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Mouse social stress induces increased fear conditioning, helplessness and fatigue to physical challenge together with markers of altered immune and dopamine function

Damiano Azzinnari [a, f, *], Hannes Sigrist [a], Simon Staehli [a], Rupert Palme [b], Tobias Hildebrandt [c], German Leparc [c], Bastian Hengerer [d], Erich Seifritz [e, f], Christopher R. Pryce [a, f, *]

[a] Preclinical Laboratory for Translational Research into Affective Disorders, Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland
[b] Institute of Medical Biochemistry, Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria
[c] Target Discovery, Boehringer Ingelheim Pharma GmbH & Co. KG., Biberach an der Riss, Germany
[d] CNS Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG., Biberach an der Riss, Germany
[e] Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland
[f] Neuroscience Center Zurich, University of Zurich and ETH Zurich, Switzerland

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ABSTRACT

In neuropsychiatry, animal studies demonstrating causal effects of environmental manipulations relevant to human aetiology on behaviours relevant to human psychopathologies are valuable. Such valid models can improve understanding of aetio-pathophysiology and preclinical discovery and development of new treatments. In depression, specific uncontrollable stressful life events are major aetiological factors, and subsequent generalized increases in fearfulness, helplessness and fatigue are core symptoms or features. Here we exposed adult male C57BL/6 mice to 15-day psychosocial stress with loss of social control but minimal physical wounding. One cohort was assessed in a 3-day test paradigm of motor activity, fear conditioning and 2-way avoid-escape behaviour on days 16-18, and a second cohort was assessed in a treadmill fatigue paradigm on days 19 and 29, followed by the 3-day paradigm on days 30-32. All tests used a physical aversive stimulus, namely mild, brief electroshocks. Socially stressed mice displayed decreased motor activity, increased fear acquisition, decreased 2-way avoid-escape responding (increased helplessness) and increased fatigue. They also displayed increased plasma TNF and spleen hypertrophy, and adrenal hypertrophy without hyper-corticoidism. In a third cohort, psychosocial stress effects on brain gene expression were assessed using next generation sequencing. Gene expression was altered in pathways of inflammation and G-protein coupled receptors in prefrontal cortex and amygdala; in the latter, expression of genes important in dopamine function were de-regulated including down-regulated *Drd2, Adora2a* and *Darpp-32*. This model can be applied to identify targets for treating psychopathologies such as helplessness or fatigue, and to screen compounds/biologics developed to act at these targets.

1. Introduction

Depression has core symptoms of low mood, fatigue and anhedonia (ICD-10, 1994). The low mood core symptom involves the patient focussing on and emphasising aversive stimuli and events. The stressful life events that precede depression, e.g. psychosocial, financial, health, are often uncontrollable (Kendler et al., 2003; Kessler, 1997). It has been proposed that learned uncontrollability of one life event (specific helplessness) can become generalized to other life events regardless of their controllability, i.e. generalized helplessness (Abramson et al., 1989, 1978; Beck et al., 1974; Diener et al., 2009; Maier and Seligman, 1976; Pryce et al., 2011). Generalized helplessness is proposed to comprise increased emotional responding to, reduced motivation to actively

* Corresponding authors. Psychiatric Hospital Zurich, August Forel-Strasse 7, 8008 Zurich, Switzerland. Tel.: +41 634 8921; fax: +41 634 8829.
E-mail addresses: damiano.azzinnari@bli.uzh.ch (D. Azzinnari), christopher.pryce@bli.uzh.ch (C.R. Pryce).

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cope with, and reduced cognitive (response-outcome) expectancy to be able to cope with/control, aversive events; these states are clearly linked to low mood. Experimental evidence for these states includes increased fear conditioning to stimuli that predict uncontrollable electroshock (Nissen et al., 2010), and increased helplessness during and after exposure to uncontrollable electroshock (Diener et al., 2009; Strigo et al., 2008), in depressed patients relative to healthy controls. The core symptom of fatigue is also complex and comprises experiencing physical and cognitive tasks as requiring extreme effort (Demyttenaere et al., 2005). The aetiopathophysiology of these important psychopathology symptoms and features is poorly understood. Understanding might be enhanced by the development and study of animal models that comprise (1) exposure to stressors that are relevant to human life events; (2) demonstrable effects of this stress on behaviour in tests that measure fear responding to aversive stimuli, helplessness when confronted with controllable aversive stimuli, and physical fatigue in an aversive environment. Such animal models would have aetiological and face validity for these depression psychopathologies (Cuthbert and Insel, 2013; Hyman, 2012; Markou et al., 2009).

There are currently two animal models of depression psychopathologies that are particularly widely used. In chronic unpredictable mild stress, rats or mice are exposed for 21–28 days to various physical and social stressors on an unpredictable schedule, and the major readouts are tests of reward sensitivity such as the sucrose preference test. Reward sensitivity is reduced, thereby providing a rodent model of stress-induced decreased interest (anhedonia) (Tye et al., 2013; Willner, 1997). In chronic social defeat (CSD), mice are exposed to ten days of daily 10-min attack by aggressive dominant mice, and otherwise there is continuous distal exposure to this psychosocial stressor. Stressed CSD mice display submissive behaviour but this fails to deter/control attacks by the dominant mice (Kudryavtseva et al., 1991). Bite wounds are common in the standard CSD protocol (Golden et al., 2011), somewhat impacting on its aetiological validity as an emotional psychosocial stressor. Readout tests of CSD effects have focussed on passive avoidance of the aggressor mouse strain in a social proximity test (Krishnan et al., 2007; Savignac et al., 2011b); that is, on increased emotional reactivity to the specific learned uncontrollable social stimulus (Russo et al., 2012).

We previously established a specific learned helplessness paradigm in mice using 2-way escape behaviour of mild, short electroshocks (Pryce et al., 2012). Mice exposed to electroshocks that can be terminated (controlled) by transfer from one side of an arena to the other readily learn this escape behaviour and maintain a high motivation (motor reactivity) across repeated electroshocks. Mice exposed to identical electroshocks in terms of duration, intensity and interval but now inescapable, i.e. no response-outcome contingency in terms of electroshock termination, exhibit progressively decreasing motivation (reduced motor reactivity) to control the aversive stimulus. When subsequently challenged with escapable shocks they exhibit few escape attempts and fail to learn from reinforced escape responses (Pryce et al., 2012). Using the same apparatus, one aim of the present study was to investigate whether CSD, which constitutes lack of social control, leads to generalized helplessness i.e. altered emotional, motivational and cognitive responding to another form of aversive stimulus, namely electroshock. Specifically, a 3-day test paradigm was used: motor activity test on day 1, contextual fear conditioning on day 2, and 2-way avoid-escape test on day 3. The motor activity test assessed whether CSD decreased activity and/or increased fear-freezing behaviour in a neutral environment; contextual fear conditioning assessed whether CSD increased fear-freezing reactivity to uncontrollable electroshock; the 2-way avoid-escape test assessed whether CSD decreased motivation to control the aversive stimulus (motor reactivity to electroshock) and decreased cognitive expectancy to control the aversive stimulus (avoid-escape learning). Mice were tested sequentially in the different tests across days, so that for the later tests it was the effects of CSD on responses to (coping with) successive physical challenges that was under study. Whilst fatigue is a core symptom of depression, there does not appear to be an animal model for psychosocial stress induced increased fatigability. Using a treadmill combined with electroshock, fatigue induced by enforced running has been demonstrated in mice, and this method has been applied to study factors regulating fatigability, included proinflammatory cytokine administration (Carmichael et al., 2006). Therefore, another aim of the present study was to investigate whether CSD induces increased fatigability under conditions of enforced treadmill running to avoid electroshock. The same mice were subsequently also investigated in the 3-day generalized helplessness paradigm.

A final aim of this study was to investigate CSD effects on specific physiological and molecular genetic parameters that might contribute to the causation of any depression-relevant behavioural effects. One hypothesis of depression aetio-pathophysiology is psychosocial stress activation of peripheral and central immune-inflammation, leading to oxidative stress and neurotoxic disruption of several neurotransmitters including serotonin, dopamine, glutamate and GABA (Dantzer et al., 2008; Felger and Miller, 2012). The proinflammatory cytokines of tumor necrosis factor (TNF) and interleukin-6 (IL-6) are increased in a majority of depression patients relative to matched controls (Dowlati et al., 2010; Maes, 2010). To render CSD aetiologically valid with respect to stress induced inflammation it was essential to introduce refinements to prevent the bite wounding that is frequent in the standard CSD protocol (Golden et al., 2011). The peripheral immune-inflammation markers of plasma TNF and IL-6 levels and spleen mass were measured. Markers of glucocorticoid function were also measured, namely adrenal gland mass and plasma and faecal corticosterone levels. Whilst a number of studies have reported increased basal plasma cortisol levels in a subset of depressed patients relative to matched controls (Brown et al., 2004), there is also evidence for decreased plasma cortisol in depression and other stress-related disorders (Silverman and Sternberg, 2012). Another rationale for studying adrenal/corticosterone status is the evidence that stress can induce glucocorticoid resistance which in turn results in attenuation of its anti-inflammatory function (Rhen and Cidlowski, 2005; Silverman and Sternberg, 2012). Next generation sequencing and canonical pathway analysis were applied to conduct a hypothesis-free, transcriptome-level analysis of effects of CSD on gene expression in specific brain regions. The regions selected for study, ventral hippocampus, medial prefrontal cortex, and central and basolateral nuclei of amygdala, are fundamental to the neurocircuitries underlying the behaviours under study here (Amat et al., 2005; Maren et al., 2013; Moscarello and LeDoux, 2013) as well as the corresponding depression psychopathologies (Capuron et al., 2007; Disner et al., 2011; Mayberg, 2003; Price and Drevets, 2010; Savitz et al., 2013; Sibille et al., 2009; Strigo et al., 2008).

2. Methods

2.1. Animals and maintenance

Breeding of C57BL/6J mice was conducted in-house. Male offspring were weaned at age 3 weeks and caged in groups of 2–3 littermates. The study was conducted with a total of 72 mice born to 26 breeding pairs. Mice were aged 10–13 weeks and weighed 24.0–30.0 g at study onset. Male CD-1 mice (Janvier, Saint-Berthevin, France) were aged 8 months, were ex-breeders, and caged singly at study onset. Mice were maintained on a reversed 12:12 h light–dark cycle (lights off 07:00–19:00 h) in an individually-ventilated caging system at 20–22 °C and 50–60% humidity. Cages were type 2 L and contained woodchips, a sleep igloo and

tissue bedding. Complete-pellet diet (Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water were available continuously. In the week prior to CSD, all C57BL/6J mice were handled and weighed on five days; body weights were used to assign mice to CSD and control (CON) groups such that mean weight per group was counterbalanced. The study was conducted under a permit (110/2009) for animal experimentation issued by the Veterinary Office, Zurich, Switzerland. All efforts were made to minimize the number of mice used and any unnecessary stress to those mice that were used, including refinement of the published protocol for CSD (Golden et al., 2011).

2.2. Chronic social defeat

A standard protocol for CSD (Golden et al., 2011) was used for the present study with refinements. Social defeat sessions were conducted under dim light on 15 consecutive days (versus 10 days in the standard protocol). On the day prior to day 1 of CSD, a CD-1 mouse demonstrated to be aggressive (Golden et al., 2011) was placed in one compartment of a cage (Indulab, Gams, Switzerland) containing a longitudinal divider made from transparent Plexiglas and perforated with multiple holes (⌀ = 10 mm) for sensory interactions. To prevent bite wounds, a concern in the standard protocol (Golden et al., 2011), the lower incisors of these CD-1 mice were trimmed using rodent tooth-cutting forceps (Precision Surgical International, USA) under brief isoflurane anaesthesia; this was repeated at 3-day intervals across CSD. Daily CSD was conducted between 14:00–17:00 h. On day 1, each CSD C57BL/6J mouse was removed from its home cage, weighed and placed singly in the compartment of a CD-1 mouse. Behaviour was observed and the duration of each physical attack was timed; mice remained together until either a cumulative total of 60-s physical attack had occurred or 10 min had elapsed (versus 10 min together regardless of attack time in the standard protocol). Also scored for each session were whether or not the CSD mouse fought back when attacked by the CD-1 mouse, and whether or not the CSD mouse displayed an upright submissive posture or submissive vocalisation. When the CSD mouse displayed submissive behaviour, it was scored whether or not the CD-1 mouse nonetheless attacked the CSD mouse. These behaviours were scored as did or did not occur per session, and the number of times per session that they occurred was not scored. The CSD mouse remained in the compartment in which it had been attacked and the CD-1 mouse was placed in the opposite compartment, allowing continuous olfactory, visual and auditory contact during the following 24 h. Because of behavioural observation and the timing of attack durations, CSD was conducted with only two cages at a time (versus 10 cages at a time in the standard protocol (Golden et al., 2011)). On day 2, the CSD × CD-1 mice pairings were rotated so that each CSD mouse was confronted with a novel CD-1 mouse and *vice versa*. The procedure was continued until day 15. Thereafter, each CSD mouse remained next to the same CD-1 mouse without any further attack sessions or rotations. Control (CON) mice remained in littermate pairs, the standard caging condition in our laboratory, and were handled and weighed daily (versus pairs of CON mice caged singly separated by dividers (Golden et al., 2011)). A pilot study comparing CON mice pairs maintained together or separated from each other by a divider demonstrated no effects in tests of motor activity, fear acquisition or two-way avoid-escape behaviour.

2.3. Experimental design

Three experiments were conducted, the designs of which are summarized in Fig. 1. Experiment A (CON = 12, CSD = 13) investigated the effects of 15-day CSD on motor activity, contextual fear acquisition, two-way avoid-escape and hot-plate pain sensitivity at days 16–20, respectively. Physiological assessments were also conducted. Experiment B (CON = 10, CSD = 13) investigated the effects of 15-day CSD on treadmill fatigue at days 19 and 29, and on motor activity, contextual fear acquisition, two-way avoid-escape and hot-plate pain sensitivity at days 30–33, respectively. Experiment C (CON = 12, CSD = 12) investigated the effects of 15-day CSD on transcriptome expression in specific brain regions at day 17.

2.4. Motor activity, contextual fear acquisition and two-way avoid-escape behaviour

Behavioural testing was conducted under dim lighting in a room adjacent to the mouse holding room, between 08:30–12:00 h except for the hot plate test (16:00–17:00 h). Motor activity, contextual fear acquisition and two-way avoid-escape testing were conducted on three consecutive days using a single system (Multi Conditioning System, TSE Systems GmbH, Bad-Homburg, Germany), details of which are given elsewhere (Pryce et al., 2012).

2.4.1. Motor activity test

The mouse was placed on the grid floor in the empty arena for 15 min, and distance moved (arbitrary units/min, a.u./min) and % time spent freezing were recorded continuously using an infrared beam movement-detection system. Freezing episodes were defined and scored as no detectable movement for at least 2 s.

2.4.2. Contextual fear acquisition

On the following day, the mouse was placed on the grid floor in the empty arena and exposed to 15 mild inescapable electroshocks of 0.15 mA × 3 s delivered at inter-trial intervals (ITIs) of 50 s. Mean reactivity to electroshock (a.u./min) and mean % time spent freezing during ITIs were calculated.

2.4.3. Two-way avoid-escape test

On the following day, the mouse was placed on the grid floor in the arena which now contained a central divider with a gate, through which the mouse could transfer between identical left and right compartments. A first stage consisted of 10 trials each of 12-s tone (5 kHz, 85 dB, conditioned stimulus, CS) followed immediately by 0.15 mA × 3 s inescapable electroshock, with ITIs of 50 s. This tone-inescapable electroshock stage was included to ensure that all mice received equivalent tone aversive conditioning prior to two-way avoid-escape testing: otherwise, as observed in a pilot study, mice that are more active transfer frequently during early trials and terminate the CS before it becomes conditioned to the electroshock. (Data for this stage are not reported.) Directly thereafter, mice proceeded to the test, comprising 30 trials each of 10-s CS followed immediately by 0.15 mA × 5 s-maximum escapable electroshock, with ITIs of 50 s. If the mouse did not transfer during tone + electroshock this was an avoid-escape failure; if the mouse transferred during the electroshock it was terminated immediately and this was an escape response; if the mouse transferred during the tone CS it was terminated immediately and the electroshock was omitted and this was an avoid response. The following measures were calculated for the two-way avoid-escape test: mean avoid-escape latency, mean % time freezing during tone and ITI, mean reactivity to electroshock, total avoid responses, total escape responses and total avoid-escape failures.

2.5. Hot plate test

To assess pain sensitivity, a hot plate test was conducted (Pryce et al., 2012). At 16:00–17:00 h the mouse was placed inside a chamber on a hot plate, and the latency (sec) from the onset of the test until the first occurrence of one of the following

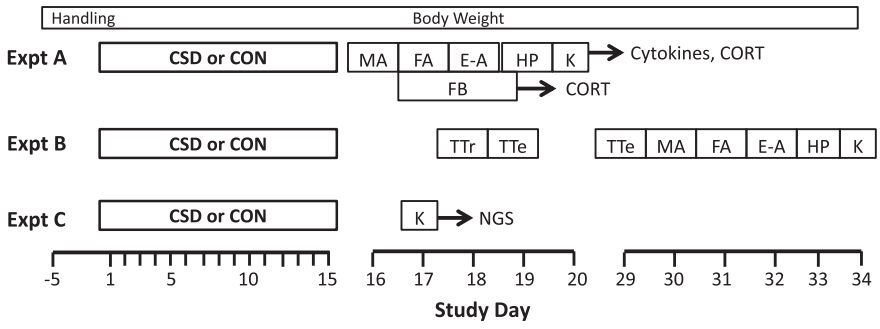

Fig. 1. Experimental designs. Three experiments were conducted with mice exposed to chronic social defeat (CSD) or control handling (CON) on days 1–15. Mice were handled prior to experiments and body weights were measured daily. Experiment A: a 3-day test paradigm of motor activity (MA), contextual fear acquisition (FA) and two-way escape-avoidance (E-A) was conducted on days 16, 17 and 18, respectively. Morning faecal boli (FB) were collected on days 17–19 in the waste tray of the behavioural test apparatus for corticosterone (CORT) metabolite determination. A hot plate test (HP) was conducted on day 19, and mice were killed (K) and physiological samples were collected on day 20, including blood samples for cytokine and CORT determinations. Experiment B: Treadmill training (TTr) and a treadmill pre-test and test (TTe) were conducted on days 18 and 19, respectively, and a second TTe was conducted on day 29. The 3-day MA, FA, E-A paradigm was conducted on days 30–32, and HP on day 33. Mice were killed and physiological samples were collected on day 34. Experiment C: Mice were killed on day 17 and brains were collected for genome-wide gene expression in ventral hippocampus, amygdala, and medial prefrontal cortex using next generation sequencing (NGS).

behaviours was scored: licking a forepaw, licking a hind paw, lifting a hind paw, jumping. The mouse was then removed immediately from the hot plate. The maximum test duration was 60 s and 60 s was the latency score given to mice that did not exhibit one of the target behaviours.

2.6. Treadmill fatigue test

The apparatus was a single lane treadmill (Panlab/Harvard Apparatus, Cornellà, Spain) inclined at 5° and with an electroshock grid at its lower end set at 0.15 mA. By running up-hill, mice could avoid the grid, or if they had stopped running and travelled back onto the grid, escape from it. Total number and duration of electroshocks were scored automatically. A pilot experiment in non-manipulated mice demonstrated that a treadmill speed of 20 cm/s was a moderate running speed, and that 25 cm/s was a fast running speed that mice could maintain for at least 20 min whilst receiving only a low number (5–15) and total duration (1–2 s) of electroshocks. A training session consisted of 2 min at 0 cm/s, 5 min at 15–20 cm/s at 1 min increments, and 5 min at 20 cm/s. A pre-test session consisted of 2 min at 0 cm/s and 5 min at 20 cm/s. Beginning immediately thereafter, a test session consisted of 20 min at 25 cm/s. Measures were total electroshock duration at pre-test and, for the test session, running duration at 25 cm/s (maximum 20 min) and total electroshock duration (maximum 10 s) i.e. the session was terminated prior to 20 min if subjects accumulated a total of 10 s electroshock at test. To ensure that differences in electroshock received could not carry over to influence behaviour in subsequent tests, mice that completed the 20 min test, i.e. received less than 10 s electroshocks, were exposed to a further short session at high speed (35 cm/s) until their total electroshock exposure reached 10 s.

2.7. Collection of faeces, blood, spleen, adrenal glands and brain

In Expt A, fresh faecal boli were collected from the waste tray after behavioural testing on days 17, 18 and 19 (mice placed in arena for 10 min without test on day 19) for corticosterone metabolite measurement, as a non-invasive marker of 24-h plasma corticosterone levels on days 16 (motor activity test, without electroshock), 17 (fear acquisition test, with electroshock) and 18 (2-way avoid-escape test, with electroshock), respectively. On day 20, mice were decapitated and trunk blood was collected in EDTA-coated tubes (Microvette 500 K3E, Sarstedt) and placed on ice. Bloods were centrifuged at 3000 rpm and 4 °C for 10 min, plasma aliquots were transferred to cryotubes (Protein LoBind, Eppendorf) and stored at −80 °C. Adrenal glands and spleen were removed, cleaned of fat and connective tissue and weighed. In Expt B, on day 33 mice were decapitated and adrenal glands and spleen were removed, cleaned of fat and connective tissue and weighed. In Expt C, on day 17 mice were decapitated and the brain was removed, rinsed with ice-cold saline, frozen on dry ice and stored at −80 °C. Adrenal glands and spleen were removed, cleaned of fat and connective tissue and weighed.

2.8. Plasma cytokine measurement

Plasma proinflammatory cytokines tumor necrosis factor (TNF) and interleukin-6 (IL-6) were measured using a multiplexed particle-based flow cytometric cytokine assay (Marques-Vidal et al., 2011). The lower limit of detection for TNF and IL-6 was 0.5 pg/mL.

2.9. Corticosterone measurement

Plasma corticosterone was measured using an EIA kit (AssayMax CORT ELISA kit; AssayPro, Saint Charles, MO, USA). Plasma was diluted 1:50 in kit diluent and heated at 90 °C for 10 min for transcortin denaturation (Pryce et al., 2001). All further steps were performed according to the manufacturer's protocol. Faecal samples were dried and powdered, 20–50 mg were extracted in 80% methanol. After centrifugation supernatants were stored at −20 °C and transferred to Vienna. The faecal corticosterone metabolite 5α-pregnane-3β,11β,21-triol-20-one was measured using an in-house EIA (Touma et al., 2003), to provide a non-invasive biomarker of blood corticosterone levels.

2.10. Brain region-specific transcriptome expression

Frozen brains from Expt C were sectioned coronally at 1 mm intervals using a stainless-steel brain matrix (model MMCS-1, Plastics One) and single-edge blades (model 10-100-063, Apollo Herkenrath, Solingen, Germany). Regions of interest were ventral hippocampus (vHIPP), amygdala (AMYG) and medial prefrontal cortex (mPFC; infralimbic cortex + ventral prelimbic cortex). These were microdissected bilaterally from corresponding sections using a brain punch (⌀ = 1 mm, model 57397, Stoelting Europe) and a mouse brain atlas (Franklin and Paxinos, 2008) (Fig. 6): mPFC (1 biopsy/hemisphere) at bregma 2.1 to 1.2 ± 0.2 mm; AMYG (1 biopsy/hemisphere) at bregma −0.7 to −1.7 ± 0.3 mm; vHIPP (2 biopsies/hemisphere) at bregma −2.8 to −3.9 ± 0.3 mm. Tissue mass was 0.6–0.8 mg per biopsy. All microdissection steps were conducted at −18 °C. Brain biopsies were stored in 1.5 ml DNA LoBind polypropylene tubes (Eppendorf, model 0030 108.051) at −80 °C.

For RNA isolation, samples were homogenised in 400 μl 1%-ß-mercaptoethanol-RLT-buffer, and total RNA was isolated from 200 μl homogenate using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The high quality of the RNA was determined using the RNA 6000 Nano kit and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Preparation of the mRNA sequencing library, separately for each sample (CSD $N = 12$, CON $N = 12$, i.e. without pooling) was conducted using 200 ng of total RNA in the TrueSeq RNA Sample Preparation Kit v2-Set B (RS-122-2002, Illumina Inc, San Diego, USA); this yielded fragments of an average size of 275 bp including the adaptors with their library-specific indices supplied by the manufacturer. Concentration of the libraries was normalised and eight libraries were multiplexed and clustered on the cBot Instrument (Illumina Inc) using the TruSeq SR Cluster Kit v3 − cBot − HS (GD-401-3001, Illumina Inc). Sequencing was performed with single reads of 52 bp using the TruSeq SBS Kit HS − v3 (FC-401-3002, Illumina Inc) on an Illumina HiSeq2000 instrument. Read alignment of the RNA-seq dataset was performed using STAR aligner software (Dobin et al., 2013). Gene expression analysis was conducted using Cufflinks software with differential expression of genes in CSD ($N = 12$) versus CON ($N = 12$) mice identified using the Cuffdiff 2 test (Trapnell et al., 2013). Briefly, the Cuffdiff 2 test assesses between-group log-fold change in gene expression against the null hypothesis of no change whilst controlling for false discovery rate (FDR) using the Benjamini-Hochberg correction for multiple-testing. The criteria used to identify differential gene expression in CSD relative to CON mice were mean reads per kilo base per million (RPKM) > 5, mean fold change > 1.4, and $q < 0.05$. Within and between the three regions of interest, those genes that met these criteria for significantly increased or decreased expression in CSD versus CON mice were investigated in terms of canonical pathways and upstream regulator networks, using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, USA). The undetectable/low level of expression of globin genes (*Hbb*) demonstrated that brain tissue contamination with blood cells was negligible.

2.11. Statistical analysis

Statistical analysis of CSD effects on behaviour and physiology was conducted using SPSS (version 20, SPSS Inc., Chicago IL, USA). In most cases a *t*-test was conducted. Analysis of variance (ANOVA) was conducted in cases of a between-subject factor of group (CSD, CON) and a within-subject factor of CSD day block (e.g. body weights). ANOVA *post hoc* testing was conducted using the Bonferroni correction for multiple comparisons. Statistical significance was set at $p \leq 0.05$. Where an estimate of variance is given this is the standard deviation (SD). In the case of behavioural and physiological measures, for CSD and CON mice separately, correlations were run to determine if any of these parameters varied together; consistent evidence that certain mice displayed high CSD effects and others low CSD effects would point towards relative susceptibility and resilience, respectively. As a partial control for multiple testing of correlations, statistical significance was set at $p \leq 0.01$.

3. Results

3.1. Chronic social defeat

In the daily social defeat sessions the mean duration of attacks received on days 1–15 by C57BL/6 CSD mice in experiments A, B and C was, respectively, 47 ± 3, 44 ± 4 and 46 ± 5 s (mean ± SD). Table 1 summarises the outcome of CSD sessions in three 5-day blocks. Attacks occurred in each CSD session and the average total duration of attacks decreased to a small extent from CSD days 1–5 to 11–15. A minority of CSD mice were observed to fight back when being attacked by a CD-1 aggressor mouse, and this was largely restricted to CSD days 1–5. Thereafter, all CSD mice were observed to exhibit submissive body postures and emit submissive vocalisations during the majority of social defeat sessions. Furthermore, all CD-1 aggressor mice were observed to attack despite these submissive signals. Therefore, CSD mice experienced repeated absence of control in an aversive social situation. CSD mice were controlled for wounding immediately after each session. Because of regular trimming of the incisor teeth of CD-1 mice, wounding was rare: per experiment, there were 1–2 surface wounds (skin abrasions) in 2–4 mice, and these were more common on CSD days 6–10 and 11–15; importantly, deep bite wounds (skin penetration) did not occur.

3.2. Motor activity, fear acquisition, helplessness and fatigue

In Expt A, in the motor activity test (MA, day 16), CSD mice exhibited decreased activity relative to CON mice (arbitrary units/min: CSD 3907 ± 1535, CON 5806 ± 2113, $t_{(23)} = 2.59$, $p < 0.02$). In

Table 1
Summary of behaviour and physical status of C57BL/6 and CD-1 mice during CSD sessions.

Experiment	Parameter	CSD Days		
		1–5	6–10	11–15
A	Sum of sessions with attack[a]	5 (5–5)	5 (5–5)	5 (5–5)
	Total attack duration (sec)[b]	50 (42–55)	47 (38–57)	44 (30–52)
	Sum of sessions with fighting back[a]	0 (0–3)	0 (0–0)	0 (0–1)
	Sum of sessions with submission + attack[a]	3 (2–5)	4 (3–5)	5 (3–5)
	Sum of sessions with surface wound[a]	0 (0–1, $n = 2$[c])	0 (0–2, $n = 2$[c])	0 (0–2, $n = 3$[c])
	Sum of sessions with deep bite wound[a]	0 (0–0)	0 (0–0)	0 (0–0)
B	Sum of sessions with attack[a]	5 (5–5)	5 (5–5)	5 (5–5)
	Total attack duration (sec)[b]	50 (40–56)	44 (34–58)	38 (31–48)
	Sum of sessions with fighting back[a]	0 (0–3)	0 (0–1)	0 (0–0)
	Sum of sessions with submission + attack[a]	3 (2–5)	4 (4–5)	5 (4–5)
	Sum of sessions with surface wound[a]	0 (0–1, $n = 1$[c])	0 (0–1, $n = 2$[c])	0 (0–2, $n = 2$[c])
	Sum of sessions with deep bite wound[a]	0 (0–0)	0 (0–0)	0 (0–0)
C	Sum of sessions with attack[a]	5 (5–5)	5 (5–5)	5 (5–5)
	Total attack duration (sec)[b]	50 (39–60)	43 (35–54)	45 (36–55)
	Sum of sessions with fighting back[a]	0 (0–2)	0 (0–0)	0 (0–0)
	Sum of sessions with submission + attack[a]	3 (3–5)	4 (4–5)	5 (4–5)
	Sum of sessions with surface wound[a]	0 (0–0)	0 (0–2, $n = 2$[c])	0 (0–1, $n = 3$[c])
	Sum of sessions with deep bite wound[a]	0 (0–0)	0 (0–0)	0 (0–0)

[a] Parameter values are the overall median and (minimum–maximum) number of the five sessions during which the behaviour occurred at least once.
[b] Parameter value indicates the overall mean and (minimum–maximum mean) duration of the behaviour during the five sessions across all mice.
[c] Denotes the number of mice that received at least 1 surface wound.

the same test, CSD and CON mice spent a similar, low % of time freezing ($p = 0.62$, Fig. 2A). In the fear acquisition test (FA, day 17), CSD mice exhibited increased mean % time fear freezing relative to CON mice ($t_{(23)} = -4.41$, $p < 0.0005$, Fig 2A). There was no effect of CSD on reactivity to electroshock ($p = 0.14$, Fig. 2B). In the 2-way avoid-escape test (E-A, day 18), CSD mice exhibited a greater mean latency to avoid-escape ($t_{(23)} = -3.00$, $p < 0.006$, Fig. 2C). CSD mice did not make more avoid-escape failures (CSD 14.2 ± 10.1, CON 8.8 ± 5.1, $p = 0.11$), but they did make less avoid responses than CON mice ($t_{(23)} = 2.70$, $p < 0.01$, Fig. 2D). The CSD mice also exhibited increased mean % time fear freezing, both during tones ($t_{(23)} = -4.18$, $p < 0.0005$, Fig. 2E) and 50-s intervals between successive tone-electroshock pairings ($t_{(23)} = -4.64$, $p < 0.0005$, Fig. 2E). Therefore, during tone CSs, CSD mice displayed more

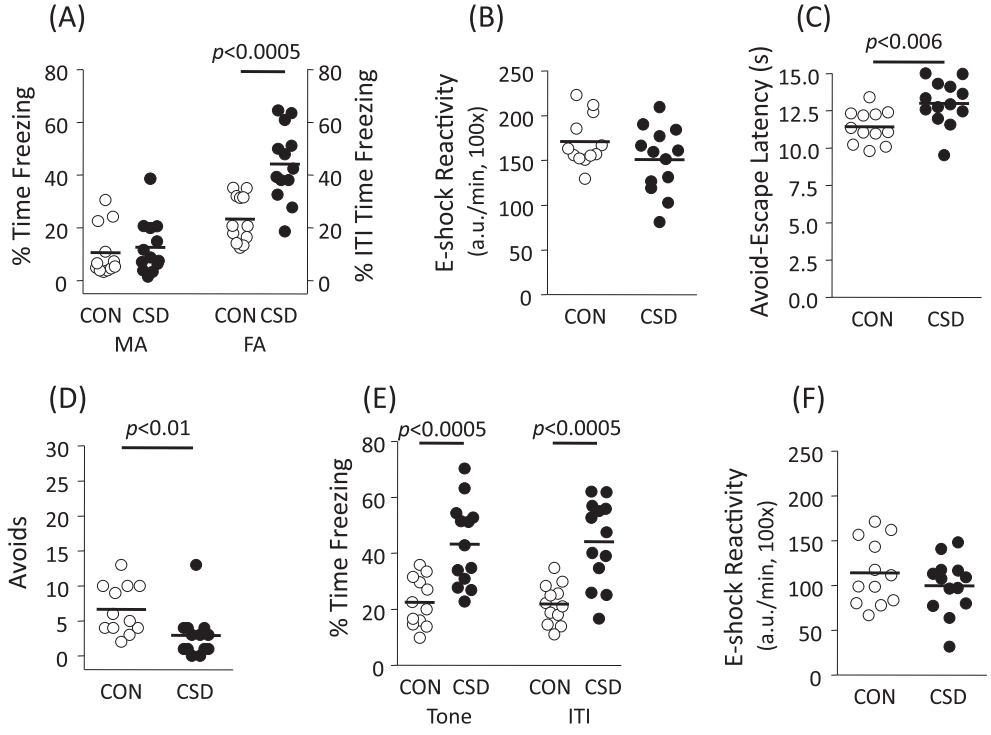

Fig. 2. Experiment A, effects of 15-day chronic social defeat (CSD, $N = 13$) versus control handling (CON, $N = 12$) in tests of motor activity, fear acquisition and two-way avoid-escape on days 16, 17 and 18, respectively. (A) Percent time freezing in the motor activity test (MA) and mean percent time freezing during 14 inter-trial intervals in the fear acquisition test (FA). (B) Mean reactivity (distance moved) to 15 inescapable electroshocks in the FA test. (C)–(F) Two-way avoid-escape test with 30 tone-escapable electroshock trials: (C) Mean latency to avoid-escape response, (D) Total avoid responses, (E) Mean percent time freezing during tones and ITIs, (F) Mean reactivity to escapable electroshocks. *p* values are for Student's *t*-test.

freezing and less avoids. There was no CSD effect on reactivity to electroshock ($p = 0.31$, Fig. 2F). In the hot plate test (day 19), there was no effect of CSD on latency to display a pain response: CSD 18.0 ± 11.5 s, CON 22.5 ± 11.8 s ($p = 0.35$). Within CSD mice specifically, % time freezing during the motor activity test (Fig. 2A) predicted % time freezing during tones ($r = 0.80$, $p < 0.0009$) and ITIs ($r = 0.76$, $p < 0.002$) in the avoid-escape test (Fig. 2E). Furthermore, the number of avoid responses (Fig. 2D) was inversely correlated with the mean duration of attacks received during CSD sessions (Table 1) ($r = -0.72$, $p < 0.006$).

In Expt B, at treadmill training (TTr, day 18), there was no CSD effect on total electroshock time (CSD 11.9 ± 10.7 s, CON 7.3 ± 2.5 s, $p = 0.25$). On day 19, during the 5 min treadmill pre-test at 20 cm/s, there was also no CSD effect on total electroshock time ($p = 0.11$, Fig. 3A). However, two outlier CSD mice received 60 s of total electroshock during this pre-test and were removed from all subsequent testing for welfare reasons. At test (TTe), CSD mice ran less than CON mice as indicated by the decreased running time achieved before delivery of a total 10 s electroshocks ($t_{(19)} = 4.40$, $p < 0.0005$, Fig. 3B). Nine of 11 CSD mice received the maximum 10-s electroshocks compared with zero of 10 CON mice. The mean duration of each electroshock did not differ between the groups (CSD 0.23 ± 0.11 s, CON 0.20 ± 0.08 s, $p = 0.58$), indicating that CSD and CON mice required a similar time to escape from the electrified grid each time they ceased running and came into contact with it. On day 29, during the 5 min pre-test at 20 cm/s, there was a CSD effect on total electroshock time ($t_{(19)} = -2.23$, $p < 0.04$, Fig. 3C). At test, running time achieved was decreased in CSD relative to CON mice ($t_{(19)} = 5.42$, $p < 0.0005$, Fig. 3D). All 11 CSD mice received the maximum 10 s electroshocks versus only one of 10 CON mice. The mean duration per electroshock was greater in CSD (0.26 ± 0.10 s) than CON mice (0.17 ± 0.05 s, $p < 0.01$), indicating that CSD mice were now slower to escape from the electrified grid each time they ceased running and came into contact with it. Within CSD mice specifically, test running time achieved on day 29 was correlated with test running time achieved on day 19 ($r = 0.73$, $p < 0.01$).

In the same mice, in the motor activity test (day 30), there was no CSD effect on activity (CSD 3429 ± 1722, CON 4682 ± 1998 a.u./min, $p = 0.14$) or % time freezing ($p = 0.11$, Fig. 4A). In the fear acquisition test (day 31), as for Expt A (Fig. 2A), CSD mice exhibited increased mean % time fear freezing relative to CON mice ($t_{(19)} = -3.55$, $p < 0.002$, Fig. 4A). In contrast to Expt A (Fig. 2B), CSD mice exhibited decreased reactivity to electroshock compared to CON mice ($t_{(19)} = 3.82$, $p < 0.001$, Fig. 4B). In the 2-way avoid-escape test (day 32), CSD mice displayed a greater mean latency to avoid-escape than CON mice ($t_{(19)} = -2.16$, $p < 0.04$, Fig. 4C). The CSD mice made more avoid-escape failures ($t_{(19)} = -2.66$, $p < 0.02$, Fig. 4D) and less escapes (CSD 9.6 ± 7.0, CON 17.2 ± 6.9, $t_{(19)} = 2.49$, $p < 0.02$) than CON mice, while there was no difference in avoids (CSD 5.6 ± 5.5, CON 7.9 ± 5.0, $p = 0.34$). There was no CSD effect on % time spent freezing during tones ($p = 0.83$, Fig. 4E) or intervals between successive tone + electroshock pairings ($p = 0.67$, Fig. 4E). CSD mice exhibited decreased reactivity to electroshock ($t_{(19)} = 3.13$, $p < 0.006$, Fig. 4F). Within CSD mice specifically, % time freezing during the motor activity test (Fig. 4A) was associated with % time freezing in the avoid-escape test, both during tones ($r = 0.83$, $p < 0.002$) and ITIs ($r = 0.73$, $p < 0.01$) (Fig. 4E). Also within CSD mice specifically, % time spent freezing during tones (Fig. 4E) was correlated with avoid-escape latency ($r = 0.91$, $p < 0.0005$, Fig. 4C) and number of avoid-escape failures ($r = 0.76$, $p < 0.007$, Fig. 4D). Therefore, in experiments A and B, CSD mice exhibited evidence of helplessness in the 2-way avoid-escape test but the specific behavioural effects differed: In Expt A (day 18) the deficits were increased avoid-escape latency due to impaired avoidance combined with increased freezing and no effect on electroshock reactivity. In Expt B (day 32, after treadmill testing) the deficits were increased avoid-escape latency due to increased avoid-escape failure, no overall effect in freezing, and decreased electroshock reactivity. In the hot plate test (day 33), there was no effect of CSD on latency to display a pain response: CSD 20.0 ± 9.4 s, CON 23.6 ± 11.3 s ($p = 0.46$). In Expt B, there were no significant correlations between measures of treadmill behaviour and measures of avoid-escape behaviour, and also no significant correlation between any of these outcome measures and the mean duration of attacks received in CSD mice.

3.3. Physiological status

In experiments A, B and C there was no effect of CSD on mean absolute body weight (BW) per 5-day block (e.g. Expt A, group × day–block interaction, $p = 0.13$; Fig. 5A). However, mean day-to-day BW delta (ΔBW) per 5-day block was increased in CSD relative to CON mice, and similarly across the three experiments: for example, in Expt A there was a main effect of group ($F(1, 27) = 9.18$, $p < 0.005$) and *post hoc* comparisons per 5-day block indicated that mean ΔBW was greater in CSD than CON mice at days 2–6 and 7–11 (Fig. 5B).

In Expt A, at day 20, relative to CON mice, CSD mice had increased basal plasma concentrations of TNF ($t_{(23)} = -2.95$, $p < 0.007$, Fig. 5C) and IL-6 (non-normal distribution, Mann–Whitney $U = 36$, $N = (12, 13)$, $p < 0.02$, Fig. 5D). They also exhibited increased spleen weight, both absolute ($t_{(23)} = -3.90$,

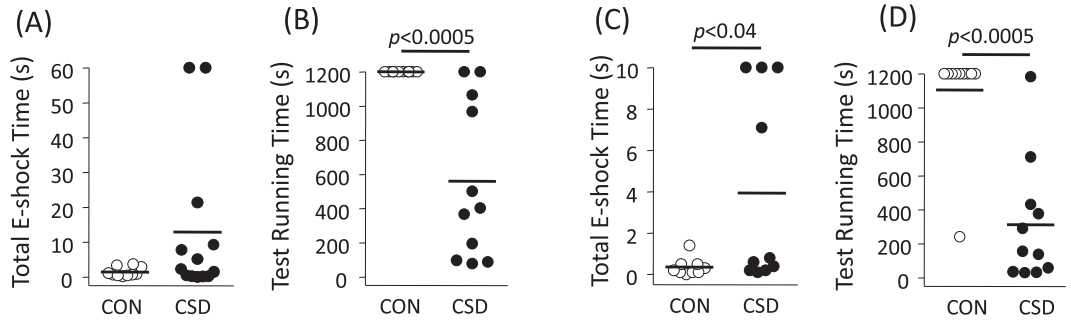

Fig. 3. Experiment B, effects of 15-day chronic social defeat (CSD, $N = 11–13$) versus control handling (CON, $N = 10$) in the treadmill fatigue test. (A) Day 19, total duration of electroshock received during 5-min pre-test at treadmill speed of 20 cm/s on. The 2 CSD mice that received 60 s of electroshock were removed from the experiment for welfare reasons. (B) Day 19, running time achieved during 20-min (maximum) test at treadmill speed of 25 cm/s. (C) Day 29, total duration of electroshock received during 5-min pre-test at treadmill speed of 20 cm/s. (D) Day 29, running time achieved during 20-min (maximum) test at treadmill speed of 25 cm/s. *p* values are for Student's *t*-test. Mice that received less than 10 s electroshocks during tests were exposed to a further short session at high speed (35 cm/s) until their total electroshock exposure reached 10 s, so that all mice received the same amount of electroshock prior to further behavioural testing.

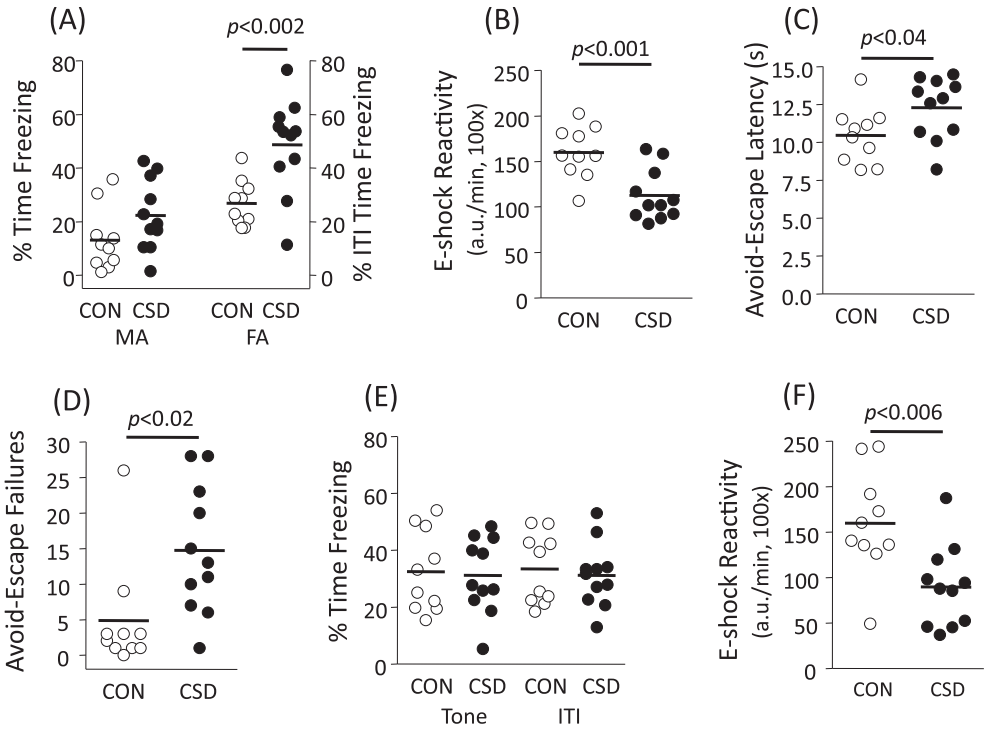

Fig. 4. Experiment B, effects of 15-day chronic social defeat (CSD, $N = 11$) versus control handling (CON, $N = 10$) in tests of motor activity, fear acquisition and two-way avoid-escape on day 30, 31 and 32, respectively. (A) Percent time freezing in the motor activity test (MA) and mean percent time freezing during 14 inter-trial intervals in the fear acquisition test (FA). (B) Mean reactivity (distance moved) to 15 inescapable electroshocks in the FA test. (C)–(F) Two-way avoid-escape test with 30 tone-escapable electroshock trials: (C) Mean latency to avoid-escape response, (D) Total avoid-escape failures, (E) Mean percent time freezing during tones and ITIs, (F) Mean reactivity to escapable electroshocks. *p* values are for Student's *t*-test.

$p < 0.001$) and relative to BW ($t_{(23)} = -3.38$, $p < 0.003$, Fig. 5E). Significant spleen hypertrophy in CSD mice was also observed in Expt B (day 34) and Expt C (day 17) (data not shown). Total adrenal gland weight was increased in CSD relative to CON mice, both absolute ($t_{(23)} = -2.30$, $p < 0.03$) and relative to BW ($t_{(23)} = -2.13$, $p < 0.04$, Fig. 5F). Significant adrenal gland hypertrophy was also observed in Expts B and C (data not shown). There was no effect of CSD on basal plasma corticosterone at day 20 ($p = 0.60$, Fig. 5G). For faecal corticosterone metabolite (Fig. 5H) there was a main effect of group ($F(1, 27) = 4.52$, $p < 0.05$) and a main effect of day ($F(2, 54) = 6.60$, $p < 0.003$). *A posteriori* analysis of CON and CSD mice separately demonstrated a significant day effect in CON mice ($F(1, 26) = 4.63$, $p < 0.02$) and not in CSD mice ($p = 0.13$); in CON mice specifically, faecal corticosterone metabolite was increased on days 18 and 19 (electroshocks received on previous day) relative to day 17 (no electroshocks on previous day) ($p < 0.02$, Fig. 5H). In CSD and CON mice analysed separately, there were no significant correlations between physiological measures and measures of fear conditioning, avoid-escape behaviour or treadmill behaviour. In CSD mice, there was no significant correlation between physiological measures and the mean duration of attacks received.

3.4. Gene expression

In Expt C (no behavioural testing), brains were collected at day 17 and differential gene expression was investigated for ventral hippocampus (vHIPP), amygdala (AMYG) and medial prefrontal cortex (mPFC) using next generation sequencing in 12 replicates per group (Fig. 6). The complete sets of genes that satisfied the expression criteria and were significantly up-regulated (↑) or down-regulated (↓) in CSD relative to CON mice are listed according to region(s) in Supplementary Tables S1 and S2, respectively. In vHIPP, CSD mice exhibited increased and decreased expression of 21 and 33 genes, respectively (Fig. 6, Tables S1 and S2). One of the up-regulated genes was *Tnfrsf25*, a member of the TNF-receptor superfamily; one of the down-regulated genes was *Cadps2*, a member of the calcium-dependent activator of secretion protein family that regulate the exocytosis of synaptic and dense-core vesicles in neurons. Using Ingenuity Pathway Analysis (IPA), there was no canonical pathway that was associated with more than one of these genes. However, upstream IPA identified the proinflammatory cytokines TNF, IL-6 and IL-3 as significant regulators of these genes. In AMYG, CSD mice exhibited increased and decreased expression of 22 and 48 genes, respectively (Fig. 6, Tables S1, S2). In terms of canonical pathways, these genes were primarily associated with "T cell receptor signalling" (4 genes: *Cd4* (↓), *Jun* (↑), *Ptprc* (↑), *Shb* (↓)), "G-protein coupled receptor signalling" (5 down-regulated (↓) genes: *Drd2*, *Adora2a*, *Chrm3*, *Htr2a*, *Pde10e*), and "C–C chemokine receptor type 5 (CCR5) signalling in macrophages" (3 genes: *Cd4* (↓), *Jun* (↑), *Gng7* (↓)). A number of the genes exhibiting altered expression in AMYG of CSD relative to CON mice encode proteins that bind dopamine and are involved in either mediating or regulating dopamine neurotransmission (Table 2). One of the genes, *Slc29a4* (↑), encodes a dopamine and serotonin reuptake protein, and expression of the serotonin receptor *Htr2a* (↓) was also responsive to CSD. Furthermore, IPA upstream analysis identified dopamine as a transcription regulator for several genes exhibiting altered expression in AMYG of CSD mice (Table 2). In mPFC, CSD mice exhibited increased and decreased expression of 6 and 14 genes, respectively (Fig. 6, Tables S1 and S2). As for AMYG, the canonical pathways primarily associated with these genes had immune-inflammatory function, namely "IL-12 signalling and production in macrophages", and "production of nitric oxide and reactive oxygen species in macrophages". The same three genes enriched both pathways, namely *Apod* (↓), *Fos* (↑) and *Prkcd* (↑).

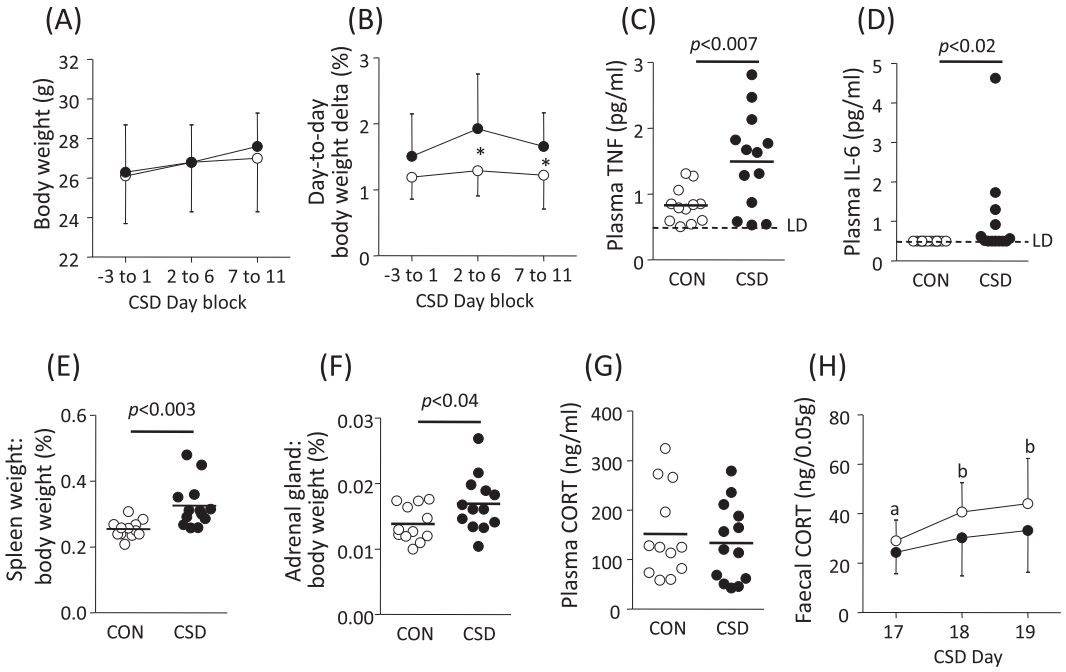

Fig. 5. Experiment A, Effects of 15-day chronic social defeat (CSD, $N = 13$) versus control handling (CON, $N = 12$) on body weight, inflammation markers and adrenal gland-corticosterone (CORT) markers. (A) Mean absolute body weight at 14:00 h in 5-day blocks (overall mean ± SD). (B) Mean percent day-to-day body weight delta (abs(BW day n−BW day n−1))/(BW day n−1)*100) (overall mean ± SD) *$p < 0.05$ for CSD versus CON mice in day block indicated, using Group × Day block ANOVA and *post hoc* tests per Day-block. End-point measures on day 20: (C) Basal plasma tumor necrosis factor (TNF). p value is for Student's t-test. (D) Basal plasma interleukin-6 (IL-6). p value is for Mann−Whitney U-test. (E) Spleen weight relative to body weight. p value is for Student's t-test. (F) Total adrenal gland weight relative to body weight. p value is for Student's t-test. (G) Basal plasma corticosterone (CORT). (H) Faecal CORT metabolite (mean ± SD) on day after motor activity test (day 17), fear acquisition test (day 18) and two-way avoid-escape test (day 19). a vs. b, $p < 0.05$ in CON mice specifically, using Group × Day block ANOVA and *a posteriori* group-specific ANOVAs.

4. Discussion

The overall aim of the present study was to investigate effects of chronic exposure to a psychosocial stressor on behavioural responses to physical stress and associated changes in physiology and brain gene expression, in adult male mice. The study design was informed by the generalized helplessness hypothesis of depression, which proposes that exposure to a specific aversive life event (stressor) that is of high emotional significance and uncontrollable leads to altered responding to all aversive life events, including increased negative emotionality, decreased motivation to actively cope with/control the adversity, and reduced cognitive expectancy to cope with/control the adversity (Abramson et al., 1989, 1978; Beck et al., 1974; Diener et al., 2009; Kendler et al., 2003; Kessler, 1997; Maier and Seligman, 1976; Pryce et al., 2011). Although it is one of the few succinct hypotheses of depression onset-maintenance, research into the neurobiology that might underlie generalized helplessness has been scant, and relevant animal models would make an important contribution to this. Here it was demonstrated that mice exposed to chronic social defeat without physical wounding developed sustained states of decreased motor activity, increased acquisition of fear, decreased active responding to an aversive conditioned stimulus or to the unconditioned stimulus, and increased fatigability, in response to a physical stressor in the form of mild electroshock. These findings are consistent with a mouse model of generalized helplessness. The behavioural effects co-occurred with peripheral and central indices of immune-inflammatory activation and, in the amygdala, changes in expression of genes important for dopamine function. Although correlational, this physiological and genetic evidence could be important to identifying the systems underlying the observed behavioural effects and, furthermore, generalized helplessness in human depression.

A modified version of the most widely-used protocol (Golden et al., 2011) for chronic social defeat was used in this study. As stated in the standard protocol, bite wounding is common and we also observed this in pilot studies. The refinements of regular trimming of the incisor teeth of CD-1 mice and restricting of attacks to 60 s/day resulted in a complete absence of skin penetration bite wounds; the majority of mice had no physical wounds and a minority received occasional surface abrasions. Therefore, in the present study CSD was primarily an emotional social stressor. The insightful first report of CSD describes that the characteristic displays of submissive behaviour by CSD mice were ineffective in controlling attacks by the aggressor mice (Kudryavtseva et al., 1991), and the same was observed here (see also Savignac et al., 2011b). That is, CSD mice experience repeated encounters with uncontrollable dominant mice and otherwise continuous exposure to the sensory stimuli associated with these social stressors. Furthermore, a different dominant mouse was encountered each day, thereby increasing the unpredictability of the stressor. Because attack duration was limited to 1 min/day, in contrast to the possible 10 min/day in the standard protocol, the chronicity of the social stressor was increased from 10 to 15 days. The control mice were maintained in our standard caging condition of littermate pairs rather than singly, so that single caging must be considered as a component of the CSD procedure in our experimental design. As stated in the Methods, a pilot study comparing CON mice pairs maintained together or separated from each other by a divider demonstrated no effects in tests of motor activity, fear acquisition or two-way avoid-escape behaviour.

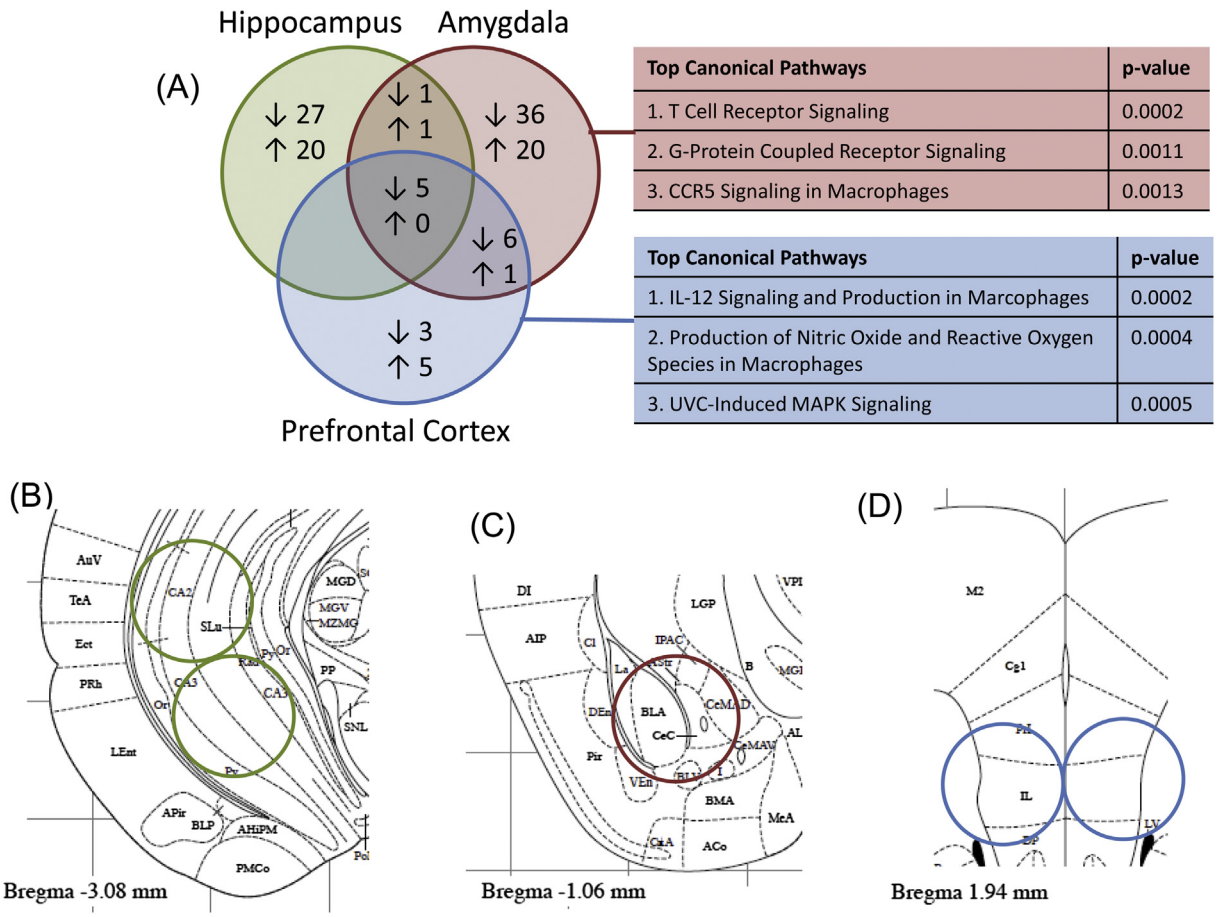

Fig. 6. Experiment C, investigation into effects of 15-day chronic social defeat on region-specific gene expression at day 17, using next generation sequencing with mRNA obtained from CSD mice ($N = 12$) and CON mice ($N = 12$). No pooling of samples was conducted i.e. for CSD and CON groups, 12 replicates were run. (A) Venn-diagram of genes that exhibited significantly decreased (↓) or increased (↑) expression in CSD relative to CON mice in one or more of ventral hippocampus, amygdala and medial prefrontal cortex. Criteria used to identify differential gene expression in CSD relative to CON mice were mean reads per kilo base per million (RPKM) > 5, mean fold change > 1.4, and $q < 0.05$. The three canonical pathways most enriched by these differentially-expressed genes, as identified using Ingenuity Pathway Analysis, are given for amygdala and medial prefrontal cortex. (B)–(D) Representative figures (from (Franklin and Paxinos, 2008)) depicting the target region for (B) ventral hippocampus, (C) amygdala and (D) medial prefrontal cortex. For the complete listing of CSD-induced altered gene expression, see Supplementary Tables S1 and S2.

With regards to behavioural effects, the 3-day paradigm was designed to allow for the assessment of CSD effects on emotional, motivational and cognitive responses to a different stressor, namely electroshock, presented in different settings. In Expt A, CSD resulted in decreased motor activity but not increased fear freezing in a neutral context. On the following day, CSD mice acquired increased fear freezing to this same context when exposed to mild inescapable electroshocks. In the two-way avoid-escape test, CSD mice exhibited a deficit in the active responding to the CS that was reinforced by CS termination and electroshock

Table 2
Genes exhibiting altered expression in CSD versus control mice in amygdala that regulate and/or are regulated by dopamine neurotransmission.

Gene		Up-/ down-regulation	Fold change[a]	Regulator of DA function	Regulated by DA function[b]	References
Adora2a	Adenosine A2a receptor	↓	1.9	+		(Boison et al., 2012)
Darpp-32	Dopamine and cAMP regulated phosphoprotein 32	↓	1.7	+	+	(Brouillet et al., 2005; Li et al., 2013)
Drd2	Dopamine receptor D2	↓	1.8	+		(Boison et al., 2012)
Gabrd	Gamma-aminobutyric acid (GABA) A receptor, delta	↓	1.6		+	(Meurers et al., 2009)
Gng7	Guanine nucleotide binding protein, gamma 7	↓	1.4	+		(Sasaki et al., 2013)
Gpr88	G protein-coupled receptor 88	↓	1.8	+		(Logue et al., 2009)
Kcnk2	Potassium channel, subfamily K, member 2	↓	1.5		+	(Meurers et al., 2009)
Pde10a	Phosphodiesterase 10A	↓	1.5		+	(Giorgi et al., 2011)
Rgs9	Regulator G-protein signaling 9	↓	2.0	+		(Celver et al., 2012; Rahman et al., 2003)
Tac1	Tachykinin, precursor 1	↓	1.9		+	(Meurers et al., 2009)
Gpr68	G protein-coupled receptor 68	↑	1.4		+	(Meurers et al., 2009)
Jun	Jun proto-oncogene	↑	1.4		+	(Luo et al., 1998)
Slc29a4	Solute carrier family 29 (nucleoside transporters), member 4	↑	1.4	+		(Engel et al., 2004)

[a] q-Values for fold change = 0.003 in all cases.
[b] Upstream analysis conducted using Ingenuity Pathway Analysis.

omission. Given that this deficit co-occurred with increased fear freezing, it could reflect either increased emotional reactivity or decreased response-outcome cognitive expectancy, or both processes. These mice did not exhibit decreased electroshock reactivity, indicating intact motivation to react to an aversive stimulus. In Expt B, the same test battery was conducted after treadmill testing and 2 weeks later than in Expt A. There was no CSD effect on motor activity in a neutral context; this contrast to Expt A was due primarily to the reduced activity of CON mice in Expt B versus A. CSD mice acquired increased fear freezing to this context when exposed to mild inescapable electroshocks (as in Expt A), although this co-occurred with decreased electroshock reactivity (not the case in Expt A); these findings are consistent with increased aversive emotionality and decreased motivation to react to an aversive stimulus, respectively. In the two-way avoid-escape test, CSD mice were deficient in active responding to the escapable electroshock, as demonstrated by increased avoid-escape failures and decreased electroshock reactivity. These deficits could reflect decreased motivation to react to or decreased expectancy to control an aversive stimulus, or both; that they were not accompanied by increased freezing suggests that fear was not increased. This behavioural profile of CSD mice in Expt B was remarkably similar to that of mice exposed repeatedly to inescapable electroshock and then tested with escapable electroshock; such mice also exhibit high escape failure i.e. specific learned helplessness, low electroshock reactivity, and no increase in fear freezing (Pryce et al., 2012). Increased stress-induced analgesia in CSD mice might have contributed to the effects observed, but two findings indicate that this was unlikely: CSD mice exhibited decreased electroshock reactivity together with increased freezing in the contextual fear test in Expt B; there was no effect of CSD in the hot plate test in Expts A or B. Comparing Expts A and B, the increased deficit in actively responding to electroshock demonstrated by CSD mice in Expt B could be due to carry over effects of an interaction between CSD and treadmill testing (see below), or to the additional 14 days between CSD and two-way avoid-escape testing; further experiments will be required to investigate this. The current findings demonstrate that mouse CSD induces altered emotional, motivational and cognitive responding to mild electroshock stimuli in accordance with the hypothesis of generalized learned helplessness. The exact protocol to be used in future studies would depend on the specific aims: a focus on generalized increased emotionality to aversive stimuli should combine CSD and fear conditioning; focus on generalized impaired cognitive control should combine CSD and two-way avoid-escape; and focus on generalized decreased motivation to react to aversive stimuli should combine CSD plus an additional challenge (e.g. treadmill) followed by two-way avoid-escape. Each of these models would be relevant to a specific depression psychopathology and to the study of its underlying aetio-pathophysiology. Additional psychopathologies will also need to be addressed in future experiments. Important among these will be whether CSD leads to deficits in extinction of fear memory, as shown for other stressors (Zhang and Rosenkranz, 2013), which would be relevant to the deficient cognitive flexibility that is a common symptom of depression (Clark et al., 2009). Furthermore, CSD effects on reward processing will need to be investigated in future studies, to examine whether the depression-relevant increase in punishment reactivity demonstrated here co-occurs with a decrease in reward sensitivity, relevant to the core depression symptom of reduced interest and pleasure. The sucrose preference test (Willner, 1997), intra-cranial self-stimulation (Slattery et al., 2007), and operant tests such as sucrose pellet reinforcement on a progressive ratio schedule (Ineichen et al., 2012), are examples of relevant readout tests.

To our knowledge treadmill testing has not been applied previously in an animal model of depression. This is surprising given the use of rodent treadmill running as a readout for fatigability in exercise physiology e.g. (Massett and Berk, 2005) and in response to inflammation e.g. (Carmichael et al., 2006), and also given that fatigue is a core depression symptom (ICD-10, 1994). Relative to CON mice, which were able to complete the tests conducted on days 19 and 29, CSD mice displayed a running deficit in both tests. At day 19, CSD mice exhibited running comparable to that of CON mice in the 20 cm/s pre-test, with the exception of two CSD mice that were unable to run consistently even at this speed and were removed from further study for welfare reasons. In the test at 25 cm/s, most CSD mice could not maintain the required running speed; two sub-groups formed in terms of mice that were or were not able to run for at least 600 s. At day 29, a sub-group of CSD mice were already unable to run consistently at the pre-test and 9 of 11 CSD mice were not able to run for at least 600 s at test. The CSD running deficit could be due to increased motor fatigability, increased emotionality, decreased motivation to respond actively to an aversive stimulus, decreased response-outcome expectancy, or a combination of these factors. Given that human fatigue in depression comprises components of physical tiredness, motor retardation, deficient motivation, emotional fatigue and cognitive fatigue (Demyttenaere et al., 2005), then strict differentiation between these components is probably artificial. The CSD-treadmill fatigue model can be applied to increase understanding of fatigue neurobiology, in terms of its separate components and their interaction.

The present findings add to those reported previously for CSD. CSD has been reported to increase expression of fear freezing to context and CS (Yu et al., 2011). As noted in the Introduction, for CSD behavioural effects the major focus to-date has been on passive avoidance by CSD mice of mice from the dominant aggressor CD-1 strain in a social proximity test (Berton et al., 2006; Haque et al., 2012; Krishnan et al., 2007; Savignac et al., 2011a). In terms of helplessness theory, this effect constitutes increased emotional reactivity to the specific stimulus that induced the CSD (Russo et al., 2012). In some studies, passive avoidance of CD-1 mice has been reported to occur in about 50% of CSD mice with the other 50% not avoiding the dominant aggressor strain (Krishnan et al., 2007). The mice that developed passive avoidance also developed a reduction in sucrose preference, whereas CSD mice that did not develop social passive avoidance also exhibited normal sucrose preference (Krishnan et al., 2007). In the present study, significant effects of CSD in terms of increased fear acquisition, decreased avoidance, increased avoid-escape failure, decreased electroshock reactivity and increased fatigue, were obtained with sample sizes of 10–13 mice without division into sub-groups. However, as described above, two sub-groups were apparent in some stages of treadmill fatigue testing. It was not the case that CSD mice which exhibited relatively large effects in terms of treadmill fatigue also exhibited relatively high fear freezing and avoid-escape failures; that is, there was no consistent evidence for relatively susceptible versus resilient mice. Although there was not a significant CSD effect on fear freezing in the neutral motor activity test, this parameter did predict fear freezing during the avoid-escape test; as such fear freezing in the neutral environment will provide a valuable marker for balanced allocation of CSD mice to drug treatment groups in future pharmacological studies with this model.

In terms of physiological effects, CSD did not impact on absolute body weight. Previous studies report that CSD increased (Bartolomucci et al., 2009), decreased (Kudryavtseva et al., 1991) or had no effect on (Savignac et al., 2011a) absolute body weight. Decreased and increased body weight are both facultative depression symptoms (ICD-10, 1994). In the present study CSD did increase day-to-day body weight variability; high variability is

associated with negative affect and low self-esteem in humans (Foreyt et al., 1995; Serdar et al., 2011). A major rationale for refining the CSD protocol to eliminate bite wounding was to render the current model compatible with the study of the immune-inflammation hypothesis of depression. Accordingly, it is interesting that plasma levels of the pro-inflammatory cytokine TNF were increased in CSD mice, as was IL-6 in a sub-group of these mice. A CSD study in BALB/c CSD mice and C57BL/6 aggressor mice also reported increased plasma levels of TNF and proinflammatory interleukins; although the standard CSD protocol was used there were no correlations between cytokine level and number of bite wounds received (Savignac et al., 2011a). In healthy humans, acute psychosocial stress causes increased blood levels of pro-inflammatory cytokines (Bierhaus et al., 2003) and blood levels of TNF and IL-6 are increased in depression (Dowlati et al., 2010). In addition, the spleens of CSD mice were hypertrophic, consistent with invasion and expansion of activated immune cells. Studies using the standard CSD protocol report either spleen hypertrophy (Bartolomucci et al., 2004) or normal spleen size (Savignac et al., 2011a) in CSD mice. Adrenal glands were also hypertrophic in CSD mice, as observed in mice exposed to chronic subordinate colony housing (Reber et al., 2007). Despite adrenal hypertrophy, basal plasma CORT levels in blood samples collected at the termination of Expt A were not increased in CSD mice. Previous CSD studies report that basal plasma CORT is increased (Perez-Tejada et al., 2013) or unchanged (Krishnan et al., 2007). Furthermore, in faecal boli collected across the 3-day paradigm in Expt A, CORT metabolite was increased on post-stress days in CON but not in CSD mice. The combination of adrenal hypertrophy, no increase in basal CORT and evidence for decreased CORT stress-reactivity, is consistent with adrenal insufficiency in terms of response to ACTH challenge, as reported for chronic subordinate colony housing (Reber et al., 2007). Therefore, CSD mice exhibited signs of immune-inflammatory activation combined with an adrenal insufficiency that would dampen anti-inflammatory corticoid activity; this would predispose them to stress-induced activation of immune-inflammatory pathways and the pathological effects thereof (Rhen and Cidlowski, 2005; Silverman and Sternberg, 2012). It was not the case that CSD mice which exhibited relatively large effects in terms of inflammatory or adrenal measures also exhibited relatively large behavioural effects of CSD; that is, there was no consistent evidence for relatively susceptible versus resilient mice.

Given the observed behavioural effects of CSD, specific brain regions important in the circuitries of fear conditioning (AMYG, vHIPP (Maren et al., 2013)), avoid-escape learning (AMYG, mPFC (Moscarello and LeDoux, 2013)), specific learned helplessness (mPFC (Amat et al., 2005; Pryce et al., 2011)) and fatigue (DeLuca et al., 2009), were investigated in terms of CSD effects on transcriptome-level gene expression, with brains collected at day 17. Importantly, these same regions exhibit altered structure-function-molecular genetic changes in depression e.g. (Disner et al., 2011; Mayberg, 2003; Price and Drevets, 2010; Savitz et al., 2013; Sibille et al., 2009). The experiment was conducted using next generation sequencing, and genes that were expressed above a threshold level and were significantly altered in their expression in CSD versus CON mice were subjected to pathway analysis to identify the processes and pathways with which they are most commonly associated and their upstream regulators. In vHIPP, for 11 of the 54 genes with altered expression in CSD mice, the pro-inflammatory cytokines TNF, IL-6 and IL-3 are major upstream regulators of expression, and the former two were increased in the plasma of CSD mice. The up-regulated genes included the TNF-receptor superfamily gene *Tnfrsf25*, the protein encoded by which stimulates NF-κβ and cell apoptosis. In AMYG, CSD led to de-regulation of 70 genes (48 ↓ and 22 ↑). Two of the three canonical pathways most enriched by these genes were "T cell receptor signalling" and "CCR5 signalling in macrophages", suggesting that CSD initiated changes in immune-inflammation transcription processes in this region. In the former pathway, *Ptprc*, an essential regulator of cytokine signalling, was up-regulated in CSD mice; in the latter pathway, *shb*, an inhibitory regulator of T cell receptor, was down-regulated in CSD mice, consistent with disinhibition of T cell signalling (Gustafsson et al., 2011). Another down-regulated gene in the CCR5 pathway was *Gng7*: Gng7 is coupled to the dopamine (DA) 1 receptor and mice deficient in Gng7 exhibit down-regulation of DA 2 receptor (D2R) (Sasaki et al., 2013). In mPFC, CSD led to de-regulation of 20 genes (14 ↓ and 6 ↑). The canonical pathways most enriched by these genes were immune-inflammatory, namely "IL-12 signalling and production of macrophages", and "production of nitric oxide and reactive oxygen species in macrophages". One of the up-regulated genes was *Prkcd*: Prkcd has been identified as a major mediator of TNF-induced degeneration of DA neurons (Gordon et al., 2012). Several recent human studies report increased expression of immune-inflammation genes in depressed relative to healthy-control probands. These include a genome-wide expression study of peripheral blood mononuclear cells that identified increased *TNF* expression in depression (Savitz et al., 2013), and a *post mortem* brain tissue microarray study reporting increased expression of pro-inflammatory and anti-inflammatory cytokine pathway genes in the mPFC in depression (Shelton et al., 2011). Specific evidence for increased density or activation of microglia was not obtained in the present study. For genes expressed by microglia, e.g. toll-like receptors, expression levels were below the threshold level (>5 RPKM) for reliable quantification. Indeed, previous studies that have demonstrated that CSD-like manipulations do activate microglia have used whole-brain cell sorting and flow cytometry to isolate microglia (e.g. Wohleb et al., 2011). This methodology will need to be applied to the current model in future studies.

Attributing function to these CSD effects on gene expression can only be done conservatively in the absence of evidence for corresponding effects at the protein level. One pathway via which increased central immune-inflammatory activity has been proposed to impact on brain function is oxidative stress and neurotoxicity leading to inhibition of dopamine and serotonin function (Felger and Miller, 2012; Haroon et al., 2012; Miller et al., 2009). In this respect it is noteworthy that for AMYG, a number of the genes de-regulated by CSD express proteins that are either mediators or regulators of DA function, including: *Drd2*, *Adora2a*, *Gpr88*, *Darpp-32*, *Rgs9*, *Slc29a4* and, as discussed above, *Gng7* (as well as *Prkcd* in mPFC) (Table 1). Dopamine is released into the basolateral (BLA) and central (CeA) AMYG in the presence of emotional stimuli, and both regions express non-overlapping populations of D1 and D2 receptors, primarily on glutamate projection neurons in BLA and GABA neurons in CeA (Perez de la Mora et al., 2012). D2R expression is high in CeA, as it is in the inter-connected structures of bed nucleus of stria terminalis and nucleus accumbens (central extended AMYG) and dorsal striatum-external pallidum (Alheid and Heimer, 1988). D2R antagonism, including in the CeA specifically, has been demonstrated to decrease two-way avoid-escape and increase fear (Perez de la Mora et al., 2010; Reis et al., 2004). The D2R interacts antagonistically with the adenosine receptor A2AR by forming heteromers (Boison et al., 2012); because expression of both *Drd2* and *Adora2a* was decreased by CSD it is difficult to predict the functional consequences; nonetheless it is noteworthy that striatum-specific *Adora2a* knockout mice exhibited decreased activity and two-way avoid-escape (Singer et al., 2013), as observed in CSD mice. Both *Adora2a* and *Gpr88* exhibit enriched expression in the central extended AMYG and dorsal striatum (Becker et al.,

2008); accordingly, the decreased expression observed in CSD mice might have occurred primarily in CeA indicating that future studies should analyse CSD effects on gene expression here and in striatal regions. In striatum, Gpr88 is expressed on GABA medium spiny neurons also expressing D2R or D1R; *Gpr88* knockout mice exhibited impaired two-way avoid-escape (Quintana et al., 2012), as observed in CSD mice. *Darpp-32* encodes a signalling phosphoprotein expressed in D2R and D1R neurons and is a major integrator of signals from neurotransmitters and neuromodulators targeting these neurons; again, it has been studied mainly in striatum but decreased expression here or in AMYG would be predicted to have important consequences for DA neuron function and behaviour (Bateup et al., 2010). *Rgs9* encodes a protein, Rgs9-2, that modulates D2R function: *Rgs9* knockout mice exhibited increased locomotor activation in response to amphetamine, indicating that Rgs9-2 normally reduces D2R signalling (Rahman et al., 2003); however, in such mice there was no change in D2R levels, whereas in CSD mice the decrease in *Rgs9* might constitute a response to decreased D2R levels. *Slc29a4* encodes a presynaptic protein expressed by monoamine neurons, including in AMYG, and catalyses monoamine reuptake (Dahlin et al., 2007); *Slc29a4* was up-regulated in the AMYG of CSD mice, suggesting that their DA and 5-HT reuptake might be increased. Finally, the current finding that CSD decreases *Drd2* expression is also significant in the light of the evidence that striatal D2R is a positive modulator of motivation (Trifilieff et al., 2013), DA-striatal dysfunction underlies fatigue (Capuron et al., 2007; Demyttenaere et al., 2005), and a polymorphism in *D2R* associated with reduced D2R density, particularly in dorsal striatum, is associated with increased risk of depression (Zou et al., 2012).

Overall, the present experiments demonstrate that chronic social defeat in adult male C57BL/6 mice leads to altered processing of aversive physical stimuli in the form of increased emotional fear, decreased motivation to respond actively to the aversive stimulus, decreased response-control cognitive expectancy, and increased fatigability. These effects are relevant to specific psychopathology symptoms and features in depression and also to the generalized helplessness theory of depression onset-maintenance. The model therefore has aetiological and face validity and can be utilised to investigate the specific processes mediating between chronic social defeat and these depression-relevant behavioural effects. In this respect, evidence is presented for potentially important roles of immune-inflammation and altered dopamine function, and these two processes could be causally linked. Future studies will investigate these hypotheses, with a view to applying the model to identify novel targets for treatment of specific psychopathologies such as generalized helplessness or fatigue, and to conduct preclinical screening of compounds/biologics developed to act at these targets.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2014.05.039.

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