swim test was significantly longer in susceptible mice than in control and resilient animals. (D) Latency to float in the forced swim test was significantly shorter in susceptible mice than in control and resilient groups. (E) In comparison to control mice, the number of rearings in an open field was significantly increased in susceptible mice and, to lesser extent, in resilient animals. Data are mean ± SEM. $^{*} = p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control animals. $^{\#} = p < 0.05$ and $^{\#\#} = p < 0.01$ compared to resilient animals.

both susceptible and resilient animals showed a significant weight loss (Fig. 2B; one-way ANOVA $F_{3,19} = 4.489$, $p < 0.05$).

Previous studies have shown that the occurrence of stress-induced anhedonia is associated with increased 'behavioural despair' in the modified Porsolt forced swim test (FST). Behavioural despair in the FST is defined as floating behaviour, the point at which animals stop trying to escape the water. Here, susceptible animals displayed a significantly decreased latency to float compared to control animals (Fig. 2C; one-way ANOVA, $F_{3,23} = 16.56$, $p < 0.001$) as well as compared to resilient animals (Fig. 2C; $F_{3,23} = 16.56$, $p < 0.01$). Similarly, the duration of floating in susceptible animals was significantly different from both control animals (Fig. 2D; one-way ANOVA, $F_{3,23} = 16.83$ $p < 0.001$) and the resilient animals (Fig. 2D; $F_{3,23} = 16.83$ $p < 0.05$).

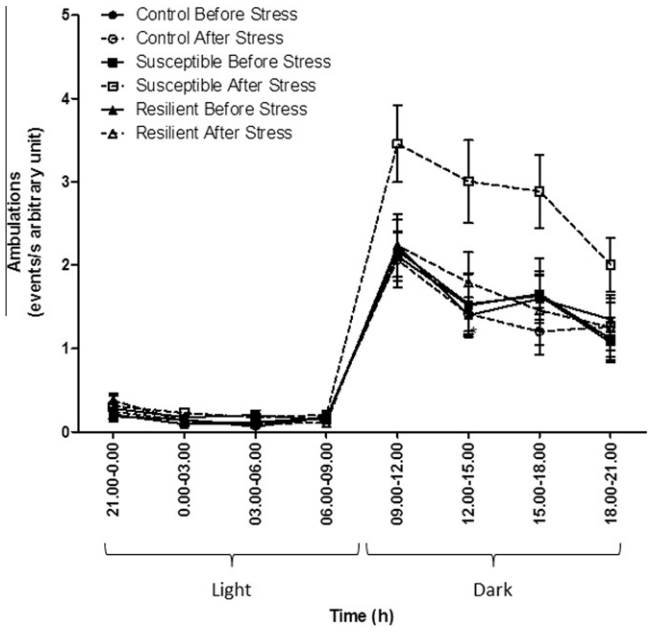

Fig. 3. Susceptible mice exhibit increased locomotor activity in the home-cage environment during the hours of darkness. Animals were subjected to chronic stress for 10 days and separated into susceptible and resilient subgroups according to sucrose preference at this time. Locomotor activity was quantified using an automated monitoring system in the home-cage environment in control animals (closed circles – before stress, open circles – after stress); susceptible animals (closed squares – before stress, open squares – after stress) and stress-resilient animals (closed triangles – before stress, open triangles – after stress). Data are mean locomotor counts/h in arbitrary units and binned into 3-h groups. Data were acquired over 3 days and averaged for each animal. Data are mean ± SEM; $n = 15$ for all groups.

Rearing in a novel environment is an exploratory behaviour and is indicative of both interest and activity. Following 10-days of stress, susceptible animals displayed an increase in rearing behaviour compared to control and acutely stressed animals (Fig. 2E; one-way ANOVA, $F_{3,18} = 21.23$, $p < 0.001$). Stress-resilient animals also displayed increased rearing behaviour compared to control and acutely stressed animals (Fig. 2E; $F_{3,18} = 21.23$, $p < 0.01$), but not when compared to susceptible animals.

3.2. Chronic stress alters home-cage activity in stress susceptible mice

As well as measuring behaviour in novel and challenging environments such as the open field and the FST, we also monitored behaviour in the home cage for 72 h prior to, and at end of the 10-day stress period. The data were averaged over the 72-h period to give overall day/night activity graphs. Control animals were inactive during the light phase of the light/dark cycle followed by an increase in activity peaking at the start of the dark phase and gradually declining across 12 h (Fig. 3). Activity in the home cage environment prior to stress in stress susceptible animals did not appear different. After the onset of stress, susceptible animals were significantly more active during the dark phase of the light/dark cycle (area under curve, $F_{2,14} = 7.96$ $p < 0.05$; Fig. 3). This increase in activity was not observed in animals that had undergone the stress procedure but had a high sucrose preference (stress-resilient animals), whose light/dark activity patterns represented those of control animals (Fig. 3).

3.3. Chronic stress causes anxiety independent of depressive-like behaviour

It was important to determine whether stress induces depression like behaviours independently of anxiety. Anxiety and depression are frequently comorbid in depressed patient and in animal models. To test this, animals were introduced into the light–dark box. After 10 days of chronic stress all animals (susceptible and resilient) showed a significantly increased latency to enter the dark box compared to control animals (Fig. 4A; one-way ANOVA, $F_{2,18} = 4.402$). Consequently, they spent significantly more time in the dark box compared to control animals (Fig. 4B; one-way ANOVA, $F_{2,16} = 5.857$, susceptible $p < 0.01$, resilient $p < 0.05$) and a decreased number of transitions between the light and dark zones (Fig. 4C; one-way ANOVA, $F_{2,18} = 3.244$, $p < 0.05$).

Stress is known to up-regulate corticosterone, and in humans, depressed patients often show dexamethasone non-suppression, indicating an overactive HPA axis and extremely high levels of circulating cortisol (Bhagwagar et al., 2002). Circulating levels of corticosterone in control animals were, on average around 10nM. In contrast, circulating corticosterone was significantly increased (~90 nM) in stress susceptible (Fig. 5; one-way ANOVA, $F_{2,16} = 7.687$, $p < 0.01$) and stress resilient (Fig. 5; $F_{2,16} = 7.687$, $p < 0.01$) animals.

3.4. Chronic stress up-regulates specific 5-HT-related genes

Given the marked behavioural differences between susceptible and resilient animals, we sought to discover whether any distinct molecular changes in the 5HT system or in pro-inflammatory cytokine expression might associate with each subset of animals. SERT expression was increased significantly in stress-susceptible animals after 10 days of chronic stress in the prefrontal cortex (one-way ANOVA: $F_{4,15} = 10.81$ $p < 0.001$), striatum ($p < 0.05$) and hippocampus ($p < 0.05$; Fig. 6B). These animals showed no significant changes in the raphe or motor cortex compared to controls. Importantly, stress resilient animals showed no significant changes in SERT expression compared to control animals. No changes in SERT expression were observed in the acutely stressed animals.

Chronic stress resulted in a significant 3-fold increase in 5-HT_{2A} expression in prefrontal cortex in all animals post-stress animals (one-way ANOVA: susceptible $F_{3,7} = 2.168$, $p < 0.01$, resilient $p < 0.05$; Fig. 6A) compared to control animals. Elsewhere, susceptible animals showed an increase in receptor expression in the striatum ($F_{3,7} = 2.785$, $p < 0.05$) compared to control animals and also showed a tendency towards increase in the raphe ($p = 0.062$). Resilient animals did not show any changes in receptor expression anywhere other than the prefrontal cortex. No changes in 5-HT_{2A} expression were observed in the acutely stressed animals.

3.5. Proinflammatory cytokines and enzymes after chronic stress

There has been increasing interest in the role of proinflammatory cytokines, and, in particular, interleukin (IL)-1 and TNF, in the aetiology and pathophysiology of major depression. Here we were interested to discover whether the expression of TNF or IL-1β mRNA might segregate with either resilient or susceptible animals. We, therefore, examined expression of these cytokines in the prefrontal cortex, striatum, hippocampus and dorsal raphe nucleus. No increase in TNF expression or IL-1β was detectable in the liver after chronic stress, which was chosen as a peripheral organ to control for evidence of infection or injury (data not shown). However, brain TNF expression was significantly increased as result of the chronic stress paradigm in the prefrontal cortex in stress susceptible animals (one-way ANOVA: $F_{3,6} = 4.02$ $p < 0.05$ Fig. 7A). TNF levels also appeared to show increases in other brain areas in stress susceptible animals, such as the motor cortex, but these increases were not statistically significant. Stress resilient animals also to show a slight increase in TNF in the striatum, but in no region were they significantly different from control animals. No significant increase in IL-1β expression was detectable in the

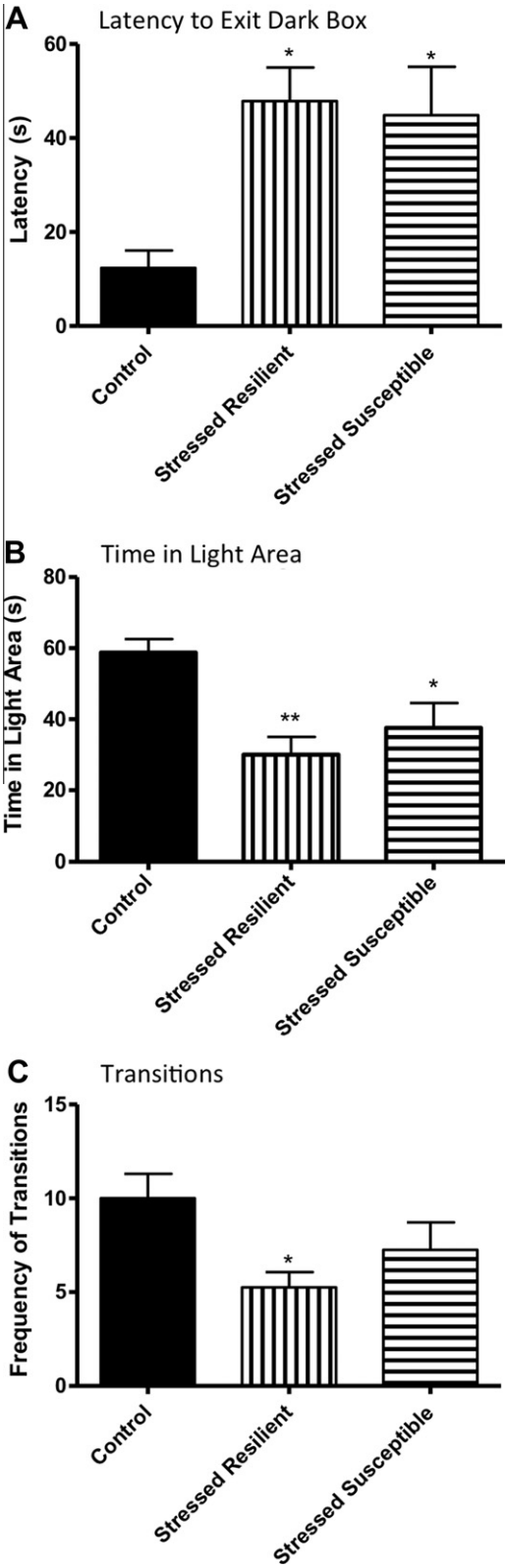

Fig. 4. Susceptible and resilient animals both display anxiety behaviour in the light–dark box. 10 days of chronic stress causes (A) an increased latency to leave the dark area in the light–dark box; (B) an increased time spent in the dark box; and (C) a decreased number of transitions between the light and dark areas in all animals. Data are mean ± SEM (control $n = 5$; stressed susceptible $n = 7$; stressed resilient $n = 7$). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control animals.

prefrontal cortex in either group. However, chronic stress likewise significantly increased IL-1β expression in the hippocampus in

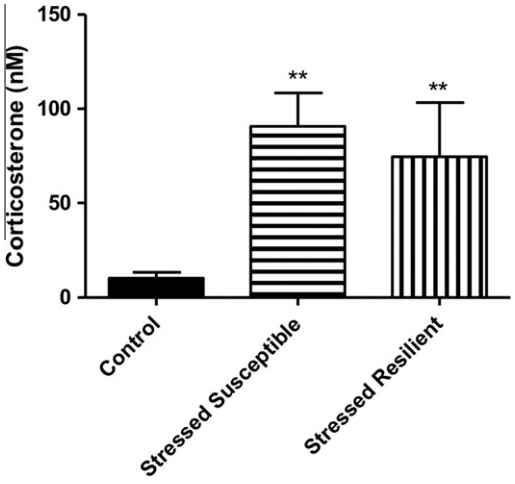

Fig. 5. Corticosterone levels following chronic stress. Plasma samples were taken after 10 days of chronic stress in control animals ($n = 8$), stress susceptible animals ($n = 5$) and stress resilient animals ($n = 6$). Corticosterone levels were assessed by HPLC and are presented as absolute concentrations in plasma. Note that there was no significant difference in corticosterone level between the susceptible and resilient animals Data are mean ± SEM; $^{***}p < 0.001$.

both stress susceptible and stress resilient animals (one-way ANOVA: $F_{3,11} = 4.986$ susceptible $p < 0.05$, resilient $p < 0.01$) (Fig. 7B). In the raphe, significant increases in IL-1β were seen in stress susceptible animals compared to controls (one-way ANOVA: $F_{3,12} = 3.562$ $p < 0.05$; Fig. 7B). Thus distinct patterns of TNF and IL-1β expression were observed. We also examined COX-1 expression since repeated social defeat increases the PGE_2 level in the subcortical region of the brain, and mice lacking COX-1, are impaired in induction of social avoidance by repeated social defeat (Tanaka et al., 2012). All stressors, including the acute stress paradigm induced COX-1 expression in the prefrontal cortex. In the raphe, susceptible animals displayed a significant increase in expression compared to the other groups, while in the striatum COX-1 was increased in the striatum. However, neither of these changes was particularly marked.

The potential role of indoleamine-2,3-dioxygenase (IDO) in the development of inflammatory-related malaise has been well documented recently (O'Connor et al., 2009) where activity of this enzyme has been shown to increase after a peripheral immune challenge as a consequence of proinflammatory cytokine induction. With this in mind we were interested to discover whether IDO mRNA expression increased after chronic stress and whether the expression profile mirrored cytokine expression. Surprisingly, no changes were observed in any region with the exception of the raphe where susceptible animals displayed a significant increase in mRNA expression (Fig. 7D).

3.6. Increased prefrontal microglial activation after chronic stress

TNF has been show to be expressed principally by microglial in the CNS (Lambertsen et al., 2009) and given the strong induction of TNF in the prefrontal cortex we were interested to discover whether this was associated with alterations in the microglial population with the tissue. Microglia was counted in the prefrontal area of animals after 10 days of chronic stress (Fig. 8A). Using Iba-1 as a microglia marker, susceptible animals showed a significant increase in the number of Iba-1-positive cells/mm^2 than control or stress-resilient animals (Fig. 8B; one-way ANOVA: $F_{2,13} = 4.308$ $p < 0.05$) in the prefrontal cortex. No differences in the number of microglia were observed between resilient or

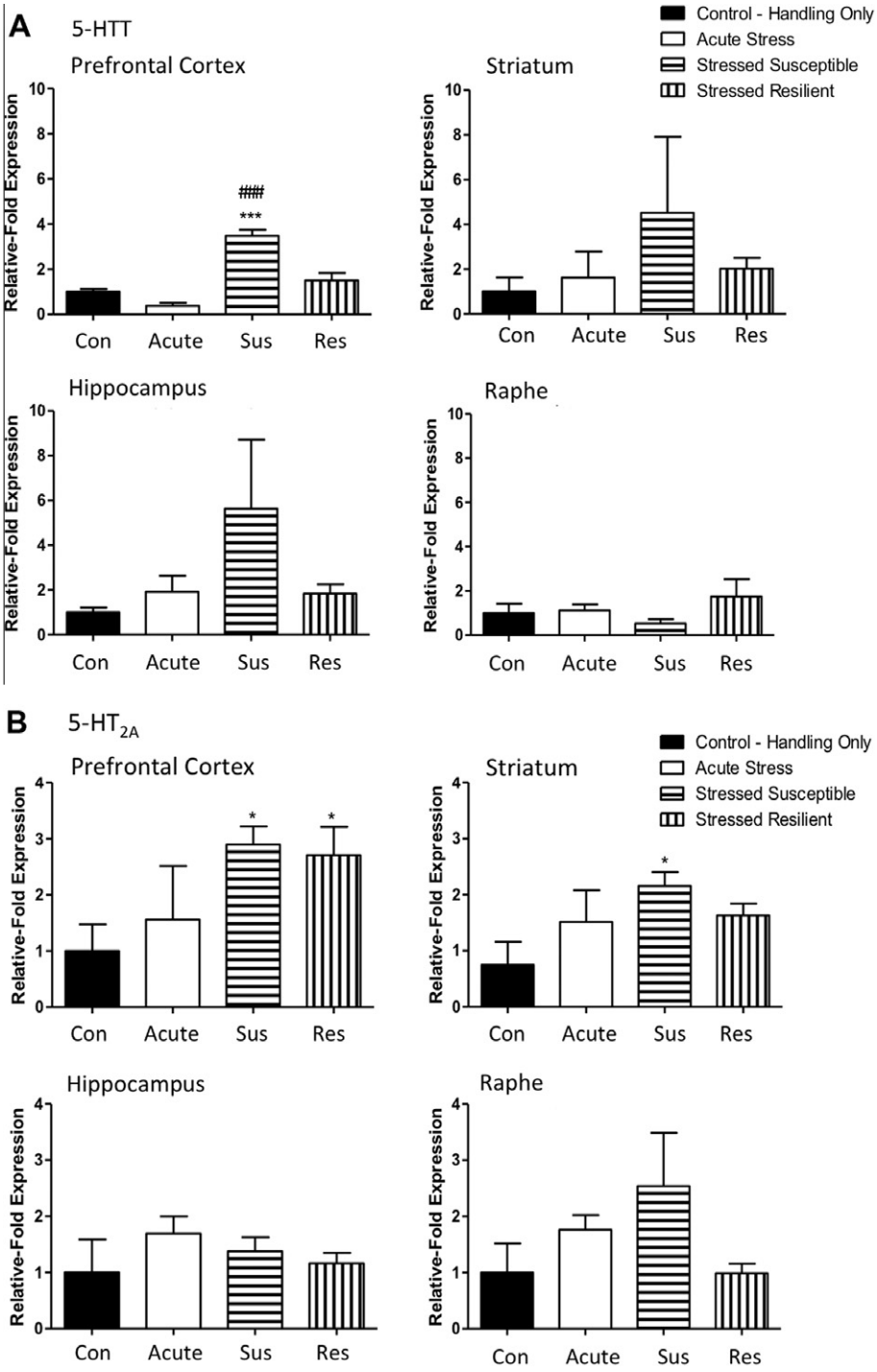

Fig. 6. Chronic stress induced significant changes in the expression of 5-HT_{2A} and SERT. (A) Relative-fold mRNA expression levels of the 5-HT transporter (SERT) in the prefrontal cortex, striatum, hippocampus and dorsal raphe nucleus after 10 days of chronic stress in control, acutely stressed, susceptible and resilient animals. (B) Relative-fold mRNA expression of the 5-HT_{2A} receptor in the prefrontal cortex, striatum, hippocampus and dorsal raphe nucleus after 10 days of chronic stress in control, acutely stressed, susceptible and resilient animals. Data are presented as relative-fold compared to control animals with all expression being normalised to the housekeeping gene GAPDH. Data are mean ± SEM ($n = 4$ for all groups). *** = $p < 0.001$ compared to control animals.

susceptible animals and controls in the lateral periaqueductal gray or in the striatum.

4. Discussion

In this study, using a chronic stress model in inbred mice, we have discovered that our animals segregate into two groups that can be described as resilient or susceptible. All animals displayed anxiety-like behaviours, but only a subset of susceptible animals developed an anhedonic phenotype and exhibit depression-like behaviour. We have explored the molecular profile of these subtypes in the brain and in the periphery. The behavioural changes are reflected by distinct changes in molecular biology, specifically changes in CNS cytokines and 5-HT-related genes. Susceptible animals exhibit increases in TNF expression in the prefrontal cortex, but resilient animals do not. All animals exhibited similar changes

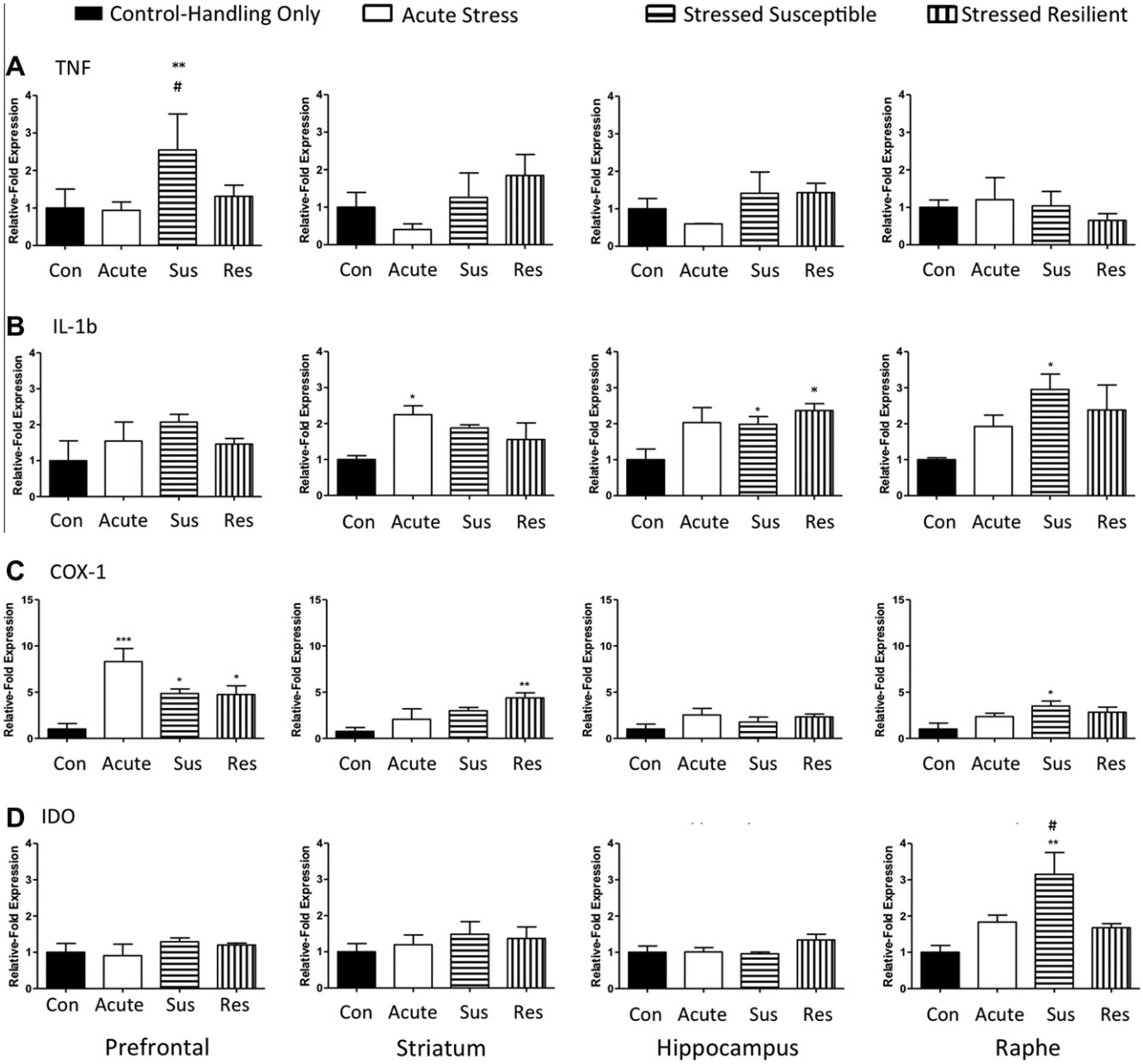

Fig. 7. Chronic stress induced mRNA expression of pro-inflammatory cytokines and COX-1 in susceptible and resilient animals. Animals were grouped into controls, acutely stressed, stress-susceptible or stress-resilient and brain tissue was dissected into the prefrontal cortex, striatum, hippocampus and raphe. Data show (A) relative-fold mRNA expression of TNFα in the prefrontal cortex, striatum, hippocampus and raphe; (B) relative-fold mRNA expression of IL-1β in the prefrontal cortex, striatum, hippocampus and raphe; (C) relative-fold mRNA expression of COX-1 in the prefrontal cortex, striatum, hippocampus and raphe; and (D) relative-fold mRNA expression of IDO in the prefrontal cortex, striatum, hippocampus and raphe. Data are presented as relative-fold compared to control animals with all expression being normalised to the housekeeping gene GAPDH. Data are mean ± SEM ($n = 4$ for all groups). $^{*}p < 0.05$; $^{**}p < 0.001$ and $^{***}p < 0.0001$ compared to control animals.

in cortisol and $5HT_{2A}$ expression, but the susceptible animals displayed greater increases in SERT expression. Thus, at a molecular level, for depression-like behaviours which are neurophysiologically uncoupled from anxiety-like behaviours the expression of TNF and SERT seem to be important determinants. In animals susceptible to chronic stress the number of Iba-1 positive microglia were also increased in the prefrontal cortex.

One of the major problems with modelling affective disorders such as depression in rodent models is the inter-individual variation that is encountered and can often appear dichotomous. Our approach here has been to recognise the existence of this variation and seek to explore the underpinning molecular mechanism. Others have found increases in depression-like behaviours similar to those we found (decreased sucrose consumption, increased floating in the FST), as well as 'anomalous' anxiolytic behaviours in response to novel environments (Mineur et al., 2006; Schweizer et al., 2009). Our experiments with the light/dark box revealed that susceptible and resilient animals performed in a similar manner, showing stress is capable of inducing anxiety in all animals. However, the significant differences in the home cage and forced swim test are consistent with a subgroup with depression-like signs. In light of these data, and taking into account our resilient animals' increased exploratory behaviour, it seems reasonable to assume that chronic stress is capable of inducing depression-like behaviours which are neurophysiologically uncoupled from anxiogenic behaviours.

Stress is well known to increase circulating cortisol and there have been numerous studies that have examined the association between cortisol levels and anxiety disorders and depression. Depressed patients frequently show dexamethasone non-suppres-

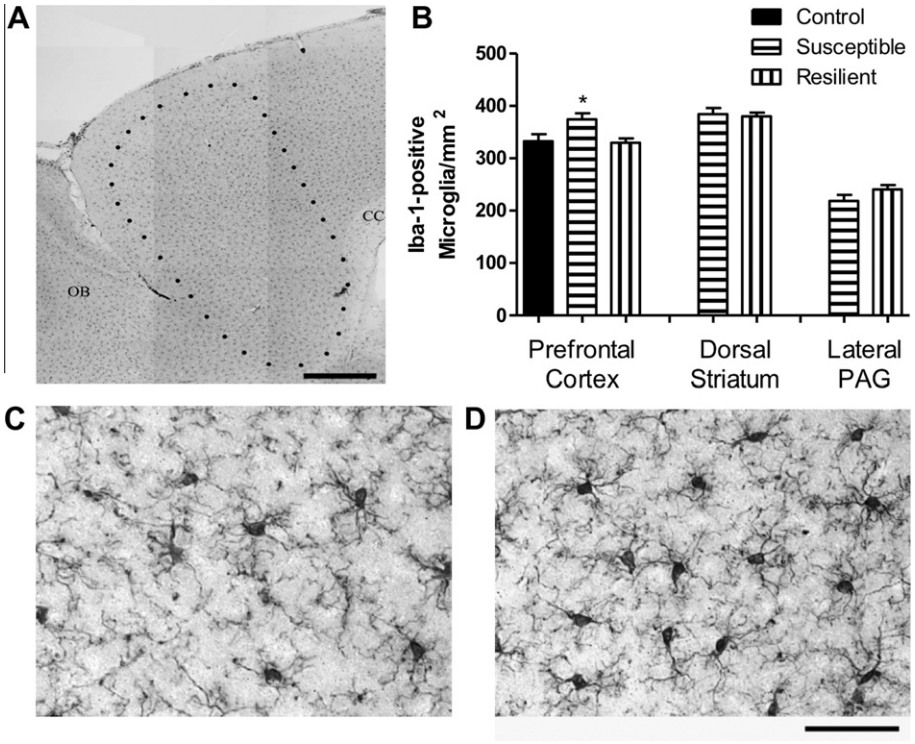

Fig. 8. Iba-1-positive microglia increased in the prefrontal cortex of susceptible animals. Mice were subjected to either handling stress prior to killing ($n = 6$), or chronic stress for 10 days. Susceptible ($n = 5$) and resilient ($n = 5$) animals were separated according to sucrose preference at day 10. Brain tissue was stained for the microglial activation marker Iba-1 and cells were counted across a 1 mm^2 grid over serial sections in each animal. (A) Micrograph and outline of the area counted within each brain. (B) Mean Iba-1 counts on three serial sections. (C) Representative photomicrograph showing Iba-1 positive cells in control animals. (D) Representative photomicrograph showing Iba-1 positive cells in susceptible animals. Scale bar is 50 μm. Data are mean ± SEM. $^*p < 0.05$ compared to control animals.

sion, suggesting hyperactivity of the HPA axis (Coppen et al., 1983). In our studies, 10 days of chronic stress results in around a 7-fold increase in serum corticosterone. Others have shown similar increases after stress in both susceptible and resilient animals (Goshen et al., 2008; Li et al., 2008). However, the absence of a clear difference in the susceptible vs. the resilient animals indicates that cortisol levels are not a useful discriminator.

Despite the failure of cortisol levels to be a good discriminator of resilient and susceptible animals, we discovered effective markers by examining the 5HT system and inflammation-related transcripts in the CNS. We have found up-regulated levels of SERT mRNA in the prefrontal cortex of mice susceptible to stress-induced anhedonia that were not found in the resilient animals. Mouse models of social defeat have also shown changes in SERT mRNA, albeit in different brain regions (Filipenko et al., 2002). The differences in these studies may reflect structural and temporal specificity of the serotoninergic response to stress over time. However, overall a change in SERT expression within the CNS in both chronic stress and depression is supported elsewhere (Murrough et al., 2011; Tordera et al., 2011; Kohut et al., 2012; Nikolaus et al., 2012). It is possible that during the first stages of depression, or experimental induction of a depressive-like state in animal models, SERT function is increased because of enhanced serotonin release, but longer-lasting over-expression of SERT in susceptible individuals' results in lower serotonin activity at later time points.

We also demonstrated a significant up-regulation of 5-HT_{2A} receptor mRNA in the prefrontal cortex of both susceptible and resilient animals, as well as a significant up-regulation in the striatum of susceptible animals. It is widely accepted that 5-HT_{2A} receptors are involved in the pathogenesis of depression and action of atypical antidepressants (Kirino, 2012). Down-regulation of 5-HT_{2A} receptor expression by mirtazapine and mianserin was shown to contribute to their antidepressant effects (Davis and Wilde, 1996; Golden et al., 1998). Here altered 5-HT_{2A} expression was not restricted to the susceptible animals and thus could not be described as discriminating in the same way as SERT. However, the data do support the use of selective 5-HT_{2A} modulators for mood disorders.

Previously, neuronal SERT expression has been shown to be dose- and time-dependently stimulated by IL-1β and TNF-α p38 MAPK-linked pathways (Zhu et al., 2010). Moreover, selective p38α MAPK deletion in serotonergic neurons results in stress resilience in a model of depression (Bruchas et al., 2011). Against this background, elevated expression of SERT and 5-HT_{2A} generally paralleled enhanced expression of TNF-α and IL-1β, this relationship was most consistent for the prefrontal cortex, but was detected in other brain structures also. However, there were clear differences in the regional expression profile and TNF expression was linked to depression-like behaviour in the susceptible, and not the resilient, animals.

Over the years most attention has focused on the role of central IL-1β expression in the generation of behavioural changes, and there is no doubt that the manipulation of central IL-1β signalling pathways can profoundly affect whether or not altered behaviour occurs after chronic stress (Goshen and Yirmiya, 2009). However, anti-IL-1β drugs such as anakinra have shown little success in combating inflammation-associated depression in patients with chronic inflammatory disorders where anti-TNF drugs have (Jiang et al., 2008; Tyring et al., 2006). While in animal models complete ablation of TNF does not result in such a robust antidepressant phenotype (Yamada et al., 2000), it could be hypothesised that signalling via the TNF receptor, and not the presence of TNF, is the critical factor in mood regulation. Tynan and colleagues have shown that SSRIs can significantly inhibit the production of both

TNF and nitric oxide in cultured microglia treated with LPS (Tynan et al., 2012). Although the target of the SSRIs and the specific effects of microglial-derived TNF on the brain serotonin microenvironment are unclear, it is becoming increasingly obvious that TNF plays a significant role in mood regulation.

Our study also revealed a general increase in COX-1 mRNA which appeared to be independent of whether the animals were resilient or susceptible. Susceptible mice did show significant elevation of COX-1 expression in the raphe, showing a general overlap between the expression profiles of both proinflammatory cytokines and SERT/$5HT_{2A}$. Selective inhibition of COX-1 in depressed patients has been shown to decrease depressive symptoms (Savitz et al., 2012). Recent data have shown that in repeated social defeat models, mice lacking COX-1 do not develop an anhedonic-phenotype (Tanaka et al., 2012). We have previously demonstrated that COX-2 is up-regulated by long-term chronic stress (Strekalova et al., 2011, Strekalova and Steinbusch, in preparation) and in various pathologies, over-expression of COX-1 was shown to precede the up-regulation of COX-2 (Yamaguchi et al., 2009; Dargahi et al., 2011). In this particular chronic stress variant, no significant up-regulation of COX-2 was found (data not shown). The elevation of COX-1 in our animals supports the use of NSAIDS for depression, but it does not represent a biomarker for anhedonic behaviours.

Of all the genes studied here, perhaps the most surprising result was the lack of an IDO response. To date, studies of the role of inflammation in depression have largely accepted the theory that cytokines increase IDO activity and decrease 5-HT levels. However, the degree of cytokine induction observed in most depressed patients is relatively small compared to that achieved with an endotoxin challenge. Recent work by Hughes and colleagues has shown that the increase in cytokines in depressed patients occurs independently of changes in plasma tryptophan (Hughes et al., 2012). Data here show that IDO expression was altered in the raphe, along with IL-1β, and it is possible that local increases in IDO activity may be contributing to altered 5HT neurotransmission. While activated microglia and astrocytes have been reported to make IDO, in dorsal raphe nucleus slice cultures, IFNγ most potently induced IDO expression in neurons, including serotonergic neurons, but not in microglia or astrocytes. Thus the altered IDO expression may reflect altered neuronal synthesis rather than glial synthesis (Hochstrasser et al., 2011).

The principal source of TNF in both the uninflamed and inflamed brain appears to be from microglia (Stellwagen and Malenka, 2006). Thus, given the upregulation of TNF in the susceptible animals we were keen to discover whether the microglia population was altered in this set of animals. The number of Iba-1-positive microglia was indeed up-regulated in the prefrontal cortex, but not in the striatum or the lateral periaqueductal grey. We suspect that the changes in activation and number of microglia in the prefrontal cortex, in susceptible animals, reflect a TNF-associated remodelling process that is likely to be more marked in the susceptible animals than in the resilient animals. This is, at least partly, confirmed by studies showing positive correlations between chronic stress, increased microglial activity and losses in working memory (Hinwood et al., 2012).

5. Conclusions

Taken together, our study suggests that a proinflammatory profile and heightened microglial activation are associated with the development of stress-induced anhedonia in specific susceptible subgroups of mice. These changes may be related to alterations in the serotonergic system induced by the proinflammatory environment. Hedonic deficit and molecular changes were accompanied by hyperlocomotion in stressful conditions and altered home cage behaviour. These features are in line with a body of clinical data on the pathophysiology of depression and, as well as highlighting a role for TNF in the prefrontal cortex, further validate this model as a construct for studying the human condition. (Bruchaset et al., 2011).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2012.12.017.

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