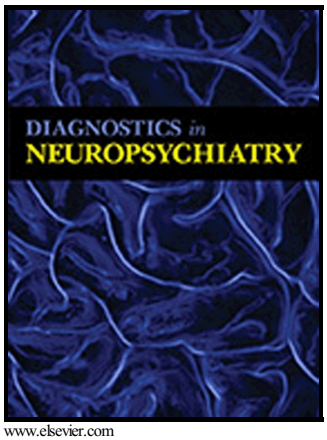

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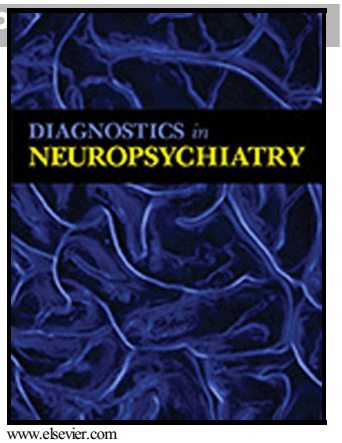

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Keywords: social defeat, immunoassay, proteomics, myelination, oligodendrocytes, inflammation

Highlights:

- Social defeat (SD) in mice is a preclinical model for major depressive disorder
- First study investigating protein differences in serum / frontal cortex in SD mice
- Resilience to SD stress could be mediated through activation of oligodendrogenesis
- Peripheral inflammation / growth-related proteins could be candidate SD biomarkers

Abstract

The social defeat mouse model is used as a preclinical model for major depressive disorder (MDD). This model is of interest, as mice subjected to chronic social defeat can be separated into stress susceptible (SS) and resilient (SR) subgroups that differ in defined behavioural and physiological characteristics. Here, we have carried out proteomic analyses of serum and brain samples from SS (n=12), SR (n=12) and unstressed control (n=12) mice, using two analytical platforms to gain insight into the underlying molecular pathways that distinguish these subgroups. Multiplex immunoassay profiling was performed using sera collected after 10 days of chronic social defeat. This analysis identified peripheral alterations in proteins mostly associated with inflammation in SS mice, whereas growth factors and hormones were changed predominantly in the SR subgroup. Label free liquid chromatography mass spectrometry (LC-MS^E) profiling of frontal cortex revealed a significant increase in myelin-associated proteins [2´,3´-cyclic-nucleotide 3´-phosphodiesterase (CN37), mylein basic protein (MBP), and myelin proteolipid protein (MYPR)] in the SR group, suggesting that resilience to social stress might be mediated through activation of oligodendrogenesis. Taken together, these results provide the first proteomic evidence of differential effects on oligodendrocyte function between susceptible and resilient subgroups in the social defeat model and suggest that enhanced of neuronal conductivity or central nervous system maintenance in the frontal cortex is involved in the adaptive response to stress. These changes appear to be reflected by serum alterations in inflammation and growth-related proteins, which could be used as biomarkers for predicting or monitoring stress response.

Introduction

Despite the profound negative effects of major depressive disorder (MDD) on public health and the associated suicide risk of 15% (1), there has been little progress in our understanding of the pathophysiological processes involved and the discovery of new therapeutic mechanisms is at a near standstill. This is partially due to the limited availability of preclinical models that accurately reflect symptoms of affective disorders (2). Nevertheless, their use is currently indispensible in psychiatric research, since ethical and practical factors restrict the ability to study MDD in human subjects.

Since MDD is a multifaceted disorder, it is unlikely that its entire symptom spectrum can be accurately recapitulated in a single preclinical model. Due to constraints in their face validity only certain affective symptom dimensions can be modelled, such as anhedonia or behavioural despair(2). Most preclinical models used in MDD research have been investigated because they show behavioural endophenotypes similar to human MDD patients. However, there have been relatively few studies which have investigated the underlying proteomics pathophysiology in these models. So far, studies in preclinical chronic mild stress models were limited to the use of two-dimensional gel electrophoresis reporting abundance changes involved in energy and protein metabolism(3-5). Further research applying advanced proteomic techniques is important to identify reproducible molecular changes which can be translated to patient studies.

Stressful life events have a substantial causal association with MDD [reviewed in (6)]. Emerging evidence suggests that a combination of genetic and lifetime stress factors may ultimately determine the vulnerability of individuals to develop psychiatric disorders such as MDD [reviewed in (7)]. One preclinical model of MDD, based on naturalistic stressors, is the social defeat (SD) model. In this paradigm, an intruder mouse is exposed repeatedly to an aggressive and dominant resident and to bouts of social subordination. The defeated animal may be returned to the aggressor's cage for repeated social defeats or placed in close proximity to the aggressor or in the vacant cage of the aggressor to reinforce the negative experience [reviewed in (6)]. After these chronic defeats, stress

susceptible (SS) mice show a range of depression-like symptoms including anhedonia and social withdrawal (8). These symptoms have been shown to be reversible by chronic but not acute antidepressant drugs (ADs) (8), demonstrating the face and predictive validity of the SD paradigm. Another advantage of the SD model is that it can be used to study factors involved in the resilience to SD stress. This is because a subset of animals of this model does not develop social avoidance behaviour. Investigations focussing on these stress resilient (SR) animals may help to elucidate why in some individuals exposure to chronic stress leads to an imbalance in physiological and behavioural homeostasis (9) which can result in MDD, while the majority of individuals appear to be resilient against such imbalances (7, 10).

In this study, we have focused on the identification of central nervous system (CNS) molecular changes in the SD mouse model subgroups, SS and SR, compared to control mice. In addition, peripheral effects were investigated, as such changes can be translated more readily to clinical studies. Therefore, blood serum and brain samples from these mice were analysed using multiplex immunoassay and liquid-chromatography mass spectrometry (LC-MSE) approaches, respectively. Our goal was to identify a translational molecular signature to evaluate the potential and relevance of this model in MDD research and in drug discovery.

Results

Serum analysis (multiplex immunoassay)

Systemic responses to stress in the SS and SR mice were measured by analysing 89 immune system and metabolic circulating markers in the serum. The full list of molecules investigated is listed in **Supplementary table 1**. Inspection of intensity data by PCA of all molecules tested with the multiplex immunoassay panels revealed no outliers and no batch effects indicating good sample and data quality. Therefore, data from all samples were used in the statistical analyses. This resulted in identification of 10 and 13 significant molecular changes in sera from SS and SR mice, respectively

(**Table 1**). Three of these molecules, including angiotensin-converting enzyme (ACE), interleukin 1 alpha (IL-1α), and matrix metalloproteinase-9 (MMP9), were altered in both subgroups, with the same direction of change and all were involved in the inflammation response.

In the SS group, myeloperoxidase (MPO), MMP-9 and MCP-3 were all changed by more than 2-fold compared to control unstressed mice. These proteins were all associated with the inflammation response. The remaining proteins which were altered in the SS mice are involved in inflammation (C-C motif chemokine 9, IL-1β, clusterin and C-reactive peptide) or stress response [adrenocorticotrophic hormone (ACTH)].

In the SR group, epidermal growth factor (EGF), vascular endothelial growth factor A (VEGFA), tumor necrosis factor alpha (TNF-α) and immunoglobulin A (IgA) showed the highest magnitude of change, as these proteins were altered by more than 2-fold. The first two of these proteins are growth factors and the latter two are associated with the inflammation response. The other proteins altered in the SR mice were either inflammation-related proteins (macrophage migration inhibitory protein 2, interferon-γ, macrophage colony-stimulating factor 1 and macrophage-derived chemokine) or hormones (insulin and progesterone).

Brain analysis (LC-MS^E profiling)

LC-MS^E analysis was carried out on frontal cortices from the SS (n=12) and SR (n=12) subgroups in comparison to control (n=12) mice. The CV of the QC samples was 17.79 ± 14.36 (average ± standard deviation). Overall, 12,127 peptides were detected in the frontal cortex which corresponded to 975 unique proteins. **Supplementary Table 3** shows the full list of altered proteins in frontal cortex ($q<0.1$)

No proteins were identified with significantly different concentrations between the SS and control mice at a strict q-value of <0.05 (**Table 2**). In contrast, 10 significantly different proteins were identified in frontal cortices from SR compared to control mice. These proteins were involved in

blood clotting (plasma protease C1 inhibitor), synaptic vesicle release (tubulin alpha 1B chain, mitogen activated protein kinase 1 and latrophilin-2), cell growth [neuromodulin and puromycin sensitive aminopeptidase (PSA)) and in myelin-associated processes (2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CN37), myelin basic protein (MBP), myelin proteolipid protein (MYPR)].

PSA showed the most marked difference with a fold change of 1.69. Additionally, five proteins (plasma protease C1 inhibitor, latrophilin, CN37, MBP, MYPR) were altered by more than 1.4-fold.

Discussion

This is the first study which investigated molecular profiling differences in serum and brain of the SD model to take into account differences between SS and SR mice. This allowed the detection of different molecular signatures between the two subgroups. The serum analysis showed several differences in the proteins altered in the two groups. Proteins mostly associated with the inflammatory response were altered in the SS mice and proteins associated with cell growth/proliferation were changed in both the SS and SR mice, whereas growth factors and hormones were changed predominantly in the SR animals. This suggests that SS mice showed signs of undergoing an enhanced inflammatory response.

Several studies have suggested that inflammatory mechanisms play an important role in MDD, with findings of elevated levels of pro-inflammatory cytokines and other inflammation-related proteins in blood, CSF and post-mortem brain tissue of MDD patients [reviewed in (11)]. Furthermore, it has been hypothesised that inflammation and MDD may share a common pathophysiological process linked to immune dysregulation (12). This is consistent with our findings since both SD subgroups showed elevated levels of pro-inflammatory or stress-related proteins, such as matrix metalloproteinase-9, ACE and IL-1-α. However, the SS group showed specific increases in additional inflammation proteins (e.g. myeloperoxidase, monocyte chemotactic protein 3, C-C motif chemokine 9, IL-1β, clusterin, C-reactive protein and ACTH), and such proteins have been shown to be also

elevated in MDD patients (13-18). The finding that the SS mice also displayed increased levels of ACTH was indicative of activation of the HPA-axis in these animals. Previous studies have shown that the release of ACTH is stimulated by pro-inflammatory cytokines [reviewed in (19)], which is consistent with the pro-inflammatory findings in the SS model discussed above. As increased ACTH levels have been previously reported in MDD patients (20, 21), these findings support the use of the SS rodents as a putative MDD animal model with associated hyperfunction of the HPA-axis. Previous studies have suggested that increased ACTH secretion occurs in depressed in-patients regardless of the cortisol-emic state and that ACTH-independent hypercortisolemia mainly results from physiological stress in severe cases of MDD (22). Interestingly, the SR mice showed specific changes in growth factors and hormones in addition to inflammation related changes. This could be an adaptive response to stress, which may require tissue repair. For example, insulin was elevated in sera from the SR mice and this hormone is known to have profound effects on neuronal functions as it modulates neurotransmitter channel activity, brain cholesterol synthesis and mitochondrial function, and the disruption of brain insulin signalling leads to impaired neuronal function and synaptogenesis (24). However, the finding that progesterone was markedly lower (-1.99 fold) in SR rats appears to be contradictory, as this hormone is also involved in synaptic growth (25) but has also been linked to social rejection and subsequent coping strategies and changes in GABAergic neurotransmission in humans and rodents (26). The SR group showed increased levels of different inflammatory proteins, such as interferon-γ, which has also been reported previously in clinical MDD studies (27, 28). Reports have shown that this protein may have an inhibitory effect on cell growth and proliferation (29-32). However, previous studies have found decreased cell proliferation in the SD model during and immediately after stress [reviewed in (33, 34). Furthermore, other studies have reported impaired cell proliferation mechanisms in brains from SD animals (8, 35). One investigation found decreased hippocampal neurogenesis in SS and SR animals immediately after the last stress episode, by measuring subgranular zone (SGZ) cells in the dentate gyrus labelled with the S-phase marker bromodeoxyuridine (BrdU) (35).

Previous studies have shown that peripheral growth factors, such as basic epidermal growth factor, and treatments affecting peripheral cytokines can induce adult brain neurogenesis and neuroprotection, as these molecules can cross the blood brain barrier and stimulate mitosis (36, 37).

Therefore, increases in cell proliferation markers seen 48 hours after stress in our study could be associated with the early phase of enhanced compensatory cell proliferation seen 4 weeks after the last defeat in SS animals by Legace *et al*. (35). Taken together, our data suggest that the effects on serum molecules in the SS and SR animals may reflect changes in neuronal compensation for trauma-induced plasticity following SD stress. It should be noted however, that the majority of proteins assessed by the multiplex immunoassay are involved in inflammatory and immune-related functions. Nevertheless, despite this fact the SS and SR mice showed different profiles in proteins associated with this molecular function, underlining the methodical need to detect a diverse range of proteins in already implicated common disease pathways in the peripheral pathophysiology of depression. One of the trade-offs of high throughput screening techniques (the same is true for the $LCMS^E$ approach) is the requirement for strict correction for multiple hypotheses. This could mask the detection of some effects of social defeat (e.g. reductions in serum testosterone or corticosterone) to be detected as not significant in the current pilot study (compare Supplementary table 2, uncorrected p-value for corticosterone in the SD group: 0.0627; FC 1.37).

Interestingly, the $LCMS^E$ profiling analysis identified no changes in the frontal cortex from the SS mice. This could reflect the possibility that these mice showed few or no fronto-cortical adaptive changes to stress, or that the SD protocol resulted in macroscopic structural changes that could not be detected using our proteomics approach. In contrast, 10 proteins were found to be altered in frontal cortex tissue from the SR mice following relative quantification and these were mostly involved in synaptic or myelin-related functions. This suggests that animals failing to up-regulate such proteins may suffer negative effects associated with SD or that animals with increased synaptic and myelin density prior to the defeat procedure are more stress resistant. MBP and CN37 are

markers of mature oligodendrocytes (38). These specialised cells are an important part of the CNS maintenance of axonal integrity(39, 40) and signal transduction(41). Frontal cortex upregulation of oligodendrogenesis has previously been linked to processes that ameliorate depression-related behaviour. Chronic unpredictable stress has been shown to decrease fronto-cortical proliferation of oligodendrocytes and to be reversible by antidepressant treatment (42). Additionally, electroconvulsive seizure treatment in rodents has been demonstrated to increase hippocampal (43) and frontal cortex (44) gliogenesis. On the other hand, chronic stress hormone exposure leads to a decrease in cortical oligodendrocyte proliferation (45). This is noteworthy since ACTH was only found to be significantly increased in the susceptible animals in our study, suggesting that only this group shows HPA axis activation and subsequently develops abnormal behaviour. Increased myelination as a marker of resilience against stressors could represent an interesting translational finding. The overall frontal cortex oligodendrocyte density in post-mortem brain of depressive patients has been found to be decreased (46) although targeted proteomic studies suggested no expression changes (47) of oligodendrocyte-related proteins. Future preclinical studies should investigate the time course of myelin-related pathologies following stress exposure and the possibility to rescue depressive phenotypes by pharmacological myelin enhancement.

Previous studies have shown that neurons of the frontal cortex are sensitive to stress and undergo remodelling following stress exposure [reviewed in (48)] which may further support our results. Additionally, the frontal cortex plays a major role in orchestrating the behavioural and systemic responses to stress and is involved in working memory, decision making, social interactions and emotional processing [reviewed in (49)], which overlap with our current molecular findings. Our results suggest the intriguing possibility that resilience in this model is associated with increased oligodendrogenesis in the frontal cortex. Recently it has been shown that stress hormones increase oligodendrogenesis in the hippocampus, a limbic brain region important for memory and emotional control (50). Since neuronal and synaptic remodelling as well as the strength of synaptic transmission has been associated with memory and learning [reviewed in (51, 52)] and the frontal

cortex has inhibitory control over the limbic system, the present findings suggest that the SR mice cope with traumata-induced stress by compensating for decreased hippocampal neurogenesis with increased fronto-cortical oligodendrogenesis. This is in line with findings that fronto-cortical integrity and increased frontal neuronal activity are mediators of antidepressant-like effects in SD mice (53). In concordance with this hypothesis Bartzokis has already suggested myelin as a central point of convergence of multiple psychotropic treatments including antidepressants(54). Alterations in network synchronicity and overall transmission speed have been suggested as the underlying pathophysiology(55, 56) . Interestingly, the frontal cortex is a particularly vulnerable brain region, as it is one of the late-myelinating areas(57).

This is the first molecular profiling study which investigated differences in serum and frontal cortex of the SD mouse model. Serum analysis showed alterations predominantly in inflammatory proteins in both SS and SR mice, whereas growth factors and hormones were also altered in SR animals. The latter finding was particularly interesting as these could be linked to different behavioural responses to stress. Frontal cortex proteomics analysis, using LC-MS^E profiling, provided evidence of differential molecular adaptations in brain tissue, particularly affecting synaptic and myelin-related proteins. This may provide novel insights into how resilient mice can avert the negative consequences of social stress, although future work has to examine whether central myelin-related or peripheral inflammation-related changes are induced by acute stress exposure or are already established prior to the SD procedure. Taken together, these central and peripheral findings suggest that further studies investigating the distinct susceptibility and resilience responses in the SD model of depression may aid in the search of novel antidepressant drug targets. An experimental agent stabilizing Axin2 (58) and natural cannabinoids (59) have already been shown to enhance myelination by influencing oligodendrocyte differentiation and maturation, opening novel therapeutic possibilities that could be explored to improve the treatment of MDD.

Materials and Methods

Animals, housing conditions and stress treatment

Male 7-9 week old C57BL/6J/6J mice (Jackson Laboratory; Bar Harbor, ME, USA) and male 9-13 month old CD1 retired breeder mice (Charles River Laboratories; Wilmington, MA, USA) were housed under constant temperature (21±1˚C), humidity (50-58%) and a 12-hour light/dark cycle with free access to food and water. Animals were allowed to acclimatise to the facility for 1 week before experimentation. C57BL/6J mice were subjected to chronic SD stress as described previously by Berton *et al.*, Krishnan *et al* and.,Golden *et al.* (8, 60, 61). In short, CD1 mice were screened for aggressive behaviour triggered by intrusion into their home cages. Mice exhibiting this trait were used in the SD model as the aggressor mouse to defeat the C57BL/6J mice. The C57BL/6J mice were placed in the home cages of CD-1 mice and subjected to a bout of SD by the aggressor mouse for 10 minutes each day. For the remainder of the day, C57BL/6J mice stayed in the cage with the aggressor but were separated by perforated Plexiglas divider to allow sensory contact but no physical interaction. This process was repeated daily for 10 consecutive days using a different CD-1 aggressor mouse each day to minimize inter-aggressor variability. Control animals were housed in divided cages with another C57BL/6J mouse.

On day 11, the mice exposed to SD stress were subjected to a social interaction test as described by Krishnan *et al.* and *Golden et al.* (8, 61). Briefly, a video-tracking system was used to score interaction/avoidance behaviours towards an unfamiliar 'social target' (male CD-1 mouse). Video recordings were performed using a camera equipped with infrared filter and lights. A white plastic open field (42 x 42cm) maintained under red light was used as the arena for the social interaction test. Each experimental C57BL/6J mouse was introduced into the open field and its trajectory tracked for two consecutive sessions of 2.5 min. Between sessions, the mice were placed into their home cages for approximately 1 minute. For the first session ('no-target' session), the mice were put into the open field containing an empty wire mesh cage (10 x 6.5cm) located at one end. For the

second session ('target' session), the mice were put into the open field under the same conditions except that an unfamiliar male CD-1 mouse was present in the wire mesh cage. Video-tracking data from both sessions were used to determine the time spent by the C57BL/6J mouse in the 'interaction zone' (8cm wide area surrounding the wire mesh cage) and the 'corners' of the open field, opposite to the location of the wire mesh cage that was used to calculate the social interaction ratio. Typical social interaction test behaviour of defeated C57BL/6J animals and control animals can be found in (8, 60).

Based on interaction score ratios of this test, the C57BL/6J mice were sorted into either susceptible (SS) (n=12) or resilient (SR) (n=12) groups (the latter showed social interaction behaviour similar to controls; n=12). A ratio equal to 1, in which equal time of the C57BL/6J mouse is spent in the presence and absence of a CD-1 mouse in the interaction zone, has been used as a threshold for dividing defeated mice into the two subgroups (<1: susceptible; >1: resilient). Finally, 48 hours after the last defeat and 24 hours after social interaction testing, all SS, SR and non-stressed control animals were decapitated and serum (trunk blood) and brain (frontal cortex) samples were taken. Samples were stored at -80°C.

Multiplex immunoassay serum profiling

A total of 89 molecules were measured in serum samples (200µL) using the multiplexed immunoassay *Rodent*MAPs® platform in the Clinical Laboratory Improvement Amendments-certified laboratory at Myriad-RBM (Austin, TX, USA). The *Rodent*MAPs® platform consisted of *Rodent*MAP®-2.0, *Rat Metabolic* MAP®-v1.0 and *Rat Kidney*MAP®-v.1.0 panels. The method has been previously described in detail (62). The full list of molecules tested is shown in **Supplementary table 1**.

Label free LC-MS proteomic profiling

Protein extraction, fractionation and digestion

All biochemical reagents were purchased from Sigma-Aldrich, Dorset, UK, unless specified otherwise. We used a label free LC-MS^E approach to profile proteins extracted from brain tissue as described previously (63). Briefly, proteins were extracted from frontal cortex (25-70mg) by adding 5 volumes of extraction buffer/tissue weight. The extraction buffer consisted of 7M urea, 2M thiourea, 4% 3-[(3-Cholamidopropyl) dimethylammonio] -1- propanesulfonate (CHAPS), 2% 3-[N,N-Dimethyl (3-myristoylaminopropyl) ammonio] propanesulfonate Amidosulfobetaine- 14 (ASB14, Calbiochem; Beeston, UK) and 70mM dithiothreitol. The samples were sonicated using a Branson Sonifier® W-150 (York, UK) for an initial 10 seconds and for another 5 seconds at 4°C. In parallel to the animal model samples, quality control (QC) samples were prepared from spare mouse frontal cortex. After fractionation of all the QC samples, the QCs were pooled and aliquoted. A QC sample was added to each sample preparation batch and prepared as it would be a normal sample.

After sonication, the samples were put on a Vortex-genie®2 shaker (VWR International; Radnor, PA, USA) at level 2 and 4°C for 30 min and then centrifuged at 17,000g for 3 min at 4°C, using a Spectrafuge™ 24D Digital microcentrifuge (Labnet International; Windsor, UK). After protein concentration measurement (Bradford reagent), 1000 µg of frontal cortex proteins were precipitated using 4 volume equivalents of -20°C cold acetone and incubated at -20°C for 16 hours. The samples were centrifuged at 13,000 rpm for 10 min at 4°C. The acetone was discarded and the pellets were resuspended in 100µL of 50mM ammonium bicarbonate. Disulfide bonds were reduced using 100 mM dithiothreitol at 60°C for 30 min and free sulfhydryl groups were alkylated with 200 mM iodoacetamide in the dark at room temperature for 30 min. The proteins were digested using trypsin (Promega; Madison, WI, USA), at a ratio of 1:50 (w/w trypsin/protein) for 17 hours at 37°C. Digestions were stopped by addition of 8.8 M HCl (ratio = 1:60) to each sample and the samples were stored at –80°C.

Liquid chromatography and mass spectrometry analysis

The LC-MS^E profiling study was carried out in expression mode using a Waters quadrupole time-of-flight (QToF)-Premier^{TM} (Waters Corporation; Milford, MA, USA) mass spectrometer, as described previously (64). Adaptations to the protocol were made as described below. The LC-MS^E analysis was carried out in duplicate for all samples, using 0.6µL of protein digest for 5µL of sample. The buffers used were: A) H_2O + 0.1% formic acid and B) acetonitrile + 0.1% formic acid. Desalting of the samples was performed online with 100% A for 2 min, using a reverse-phase C18 trapping column (180µm i.d., 20mm length, 5µm particle size) (Waters). Peptides were separated using an analytical C18 BEH nano-column (75µm i.d., 200mm length, 1.7µm particle size) (Waters) at 0.3µL/min, using a 131 min gradient. The gradient started with an initial concentration of 3% B (97% A), followed by 3-30% B (90 min), 30-90% B (25 min), 90-97% B (5 min), constant 97% B (10 min), and 97-3% B (1 min).

The resulting data were processed using the ProteinLynx Global Server (PLGS) v.2.4 (Waters) and Rosetta Inpharmatics Biosoftware Elucidator v3.3 (Seattle, WA, USA) (65). A mouse Swiss-Prot database (version 57.4, 16 140 entries) search analysis was performed using PLGS with the ion accounting algorithm described previously (66). The criteria for protein identification were set to $\geq$3 fragment ions/peptide, and $\geq$7 fragment ions and 2 peptides/protein. The maximum false identification rate was 4% using a randomized version of the database. Only peptides detected in both replicates and in 60% of all the samples were included in the further analysis. Search results were imported into Elucidator for annotation of aligned features, resulting in a matrix that included intensities for each sample and peptide. Only proteins represented by 2 or more peptides were included in the final result lists. Note that the PLGS search algorithm and the subsequent data integration does not specifically distinguish between splice variants of proteins. Only the most commonly accepted “canonical” splice variant is usually integrated in the Swiss-Prot database and peptides that are not unique for a given protein or group of isoforms are removed from the analysis process in order to remove redundancy.

Statistical analyses

The quality of recorded multiplex immunoassay and LC-MS^E data was assessed by principal component analysis (PCA) using Prism version 5 (GraphPad Software, La Jolla, CA, USA). The PCA is particularly suited for the visual display of multi-dimensional data as the dataset is projected to few (mostly two) dimensions that account for the greatest variance (information) in the dataset, and thereby can be used to identify possible outliers or batch effects. For multiplex immunoassay analyses, values under the detection limit were replaced as described previously (67). Furthermore, molecules which had more than 20% of values outside the detection limit within a group were not included in the final list of changed molecules or otherwise indicated. For all LC-MS^E studies, coefficients of variation (CVs) were calculated using the QC measured intensities. The overall study CV was calculated as the mean of total protein CVs to assess the technical variation.

The non-parametric Kruskal-Wallis one way ANOVA (analysis of variance) with Mann Whitney U test as post-hoc test were applied for statistical analyses of the multiplex immunoassay and LC-MS^E data. False discovery rate (FDR) was used to control for multiple hypothesis testing (q-value) and a q-value of each molecule was calculated according to the method of Benjamini and Hochberg (68). All analyses were performed using the statistical software package R 2.12 (69). All ratios which had a Kruskal-Wallis one way ANOVA $q < 0.05$ and Mann Whitney U $q < 0.05$ (for at least one comparison) were considered as significantly different.

Fold change values (FC) were calculated for each molecule as the mean value of the stressed animals divided by mean value of controls (SS/C and SR/C).

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References

1. Guze SB & Robins E (1970) Suicide and primary affective disorders. *Br J Psychiatry* 117(539):437-438.
2. Nestler EJ & Hyman SE (2010) Animal models of neuropsychiatric disorders. *Nat Neurosci* 13(10):1161-1169.
3. Bisgaard CF*, et al.* (2007) Proteomic investigation of the ventral rat hippocampus links DRP-2 to escitalopram treatment resistance and SNAP to stress resilience in the chronic mild stress model of depression. *Journal of molecular neuroscience : MN* 32(2):132-144.
4. Kedracka-Krok S*, et al.* (2010) Effect of chronic mild stress and imipramine on the proteome of the rat dentate gyrus. *Journal of neurochemistry* 113(4):848-859.
5. Liu Y*, et al.* (2011) Dynamic proteomic analysis of protein expression profiles in whole brain of Balb/C mice subjected to unpredictable chronic mild stress: implications for depressive disorders and future therapies. *Neurochemistry international* 58(8):904-913.
6. McArthur R & Borsini F (2006) Animal models of depression in drug discovery: a historical perspective. *Pharmacol Biochem Behav* 84(3):436-452.
7. Charney DS & Manji HK (2004) Life stress, genes, and depression: multiple pathways lead to increased risk and new opportunities for intervention. *Sci STKE* 2004(225):re5.
8. Krishnan V*, et al.* (2007) Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* 131(2):391-404.
9. McEwen BS (2007) Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiol Rev* 87(3):873-904.
10. Yehuda R & LeDoux J (2007) Response variation following trauma: a translational neuroscience approach to understanding PTSD. *Neuron* 56(1):19-32.
11. Raedler TJ (2011) Inflammatory mechanisms in major depressive disorder. *Curr Opin Psychiatry* 24(6):519-525.
12. Vaccarino V*, et al.* (2008) Association of major depressive disorder with serum myeloperoxidase and other markers of inflammation: a twin study. *Biol Psychiatry* 64(6):476-483.
13. Erdem M*, et al.* (2011) Serum haptoglobin levels in patients with melancholic and nonmelancholic major depression. *Prog Neuropsychopharmacol Biol Psychiatry* 35(4):944-947.
14. Maes M*, et al.* (1997) Acute phase proteins in schizophrenia, mania and major depression: modulation by psychotropic drugs. *Psychiatry Res* 66(1):1-11.
15. Howren MB, Lamkin DM, & Suls J (2009) Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis. *Psychosom Med* 71(2):171-186.
16. Ford DE & Erlinger TP (2004) Depression and C-reactive protein in US adults: data from the Third National Health and Nutrition Examination Survey. *Arch Intern Med* 164(9):1010-1014.
17. Lanquillon S, Krieg JC, Bening-Abu-Shach U, & Vedder H (2000) Cytokine production and treatment response in major depressive disorder. *Neuropsychopharmacology* 22(4):370-379.
18. Karlovic D, Serretti A, Vrkic N, Martinac M, & Marcinko D (2012) Serum concentrations of CRP, IL-6, TNF-alpha and cortisol in major depressive disorder with melancholic or atypical features. *Psychiatry Res*.
19. Pariante CM & Miller AH (2001) Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. *Biol Psychiatry* 49(5):391-404.
20. Breier A, Wolkowitz OM, Doran AR, Bellar S, & Pickar D (1988) Neurobiological effects of lumbar puncture stress in psychiatric patients and healthy volunteers. *Psychiatry Res* 25(2):187-194.

21. Charlton BG, Leake A, Wright C, Griffiths HW, & Ferrier IN (1987) A combined study of cortisol, ACTH and dexamethasone concentrations in major depression. Multiple time-point sampling. *Br J Psychiatry* 150:791-796.
22. Carroll BJ*, et al.* (2007) Pathophysiology of hypercortisolism in depression. *Acta Psychiatr Scand Suppl* (433):90-103.
23. Hodes GE*, et al.* (2014) Individual differences in the peripheral immune system promote resilience versus susceptibility to social stress. *Proceedings of the National Academy of Sciences of the United States of America* 111(45):16136-16141.
24. Kleinridders A, Ferris HA, Cai WK, & Kahn CR (2014) Insulin Action in Brain Regulates Systemic Metabolism and Brain Function. *Diabetes* 63(7):2232-2243.
25. Zhao YZ*, et al.* (2011) Progesterone Influences Postischemic Synaptogenesis in the CA1 Region of the Hippocampus in Rats. *Synapse* 65(9):880-891.
26. Wirth MM (2011) Beyond the HPA Axis: Progesterone-Derived Neuroactive Steroids in Human Stress and Emotion. *Frontiers in endocrinology* 2:19.
27. Dowlati Y*, et al.* (2010) A meta-analysis of cytokines in major depression. *Biol Psychiatry* 67(5):446-457.
28. Maes M*, et al.* (1994) Increased neopterin and interferon-gamma secretion and lower availability of L-tryptophan in major depression: further evidence for an immune response. *Psychiatry Res* 54(2):143-160.
29. Walter J*, et al.* (2011) A new role for interferon gamma in neural stem/precursor cell dysregulation. *Mol Neurodegener* 6:18.
30. Kim IJ, Beck HN, Lein PJ, & Higgins D (2002) Interferon gamma induces retrograde dendritic retraction and inhibits synapse formation. *J Neurosci* 22(11):4530-4539.
31. Loukinova E*, et al.* (2000) Growth regulated oncogene-alpha expression by murine squamous cell carcinoma promotes tumor growth, metastasis, leukocyte infiltration and angiogenesis by a host CXC receptor-2 dependent mechanism. *Oncogene* 19(31):3477-3486.
32. Robinson S, Tani M, Strieter RM, Ransohoff RM, & Miller RH (1998) The chemokine growth-regulated oncogene-alpha promotes spinal cord oligodendrocyte precursor proliferation. *J Neurosci* 18(24):10457-10463.
33. Gould E & Tanapat P (1999) Stress and hippocampal neurogenesis. *Biol Psychiatry* 46(11):1472-1479.
34. Schmidt HD & Duman RS (2007) The role of neurotrophic factors in adult hippocampal neurogenesis, antidepressant treatments and animal models of depressive-like behavior. *Behav Pharmacol* 18(5-6):391-418.
35. Lagace DC*, et al.* (2010) Adult hippocampal neurogenesis is functionally important for stress-induced social avoidance. *Proceedings of the National Academy of Sciences of the United States of America* 107(9):4436-4441.
36. Aharoni R, Arnon R, & Eilam R (2005) Neurogenesis and neuroprotection induced by peripheral immunomodulatory treatment of experimental autoimmune encephalomyelitis. *J Neurosci* 25(36):8217-8228.
37. Wagner JP, Black IB, & DiCicco-Bloom E (1999) Stimulation of neonatal and adult brain neurogenesis by subcutaneous injection of basic fibroblast growth factor. *J Neurosci* 19(14):6006-6016.
38. Nishiyama A, Komitova M, Suzuki R, & Zhu X (2009) Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. *Nature reviews. Neuroscience* 10(1):9-22.
39. Nave KA (2010) Myelination and the trophic support of long axons. *Nature reviews. Neuroscience* 11(4):275-283.
40. Nave KA (2010) Myelination and support of axonal integrity by glia. *Nature* 468(7321):244-252.
41. Poliak S & Peles E (2003) The local differentiation of myelinated axons at nodes of Ranvier. *Nature reviews. Neuroscience* 4(12):968-980.

42. Czeh B*, et al.* (2007) Chronic social stress inhibits cell proliferation in the adult medial prefrontal cortex: hemispheric asymmetry and reversal by fluoxetine treatment. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 32(7):1490-1503.
43. Wennstrom M, Hellsten J, Ekstrand J, Lindgren H, & Tingstrom A (2006) Corticosterone-induced inhibition of gliogenesis in rat hippocampus is counteracted by electroconvulsive seizures. *Biological psychiatry* 59(2):178-186.
44. Ongur D*, et al.* (2007) Electroconvulsive seizures stimulate glial proliferation and reduce expression of Sprouty2 within the prefrontal cortex of rats. *Biological psychiatry* 62(5):505-512.
45. Banasr M*, et al.* (2007) Chronic unpredictable stress decreases cell proliferation in the cerebral cortex of the adult rat. *Biological psychiatry* 62(5):496-504.
46. Hayashi Y*, et al.* (2011) A novel, rapid, quantitative cell-counting method reveals oligodendroglial reduction in the frontopolar cortex in major depressive disorder. *Molecular psychiatry* 16(12):1155-1158.
47. Wesseling H GM, Bahn S (Targeted Multiplexed Selected Reaction Monitoring Analysis Evaluates Protein Expression Changes of Molecular Risk Factors for Major Psychiatric Disorders. *Int J Neuropsychopharmacol*.
48. Holmes A & Wellman CL (2009) Stress-induced prefrontal reorganization and executive dysfunction in rodents. *Neurosci Biobehav Rev* 33(6):773-783.
49. Cerqueira JJ, Almeida OF, & Sousa N (2008) The stressed prefrontal cortex. Left? Right! *Brain Behav Immun* 22(5):630-638.
50. Chetty S*, et al.* (2014) Stress and glucocorticoids promote oligodendrogenesis in the adult hippocampus. *Molecular psychiatry* 19(12):1275-1283.
51. Gould E, Tanapat P, Hastings NB, & Shors TJ (1999) Neurogenesis in adulthood: a possible role in learning. *Trends Cogn Sci* 3(5):186-192.
52. Bliss TV & Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361(6407):31-39.
53. Covington HE, 3rd*, et al.* (2010) Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. *J Neurosci* 30(48):16082-16090.
54. Bartzokis G (2012) Neuroglialpharmacology: myelination as a shared mechanism of action of psychotropic treatments. *Neuropharmacology* 62(7):2137-2153.
55. Bartzokis G (2011) Neuroglialpharmacology: white matter pathophysiologies and psychiatric treatments. *Frontiers in bioscience* 16:2695-2733.
56. Bartzokis G*, et al.* (2010) Lifespan trajectory of myelin integrity and maximum motor speed. *Neurobiology of aging* 31(9):1554-1562.
57. Bartzokis G*, et al.* (2001) Age-related changes in frontal and temporal lobe volumes in men: a magnetic resonance imaging study. *Archives of general psychiatry* 58(5):461-465.
58. Fancy SP*, et al.* (2011) Axin2 as regulatory and therapeutic target in newborn brain injury and remyelination. *Nature neuroscience* 14(8):1009-1016.
59. Solbrig MV, Fan Y, Hermanowicz N, Morgese MG, & Giuffrida A (2010) A synthetic cannabinoid agonist promotes oligodendrogliogenesis during viral encephalitis in rats. *Experimental neurology* 226(1):231-241.
60. Berton O*, et al.* (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science* 311(5762):864-868.
61. Golden SA, Covington HE, 3rd, Berton O, & Russo SJ (2011) A standardized protocol for repeated social defeat stress in mice. *Nature protocols* 6(8):1183-1191.
62. Bertenshaw GP*, et al.* (2008) Multianalyte profiling of serum antigens and autoimmune and infectious disease molecules to identify biomarkers dysregulated in epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 17(10):2872-2881.

63. Ernst A*, et al.* (2012) Molecular Validation of the Acute Phencyclidine Rat Model for Schizophrenia: Identification of Translational Changes in Energy Metabolism and Neurotransmission. *J Proteome Res* 11(7):3704-3714.
64. Levin Y, Schwarz E, Wang L, Leweke FM, & Bahn S (2007) Label-free LC-MS/MS quantitative proteomics for large-scale biomarker discovery in complex samples. *J Sep Sci* 30(14):2198-2203.
65. Krishnamurthy D*, et al.* (2011) Analysis of the human pituitary proteome by data independent label-free liquid chromatography tandem mass spectrometry. *Proteomics* 11(3):495-500.
66. Li GZ*, et al.* (2009) Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. *Proteomics* 9(6):1696-1719.
67. Stelzhammer V*, et al.* (2011) Proteomic changes induced by anaesthesia and muscle relaxant treatment prior to electroconvulsive therapy. *Proteomics Clin Appl*.
68. Benjamini Y & Hochberg Y (1995) Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B-Methodological* 57(1):289-300.
69. Team RDC (2009) R: A language and environment for statistical computing. R Foundation for Statistical ComputingR: A language and environment for statistical computing. R Foundation for Statistical Computing , Vienna, Austria).

Table 1. Multiplex immunoassay profiling results of serum from social defeat susceptible (SS, n=12) and resilient (SR, n=12) mice. The table shows 20 significantly changed proteins along with their fold changes (FC). FC values are calculated based on average (stressed/control) or median (indicated with ^), p-values [Mann Whitney U test, post hoc test for Kruskal-Wallis one-way ANOVA ($p<0.05$)] and q-values (corrected Mann Whitney U test p-value). A complete list of proteins with $p<0.05$ is shown in **Supplementary Table 2**.

	Stress susceptible (SS)			Stress resilient (SR)		
Protein	**FC**	**p-value**	**q-value**	**FC**	**p-value**	**q-value**
Myeloperoxidase	**2.60**	**0.0006**	**0.0140**	1.46	0.0102	0.4733
Matrix metalloproteinase-9	**2.38**	**0.0016**	**0.0130**	**1.21**	**0.0208**	**0.0353**
Monocyte chemotactic protein 3	**2.06**	**0.0005**	**0.0204**	1.01	0.7289	1.0000
C-C motif chemokine 9	**1.66**	**0.0006**	**0.0058**	1.12	0.1408	0.3441
Interleukin-1 beta	**1.52**	**0.0047**	**0.0018**	1.30	0.1578	0.4223
Clusterin	**1.45**	**0.0003**	**0.0009**	-1.12	0.5825	0.1584
C-reactive peptide	**1.37**	**0.0056**	**0.0009**	1.04	0.6297	1.0000
Adrenocorticotrophic hormone	**1.37**	**0.0019**	**0.0140**	1.14	0.1147	0.1050
Angiotensin-converting enzyme	**1.32**	**0.0045**	**0.0029**	**1.56**	**0.0018**	**0.0003**
Interleukin-1 alpha	**1.22**	**0.0398**	**0.0135**	**1.64**	**0.0099**	**0.0055**
Epidermal growth factor	2.50	0.7717	0.0078	**3.50**	**0.0054**	**0.0018**
Vascular endothelial growth factor A	1.26	0.0026	1.0000	**3.14^**	**0.0006**	**0.0162**
Tumour necrosis factor-α	1.65	0.3486	1.0000	**2.70**	**0.0082**	**0.0352**
Macrophage migration inhibitory protein 2	2.37	0.0045	1.0000	**1.72**	**0.0032**	**0.0357**
Interferon-γ	1.15	0.8024	0.3566	**1.64^**	**0.0176**	**0.0199**
Macrophage colony-stimulating Factor 1	1.04	0.7290	0.0135	**1.29**	**0.0056**	**0.0097**
Insulin	1.15	0.1324	1.0000	**1.24**	**0.0178**	**0.0049**
Macrophage-derived chemokine	-1.11	0.1186	0.0059	**-1.41**	**0.0005**	**0.0453**
Progesterone	-1.36	0.0303	0.2356	**-1.99**	**0.0002**	**0.0212**
Immunoglobulin A	1.03	0.8428	0.0494	**-2.36**	**0.0016**	**0.0004**

Table 2: Significant protein changes in frontal cortex from social defeat model subgroups, social susceptible (SS) and social resilient (SR) animals, by LC-MSE. The table shows the 10 most significant proteomic changes (q<0.05, and FC> 10%) in SS animals(n=11) and SR animals (n=11) versus controls. The full list can be viewed in Supplementary table 2. The changes are sorted 1st by group and 2nd by highest to lowest fold change. The table includes short name (Name) and description of protein, Uniprot accession number (Acc.No.), peptide count (Pep), fold change (FC) which are calculated based on average (stressed/control), p-values (Mann Whitney U test, post hoc test for Kruskal-Wallis one-way ANOVA (p<0.05)), q-values (FDR correction of Mann Whitney U test) and sequence coverage (SC). Significant values are highlighted in bold and proteins indicated with '-' were detected in the study but had a p-value >0.1. P-values were not shown for these proteins for better clarity of table. A complete list of proteins with p<0.05 is shown in **Supplementary Table 3**.

			Stress susceptible (SS)			Stress resilient (SR)		
Code	**Protein name**	**Pep**	**FC**	**p-value**	**q-value**	**FC**	**p-value**	**q-value**
PSA	Puromycin sensitive aminopeptidase	3	1.14	0.0557	1.0000	**1.69**	**0.0008**	**0.0356**
IC1	Plasma protease C1 inhibitor	2	1.12	0.1164	1.0000	**1.52**	**0.0002**	**0.0334**
MYPR	**Myelin proteolipid protein**	24	1.05	0.4779	0.0250	**1.47**	**0.0004**	**0.0335**
MBP	**Myelin basic protein**	33	1.01	1.0000	0.8109	**1.42**	**0.0004**	**0.0335**
CN37	**2 3 cyclic nucleotide 3 phosphodiesterase**	51	1.05	0.2169	1.0000	**1.40**	**0.0003**	**0.0335**
MK01	Mitogen activated protein kinase 1	23	-1.07	0.0759	0.0577	**-1.11**	**0.0014**	**0.0438**
SV2B	Synaptic vesicle glycoprotein 2B	9	1.04	0.2169	1.0000	**-1.14**	**0.0008**	**0.0354**
TBA1B	Tubulin alpha 1B chain	12	-1.03	0.2703	0.0199	**-1.14**	**0.0008**	**0.0354**
NEUM	Neuromodulin	20	-1.07	0.1513	1.0000	**-1.17**	**0.0010**	**0.0374**
LPHN2	Latrophilin-2	2	-1.29	0.1014	1.0000	**-1.45**	**0.0006**	**0.0335**