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Original Research

Major Depression-Associated *NEGR1* Gene is Modulated in Stress-Susceptible Male Mice

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Abstract

Background: Neuronal growth regulator 1 (*NEGR1*) is an IgLON cell adhesion molecule significantly associated with depression risk in genome-wide association studies. Since the role of NEGR1 in depression pathophysiology remains incompletely understood, we investigated changes in *NEGR1*-associated gene expression levels in stress-susceptible male mice exposed to chronic restraint stress. **Methods**: Mice were subjected to 21 consecutive days of restraint stress, and stress-induced maladaptive phenotypes were evaluated by tail suspension, forced swim, splash, and open field tests. After sacrifice, the hippocampi were collected, and the levels of *NEGR1*-associated genes were assessed by quantitative polymerase chain reaction (qPCR). **Results**: In the stress-exposed group, weight was significantly reduced, and immobility time was significantly higher in the tail suspension and the forced swim tests, while grooming bouts in the splash test were reduced. No changes were observed in the open field test. A z-score normalization integrating all behavioural parameters was applied to classify the animals as resilient or susceptible to restraint stress. In stress-susceptible mice, *NEGR1*, Fibroblast Growth Factor Receptor 2 (*FGFR2*), Limbic System-Associated Membrane Protein (*LSAMP*), and Neurotrimin (*NTM*) mRNA levels were significantly higher compared to controls, while ADAM Metallopeptidase Domain 10 (*ADAM10*), a metalloprotease releasing NEGR1 from neuronal membranes, was significantly reduced. Interestingly, *ADAM10* expression negatively correlated with the behavioural z-score, whereas *NEGR1* and *LSAMP* expression showed positive correlations. **Conclusions**: These findings indicate a potential role for NEGR1 in depressive-like behaviors elicited in a stress-susceptible phenotype. Considering *NEGR1* genetic association with depression, our results suggest that the NEGR1 pathway may contribute to depression pathophysiology by modulating the interplay between genetic predisposition and exposure to stress as a crucial environmental precipitating factor.

Keywords: NEGR1 protein, mouse; cell adhesion molecules, neuronal; major depressive disorder; gene expression; stress; ADAM10 protein

1. Introduction

Major depression is a severe and debilitating disorder affecting a large number of individuals worldwide, with a heavy impact on quality of life, morbidity, and mortality [1]. Although the object of systematic and extensive investigations in the last decades, the neurobiological basis of the disease is still imperfectly understood, thus hampering the identification of novel targets for pharmacological interventions [2,3].

A complex interplay between genetic vulnerability and environmental factors is recognised as crucial for the disease onset, with a major role for stress exposure among the latter [4–6]. Stress, and especially chronic stress exposure, represents a significant risk factor for the development of depression. While a physiological stress response allows resilience to stress and adaptation, individual factors may increase stress susceptibility, thus predisposing to allostatic load and the risk of psychiatric disorders [6–8]. As for genetic vulnerability, family studies have shown an increased risk for depression in patients' first-degree relatives, but the genetic component is comprised of a relatively high number of genes, each providing comparatively small contributions, making it challenging to identify disease-associated genes [9].

Ultimately, when meta-analyses of genome-wide association studies comprising large numbers of samples became available, the identification of depression-associated genes with robust significance took place [10]. Among them, the Neuronal Growth Regulator 1 (*NEGR1*) gene has repeatedly demonstrated a solid association with depression risk as one of the most significant signals [11–15]. The relevance of *NEGR1* for depression is reinforced by its in-

creased levels in cerebrospinal fluid specimens and differential expression in brain samples derived from depressed patients [16–18].

NEGR1 belongs to a subfamily of immunoglobulin-like cell adhesion molecules dubbed IgLON, comprising four additional members beyond NEGR1 termed Limbic System Associated Membrane Protein (LSAMP), Opioid Binding Protein/Cell Adhesion Molecule Like (OPCML), neurotrimin (NTM), and IgLON Family Member 5 (IgLON5) [19–23]. The IgLON family members share structural similarities, including three immunoglobulin-like domains in their N-terminal portion, while a glycosylphosphatidylinositol segment anchors them to the cell membrane in the absence of membrane-spanning domains [24]. IgLONs also share the ability to control neurite outgrowth and neural connectivity during development, as well as promoting the genesis and maintenance of synaptic connections [25]. In addition to the cell-adhesion molecule function as full-length proteins, IgLON proteins can be cleaved by metalloproteases to generate soluble forms which interact with transmembrane receptors, thus starting intracellular signal transduction pathways potentially relevant for their functions [26,27]. Full-length NEGR1 is cleaved by the metalloprotease ADAM Metallopeptidase Domain 10 (ADAM10) to a soluble form, binding as an agonist to the Fibroblast Growth Factor Receptor 2 (FGFR2) receptor on the cell membrane and starting a signal transduction pathway involved in neurite outgrowth [28].

Available evidence implies a role for IgLON proteins in neuropsychiatric disorders [25]. In particular, a significant association between depression and *OPCML* was discovered in a genome-wide linkage analysis from a Dutch family-based study [29]. Moreover, the *LSAMP* gene has been associated with depression [30], and suicidal behaviour [31], whereas preclinical studies have revealed links with depressive- and anxiety-like behaviours and synaptic dysfunction [32–35] as well as with response to antidepressant treatment [36].

Notwithstanding the solid evidence of a bona fide link between depression and *NEGR1*, the role of the protein encoded by this gene in the pathophysiology of depression still requires elucidation. Here, we used a mouse model of depression based on chronic restraint stress in male mice to investigate the modulation of IgLON genes in the hippocampus associated with behavioural susceptibility to stress.

2. Materials and Methods

2.1 Animals

Experiments were performed in accordance with the European Community Council Directive 2010/63/EU and approved by the Italian legislation on animal experimentation (Decreto Legislativo 26/2014). The protocol was authorised by the Ethical Committee of the Italian Ministry for Health (authorization N 103/2022-PR, prot. FB7CC.55 for mice). The experimental design was compliant with the 3R principles and with the ARRIVE guidelines [37].

A total of 20 adult male C57BL/6 mice (Charles River, Calco, Italy) were used in the study. Animals were 7–8 weeks old at the beginning of the study. All animals were maintained under standard animal facility conditions (at 20–22 °C, 12 h light/dark cycle—light on at 7:00 AM, off at 7:00 PM with water and food ad libitum, except when required for the stress protocol).

2.2 Experimental Design

All mice were weighed before the beginning of the stress protocol and twice a week afterward; they were also subjected to behavioural tests to monitor depressive-like and anxiety-like phenotypes: the tail suspension test (TST) was performed once a week, on days 5, 12, and 20 of stress; the splash test (ST) and forced swim test (FST) on day 21 of stress; and the open field test (OFT) on day 19 of stress. Animals were sacrificed by decapitation immediately after the FST and the hippocampi were quickly dissected, frozen on dry ice, and stored at –80 °C (Fig. 1).

2.3 Chronic Restraint Stress (CRS) Protocol

Mice were randomly divided into two groups: stressed (CRS = stressed, n = 10) and non-stressed animals (CNT = controls, n = 10). No specific randomisation method was applied: the animals were simply divided by chance into the experimental cages. The stressed mice were subjected to 21 consecutive days of the CRS protocol, while CNT animals were left undisturbed in their home cages except for weight measurement and behavioural testing. For CRS, animals were placed in an air-accessible Falcon tube of 5 cm diameter for 2 h, unable to turn around but able only to move their head and paws, as previously reported [38]. After each session of CRS, animals were placed back in their home cages.

2.4 Behavioural Tests

For the TST, mice were hung by their tails with adhesive tape from a horizontal bar one metre above the ground for 6 minutes [39]. The total time of immobility during the last 5 minutes was evaluated.

For the ST, mice were placed into an empty cage and sprayed with a 10% sucrose solution on the dorsal coat. The sucrose solution dirties the fur and induces self-care behaviour (grooming). The ST was conducted for 5 minutes and the time spent grooming, the number of bouts, and the latency to groom (time between spray and initiation of grooming) were measured [39].

For the FST, mice were forced to swim in a Plexiglas cylinder (20 cm in diameter and 40 cm in height) filled with room-temperature water for 5 minutes. Fresh water was used for each mouse. The total time of immobility was measured [39].

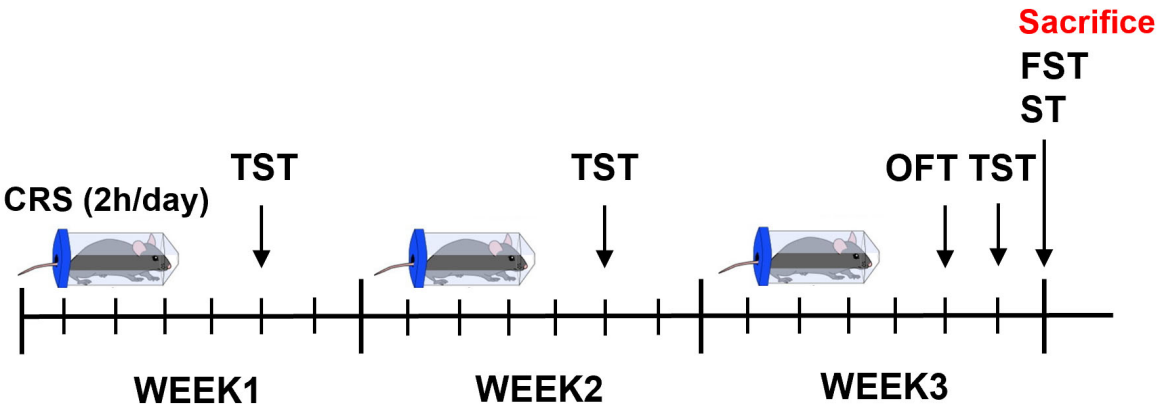

Fig. 1. Experimental design. Mice were exposed to chronic restraint stress (CRS) for 2 h every day for 21 consecutive days. Animals were weighed twice a week, and behavioural tests were performed at different time points. TST, tail suspension test; OFT, open field test; FST, forced swim test; ST, splash test.

For the OFT, mice were placed inside an arena (40 × 40 cm square and 20 cm height) with a highly illuminated centre for a total of 6 minutes (1 min of habituation and 5 of testing). Lamps with 100 W bulbs were used to increase the lighting in the centre of the arena, while the rest of the room was kept in the dark. Mice were placed in the central square (15 × 15 cm) and allowed to freely explore the field. The time spent in the centre was calculated, as well as the number of entries into the central zone [39].

The analysis of the behavioural tests was performed by blinded experimenters. Differences in sample size are due to the removal of outliers and are reported in the caption of each figure.

2.5 Identification of Stress Resilient and Susceptible Mice

An integrated behavioural z-score normalisation was adopted to identify animals resilient/susceptible to CRS. Scores from the OFT, ST, FST, and TST on day 20 of stress were integrated, and animals were classified as resilient if they had a z-score within 1.5 standard deviation (SD) of the CNT group.

2.6 qPCR

Total RNA was extracted with Tri Reagent (T9424, Merck, Milano, Italy) and the Direct-zol RNA MiniPrep kit (R2050, Zymo Research Europe, Freiburg, Germany), according to the manufacturer's instructions. cDNA was synthesised using the iScript Advanced cDNA Synthesis kit (#1725037, Bio-Rad, Hercules, CA, USA). Real-time PCR was performed in an Applied Biosystems 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using SYBR Green technology and the SSO Advanced Universal SYBR Green Supermix (#1725270, Bio-Rad), according to the following conditions: stage 1: 95 °C for 20 s; stage 2: 40 × (95 °C for 3 s; 60 °C for 30 s). The following primers were selected with NCBI tools and obtained from Eurofins Genomics (Ebersberg, Germany): Brain-Derived Neurotrophic Factor (*BDNF*) forw. 5′-GGCTGACACTTTTGAGCACGTC-3′; rev. 5′-CTCCAAAGGCACTTGACTGCTG-3′; Tropomyosin receptor kinase B (*TRKB*) forw. 5′-TGAGGAGGACACAGGATGTTGA-3′; rev. 5′-TTCCAGTGCAAGCCAGTATCTG-3′; Postsynaptic density protein 95 (*PSD95*, also known as *DLG4*) forw. 5′-GGTGACGACCCATCCATCTTTATC-3′; rev. 5′-CGGACATCCACTTCATTGACAAAC-3′; *ADAM10* forw. 5′-CGTGCCAAACGAGCAGTCTC-3′; rev. 5′-AGGGAAGTGTCCCTCTTCATTCG-3′; *FGFR2* forw. 5′-CACCATGGCAACCTTGTCCC-3′; rev. 5′-GCAACTCTAGCGATTCCCCG-3′; *IgLON5* forw. 5′-CTGGAGACAGCTCCGAGACG-3′; rev. 5′-ACATCTGTGATGGTCGGGGG-3′; *LSAMP* forw. 5′-CCGCTGGTCCTACTGAGACT-3′; rev. 5′-CGAAGATGATGCCAGAGCGG-3′; *NEGR1* forw. 5′-GCCCCTCAACCCTCCAAGTA-3′; rev. 5′-TGGATCCAGCCATCAGCACT-3′; *NTM* forw. 5′-TCTCGTGGGCAATCTTCACG-3′; rev. 5′-CGGGTGACTCGGTTGTCAAT-3′; *OPCML* forw. 5′-AGAACAAAGGCCGCATATCCA-3′; rev. 5′-ACTGCTCCAGGCCCATACAG-3′; *GAPDH* forw. 5′-GCCAAGGTCATCCATGACAACT-3′; rev. 5′-GAGGGGCCATCCACAGTCT-3′; *YWHAZ* forw. 5′-TAGGTCATCGTGGAGGGTCG-3′; rev. 5′-GAAGCATTGGGGATCAAGAACTT-3′. A dissociation curve in the 60–95 °C range was built to examine the specificity of amplification products. Missing values are due to sample analysis failures.

2.7 Statistical Analysis

The data are presented as mean values ± standard error of the mean (SEM). Absolute weight and weight gain were evaluated by two-way repeated measures analysis of variance (ANOVA) with time and stress as independent variables, followed by Bonferroni's post-hoc test. The TST

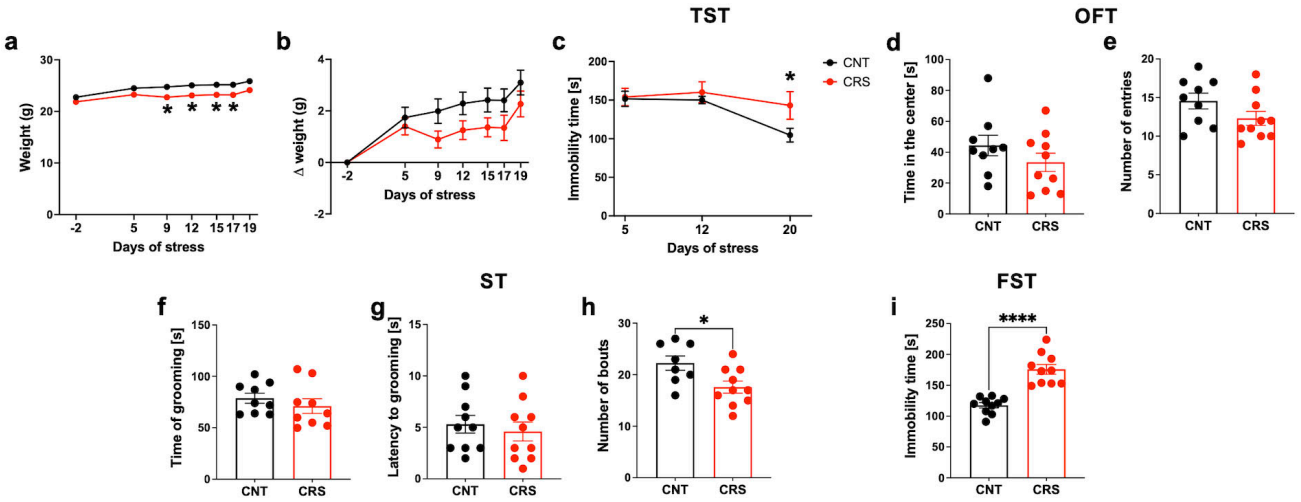

Fig. 2. Behavioural characterization of control and CRS mice. (a) Absolute body weight in grams of control (CNT) and CRS mice throughout the 21 days of CRS. Two-way repeated measures ANOVA (time: F (6, 96) = 25.61, $p < 0.0001$; stress: F (1, 16) = 8.438, $p < 0.05$; time × stress: F (6, 96) = 1.44, $p > 0.05$) followed by Bonferroni's post-hoc test: $*p < 0.05$. (b) Weight gain in grams of control and CRS mice throughout the 21 days of CRS. Two-way repeated measures ANOVA (time: F (2.227, 35.63) = 25.61, $p < 0.0001$; stress: F (1, 16) = 1.761, $p > 0.05$; time × stress: F (6, 96) = 1.644, $p > 0.05$). Δ: weight difference in grams (g) between day N and day-2. (c) Immobility time of control and CRS mice in the TST at days 5, 12 and 20. Non-parametric mixed-effects model (time: F (2, 26) = 4.728, $p < 0.05$; stress: F (1, 14) = 7.466, $p < 0.05$; time × stress: F (2, 26) = 3.771, $p < 0.05$) followed by Fisher's LSD test: $*p < 0.05$. (d) Time and (e) number of entries into the centre of the arena for control and CRS mice during the OFT. Student's unpaired *t*-test. (f) Time of grooming, (g) latency to groom, and (h) number of bouts for CNT and CRS mice in the ST. Student's unpaired *t*-test: $*p < 0.05$. (i) Immobility time of CNT and CRS mice in the FST. Student's unpaired *t*-test: $****p < 0.0001$. N = CNT 8/10, CRS 8/10. Different N values result from the removal of outliers. Means ± SEM are plotted. TST, tail suspension test; OFT, open field test; ST, splash test; FST, forced swim test; ANOVA, analysis of variance; SEM, standard error of the mean.

was evaluated by a non-parametric mixed-effects model followed by Fisher's LSD test. For behavioural tests and molecular evaluations, data were analysed using the unpaired Student's *t*-test. For molecular evaluations, since samples were analysed in different qPCR plates following a complete block design, a blocking factor "plate" was included in the statistical model to account for any plate-to-plate variability [40]. Data were analysed with the delta-delta-Ct ($2^{-\Delta\Delta Ct}$) method by normalising to the geometric average of the two reference genes, *GAPDH* and *YWHAZ* [41] and converting to a relative ratio for statistical analysis [42], as previously reported [43]. Correlation analysis was performed using the two-sided Pearson's product moment correlation coefficient. All analyses were performed with GraphPad Prism 10 (GraphPad Software Inc., San Diego, CA, USA). A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1 Behavioural Characterization of Control and Stressed Mice

Mice were exposed to a chronic restraint stress protocol for three weeks, and weight gain and behavioural phenotypes were assessed (Fig. 1). We observed a significant reduction in the absolute weight of CRS mice at 9, 10, 15, and 17 days of stress, but not at 19 days (Fig. 2a). Accordingly, the evaluation of weight gain compared to baseline showed a significant effect of time × stress interaction ($p < 0.05$), although with no significant point by point post-hoc tests, suggesting moderate and transient changes (Fig. 2b).

Stress-induced maladaptive behavioural phenotypes were assessed by applying a battery of behavioural tests. Animals were exposed to the TST once a week to monitor behavioural changes throughout the three weeks of stress. The data show that immobility time was increased in CRS mice only in the last test (day 20, Fig. 2c). No significant changes were observed in the time spent in the centre or in the number of entries into the central zone in the OFT on day 19 (Fig. 2d,e, respectively), nor in the time and latency to groom in the ST on day 21 (Fig. 2f,g, respectively). Conversely, the number of bouts in the ST was significantly reduced in CRS mice (Fig. 2h), while the time of immobility in the FST performed on day 21 was significantly increased (Fig. 2i).

We then applied a z-score normalisation integrating all the parameters evaluated in the behavioural tests performed in the last days of stress to classify the animals as resilient or susceptible to CRS (chronic restraint stress-resilient mice [CRS-R] and chronic restraint stress susceptible mice [CRS-S], respectively). By defining as resilient

the animals with a z-score within 1.5 SD of controls, we identified 2 resilient mice (Fig. 3) which, considering the small number, were excluded from further evaluations to avoid misinterpretation of the results.

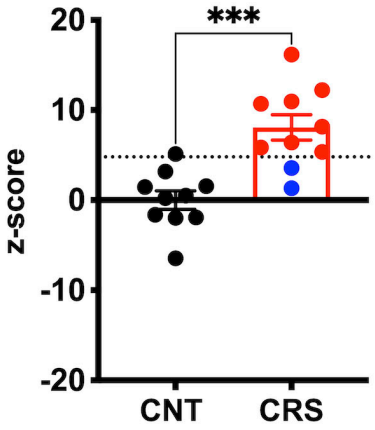

Fig. 3. Classification of the animals in resilient or susceptible to CRS. Z-score normalisation of the parameters evaluated in the behavioural tests (immobility in the TST on day 20, time in the centre and number of entries in the OFT, immobility in the FST, time of grooming, latency to groom, and number of bouts in the ST). Student's unpaired *t*-test: ***$p < 0.001$. N = CNT 10 (in black), CRS-R 2 (in blue), CRS-S 8 (in red). Means ± SEM are plotted. CRS-R, chronic restraint stress-resilient mice; CRS-S, chronic restraint stress susceptible mice.

Fig. 4 shows the same weight and behavioural evaluations reported in Fig. 2, but after the separation of CRS mice into resilient and susceptible groups. The results show that significant changes in absolute weight (Fig. 4a), TST (Fig. 4c) and FST (Fig. 4i) were detected in susceptible animals. We also observed a significant reduction in the number of entries into the central area in the OFT for CRS-S mice compared to controls (Fig. 4e; CRS-S vs. CNT, $p = 0.043$). No significant changes were found for weight gain (Fig. 4b), time spent in the centre during the OFT (Fig. 4d), or ST parameters (Fig. 4f–h).

3.2 Gene Expression Findings

We evaluated the expression profile of *NEGR1*-associated genes in the hippocampus of control and CRS-S mice. Intriguingly, we found significant changes in the CRS-S group. *NEGR1* mRNA levels were significantly higher in CRS-S mice (Fig. 5a), while the metalloprotease *ADAM10* was significantly reduced in CRS-S mice (Fig. 5b). The *FGFR2* receptor was increased by CRS in susceptible mice (Fig. 5c).

We also investigated whether other IgLON cell adhesion molecules were affected by chronic stress exposure. Both *LSAMP* and *NTM* levels were significantly higher in the hippocampus of CRS-S mice (Fig. 5d,e, respectively), thus reproducing a similar pattern to that observed for *NEGR1*. No significant changes were instead measured in the expression levels of *IgLON5* (Fig. 5f), *OPCML* (Fig. 5g), *BDNF* (Fig. 5h), *TRKB* (Fig. 5i), and *PSD95* (Fig. 5j). Exploratory evaluations in CRS-R mice suggest no changes in the expression of any of the genes analysed compared to controls (data not shown).

Finally, to evaluate whether hippocampal molecular changes in *NEGR1*-associated genes were correlated with behavioural readouts, a correlation analysis between gene expression and the behavioural z-score in the same animal was carried out (Fig. 6). All animals, including controls, CRS-S, and CRS-R, were included in the analysis. We found a negative correlation between *ADAM10* expression and the behavioural z-score, suggesting that higher expression of the ADAM10 enzyme correlates with lower behavioural alteration. Conversely, although not reaching statistical significance, the gene expression of *NEGR1* and *LSAMP* showed positive trends of correlation with the behavioural z-score, suggesting that the increased expression of these genes might be associated with stronger behavioural alterations.

4. Discussion

In this study, we investigated the modulation of the pathway of the depression risk gene *NEGR1* in a model of susceptibility to chronic stress. Exposure to stressful life events is recognised as the major environmental risk factor for depression, with ample literature showing that depression episodes are preceded by stressful life events, among which a substantial contribution is provided by chronic stress exposure [6,8]. Since a considerable number of people experience the same stressful events without developing psychiatric symptoms, it has been postulated that stress exposure acts on a vulnerability based on genetic and epigenetic risk variants to confer depression susceptibility, in a gene x environment interaction model [4–7]. The ability to successfully adapt to aversive or challenging life experiences is referred to as resilience, a concept that has gained much relevance for its potential in elucidating the neurobiology of psychiatric disorders and in contributing to therapeutic approaches. Indeed, recent findings support the notion that stress resilience is an active process triggering specific signal transduction pathways and transcriptional modulations, with the implication of limbic circuits as the most relevant brain regions [44–46].

In the present study, we used chronic restraint stress in mice, a validated model of depression known to elicit depressive-like behaviours and neurobiological alterations related to depression- and anxiety-like behaviours [47]. Our findings confirm the development of depressive-like behaviours, as far as they could be defined based on the limitations of the adopted behavioural tests, which were elicited in most, but not all, mice. Given the growing interest in studying individual responses to stress in multiple animal

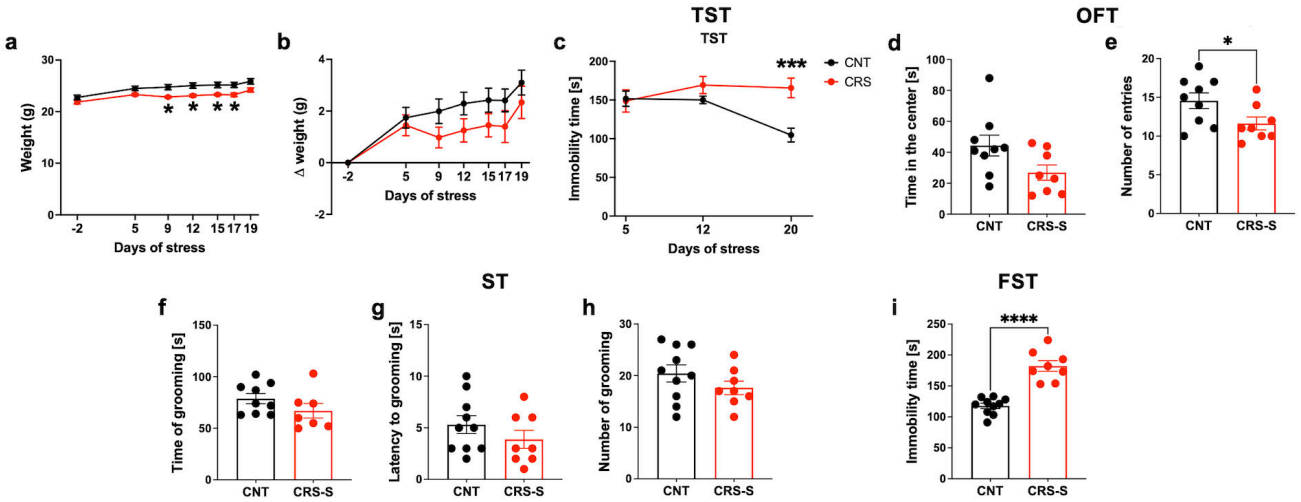

Fig. 4. Behavioural characterization of CRS resilient and susceptible mice. (a) Absolute body weight in grams. Two-way repeated measures ANOVA (time: $F(6, 96) = 25.61$, $p < 0.0001$; stress: $F(1, 16) = 8.44$, $p < 0.05$; time × stress: $F(6, 96) = 1.64$, $p > 0.05$) followed by Bonferroni's post-hoc test: $^{*}p < 0.05$. (b) Weight gain in grams. Two-way repeated measures ANOVA (time: $F(2.227, 35.63) = 25.61$, $p < 0.0001$; stress: $F(1, 16) = 1.761$, $p > 0.05$; time × stress: $F(2.227, 35.63) = 1.644$, $p > 0.05$). Δ: weight difference in grams (g) between day N and day-2. (c) Immobility time in the TST at days 5, 12 and 20. Non-parametric mixed-effects model (time: $F(2, 29) = 3.509$, $p < 0.05$; stress: $F(1, 15) = 6.544$, $p < 0.05$; time × stress: $F(2, 29) = 5.803$, $p < 0.01$) followed by Fisher's LSD test: $^{***}p < 0.001$. (d) Time and (e) number of entries into the centre of the arena during the OFT. Student's unpaired *t*-test: $^{*}p < 0.05$. (f) Time of grooming, (g) latency to grooming (h) and number of bouts in the ST. Student's unpaired *t*-test. (i) Immobility time in the FST. Student's unpaired *t*-test: $^{****}p < 0.0001$. N = CNT 9/10, CRS-S 7/8. Different N values result from the removal of outliers. Means ± SEM are plotted.

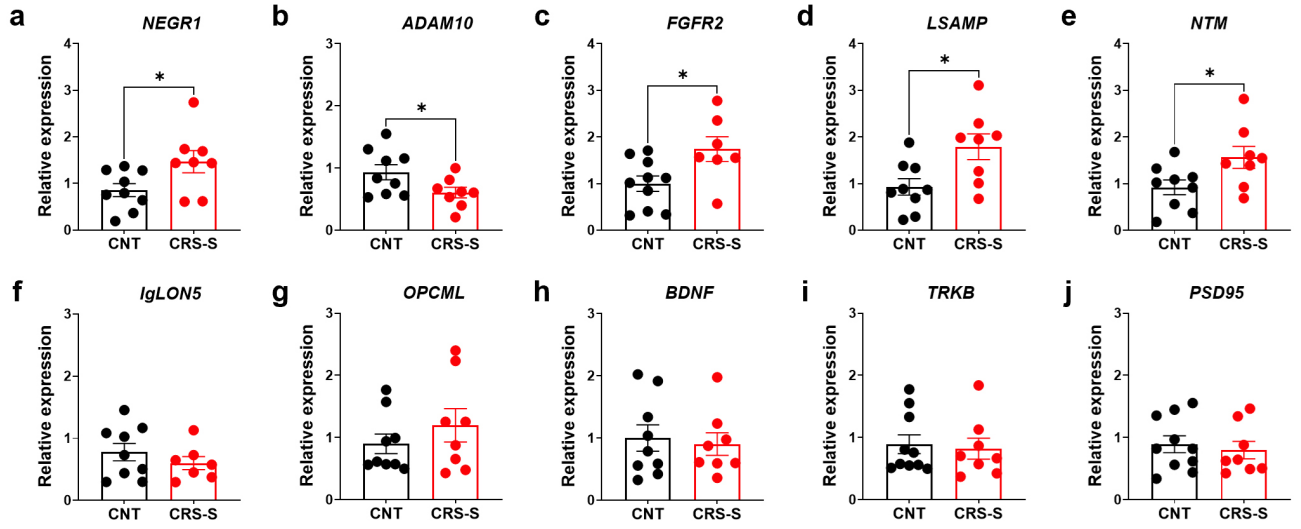

Fig. 5. Hippocampal relative mRNA levels of the NEGR1 pathway and IgLON members in CRS susceptible mice. (a) *NEGR1*, (b) *ADAM10*, (c) *FGFR2*, (d) *LSAMP*, (e) *NTM*, (f) *IgLON5*, (g) *OPCML*, (h) *BDNF*, (i) *TRKB*, and (j) *PSD95* mRNA relative expression in the hippocampus of CNT and CRS-S mice. Student's unpaired *t*-test: $^{*}p < 0.05$. N = CNT 9/10, CRS-S 7/8. Means ± SEM are plotted. *NEGR1*, Neuronal growth regulator 1; *ADAM10*, Disintegrin and metalloproteinase domain-containing protein 10; *FGFR2*, Fibroblast growth factor receptor 2; *LSAMP*, Limbic System Associated Membrane Protein; *NTM*, Neurotrimin; *IgLON5*, IgLON Family Member 5; *OPCML*, Opioid Binding Protein/Cell Adhesion Molecule Like; *BDNF*, Brain-Derived Neurotrophic Factor; *TRKB*, Tropomyosin receptor kinase B; *PSD95*, Postsynaptic density protein 95.

models to understand the mechanisms associated with psychopathological risk [48–51], we applied a z-score normalisation integrating all behavioural parameters to classify the animals into stress resilient and susceptible groups, to gain

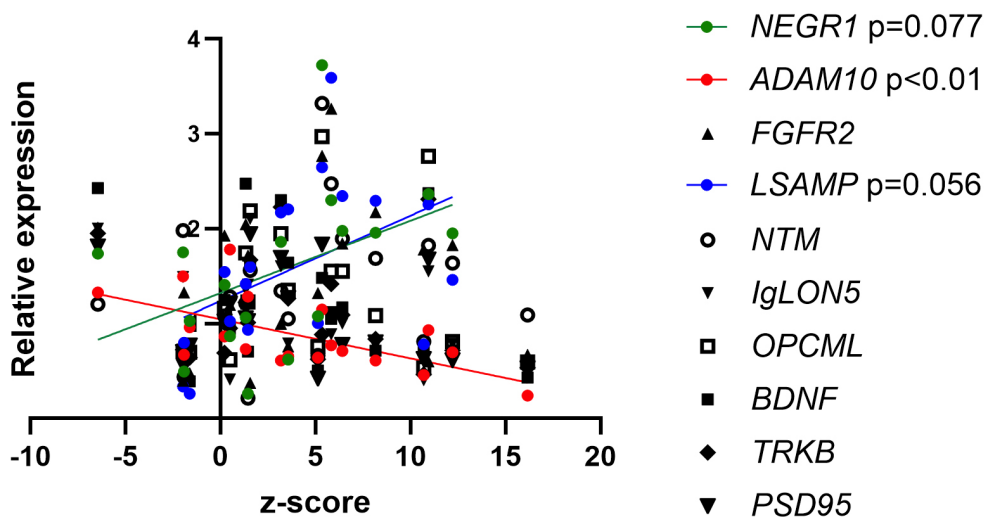

Fig. 6. Correlation of *NEGR1*-associated genes molecular changes with stress-induced maladaptive behavioural phenotypes. Two-sided Pearson's product-moment correlation coefficient (*NEGR1*: $r = 0.4404$, $p = 0.077$; *ADAM10*: $r = –0.6191$, $p < 0.01$; *FGFR2*: $r = 0.06532$, $p > 0.05$; *LSAMP*: $r = 0.4576$, $p = 0.056$; *NTM*: $r = 0.4812$, $p > 0.05$; *IgLON5*: $r = –0.3818$, $p > 0.05$; *OPCML*: $r = 0.05437$, $p > 0.05$; *BDNF*: $r = –0.2795$, $p > 0.05$; *TRKB*: $r = –0.1588$, $p > 0.05$; *PSD95*: $r = –0.2741$, $p > 0.05$). N = 20.

insight into potential molecular signatures associated with behavioural phenotypes. Considering the small number of mice identified as resilient, we excluded them from molecular analyses. More studies are warranted to characterize CMS-R animals at a molecular point of view.

Interestingly, our findings suggest higher *NEGR1* mRNA levels in the hippocampus of stress-susceptible mice, with a preliminary indication of no changes in resilient animals. Previous findings demonstrated an association between the *NEGR1* risk allele in depressed patients and increased *NEGR1* gene expression [16,52], as well as higher mRNA *NEGR1* levels in the cerebral cortex [53] and increased NEGR1 protein in the cerebrospinal fluid of depressed patients [17]. We thus may speculate that increased *NEGR1* expression in our model may contribute to eliciting depressive-like behaviours. Accordingly, it has been recently shown that *NEGR1* overexpression in the ventral hippocampus of mice led to working memory impairment and anxiety- and depression-like behaviours [35]. From a mechanistic point of view, since NEGR1 function has been implicated in synaptic formation, plasticity, and neurite outgrowth [19,26,54], it is conceivable that increased *NEGR1* expression may contribute to synaptic remodelling occurring as a consequence of stress exposure. The hippocampal region is indeed known to undergo extensive remodelling as a consequence of stress exposure, with dendritic shrinkage and retraction, and a reduction of dendritic spines and mature synapses [55]. In line with this hypothesis, *NEGR1* overexpression in the ventral hippocampus led to dendritic spine loss and synaptic ultrastructure abnormalities [35].

On the other hand, the observed reduced levels of *ADAM10* may reflect a decrease in shed NEGR1, although in the presence of higher overall levels, with a resulting lower activation of FGFR2. The reduction of *ADAM10* expression in susceptible animals and its inverse correlation with stress-induced maladaptive behavioural phenotypes may be suggested to contribute to the maladaptive plasticity associated with stress susceptibility. Accordingly, ADAM10 is an anti-amyloidogenic secretase with known neurotrophic and neuroprotective functions [56]; thus, the reduction of its expression may contribute to the neurotoxic effects of chronic stress in line with previous evidence [57].

During development, NEGR1 interacts with FGFR2, promoting the activation of intracellular signalling neurotrophic pathways involving the extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) thus promoting neuron migration and an increase in spine density [28,58]. Although more studies are required to understand the functional consequences of increased *FGFR2* expression in stress-susceptible mice, our findings are consistent with an adaptive process aimed at favouring neuroplasticity processes.

IgLON cell adhesion molecules are reported to form heterodimers that exert different biological activities depending on the dimer composition [24,59]. Since *NTM* and *LSAMP* expression was also increased in CRS-S mice, it is suggested that these molecules make a contribution to the responses induced by stress, with potential roles in synaptic remodelling.

5. Limitations

Besides the above-mentioned strengths, a number of limitations characterise the present study. Firstly, the small number of stress-resilient mice prevented a statistical evaluation of this group, thus evidence could not be derived about resilience-associated behavioural and molecular al-

terations. In addition, this work focuses on the hippocampus, because alterations in hippocampal proteins, circuits, structure, and networking are known to play major roles in physiological and pathological responses to chronic stress exposure [6,55,60]. Nevertheless, additional brain structures, including the amygdala, prefrontal cortex, and nucleus accumbens, are recognised as providing a significant contribution to overall stress responses [6,61,62]; therefore, further studies will be required to elucidate IgLON alterations in these brain regions. Also, only male mice were investigated, whereas previous reports demonstrate sex-specific behavioural and molecular adaptations to stress [63,64]; thus, this point will be addressed in future studies, keeping in consideration that MDD has different prevalences in men and women [65].

6. Conclusions

Although more studies are warranted to unveil the role of IgLON proteins in the molecular underpinning of stress-induced behavioural alterations, our findings suggest that specific changes in the expression levels of these molecular mediators could be related to processes associated with chronic stress susceptibility.

Overall, our findings strengthen the importance of NEGR1 in the pathophysiology of major depression and implicate this pathway in the interplay between genetic and environmental predisposing conditions.

Abbreviations

ADAM10, Disintegrin and metalloproteinase domain-containing protein 10; ANOVA, Analysis of Variance; BDNF, Brain-Derived Neurotrophic Factor; CNT, controls; CRS, Chronic Restraint Stress; CRS-R, Chronic Restraint Stress Resilient mice; CRS-S, Chronic Restraint Stress Susceptible mice; FGFR2, Fibroblast growth factor receptor 2; FST, forced swim test; IgLON5, IgLON Family Member 5; LSAMP, Limbic System Associated Membrane Protein; NEGR1, Neuronal Growth Regulator 1; NTM, neurotrimin; OFT, Open Field test; OPCML, Opioid Binding Protein/Cell Adhesion Molecule Like; PSD95, Postsynaptic density protein 95; SD, standard deviation; SEM, standard error of the mean; ST, splash test; TRKB, Tropomyosin receptor kinase B; TST, tail suspension test.

Availability of Data and Materials

All data reported in this paper will be shared by the lead contact upon request.

Author Contributions

LC and LM conceived and designed the research study. JM, MS, LM, and LC performed the research. RR, LM, and LC analysed the data. LC and LM wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Experiments were performed in accordance with the European Community Council Directive 2010/63/EU and approved by the Italian legislation on animal experimentation (Decreto Legislativo 26/2014). The protocol was authorised by the Institutional Review Board of the University of Milano-Bicocca and by the Italian Ministry of Health (authorization N 103/2022-PR, prot. FB7CC.55 for mice). The experimental design was compliant with the 3R principles and this report adhered to the ARRIVE guidelines.

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Conflict of Interest

The authors declare no conflict of interest. Given her role as the Guest Editor, Lucia Carboni had no involvement in the peer-review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Hongquan Wang and Xudong Huang. LM is affiliated with Fondazione IRCCS San Gerardo dei Tintori. The judgement in data interpretation and manuscript writing were not influenced by this affiliation.

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