

ORIGINAL RESEARCH REPORT

Altered emotionality, hippocampus-dependent performance and expression of NMDA receptor subunit mRNAs in chronically stressed mice[†]João Costa-Nunes^{1,2*}, Olga Zubareva^{3*}, Margarida Araújo-Correia^{1*}, Andreia Valença^{1*}, Careen A. Schroeter⁴, Jodi L. Pawluski^{1,5}, Julie Vignisse^{1,5}, Hellen Steinbusch¹, Denise Hermes¹, Marjan Phillipines¹, Harry M. W. Steinbusch¹, and Tatyana Strekalova^{1,2,4*}¹Department of Neuroscience, School for Mental Health and Neuroscience, Maastricht University, Maastricht, the Netherlands, ²Institute for Hygiene and Tropical Medicine, New University of Lisbon and Centre of Environmental Biology, Lisbon University, Lisbon, Portugal, ³Institute of Experimental Medicine, Russian Academy of Medical Sciences, St. Petersburg, Russia, ⁴Maastricht Medical Centre in Annadal, Maastricht, the Netherlands, and ⁵GIGA Neuroscience, Liège University, Liège, Belgium**Abstract**

N-Methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission in the hippocampus is implicated in cognitive and emotional disturbances during stress-related disorders. Here, using quantitative RT-PCR, we investigated the hippocampal expression of NR2A, NR2B and NR1 subunit mRNAs in a mouse stress paradigm that mimics clinically relevant conditions of simultaneously affected emotionality and hippocampus-dependent functions. A 2-week stress procedure, which comprised ethologically valid stressors, exposure to a rat and social defeat, was applied to male C57BL/6J mice. For predation stress, mice were introduced into transparent containers that were placed in a rat home cage during the night; social defeat was applied during the daytime using aggressive CD1 mice. This treatment impaired hippocampus-dependent performance during contextual fear conditioning. A correlation between this behavior and food displacement performance was demonstrated, suggesting that burrowing behavior is affected by the stress procedure and is hippocampus-dependent. Stressed mice ($n = 22$) showed behavioral invigoration and anomalous anxiolytic-like profiles in the O-maze and brightly illuminated open field, unaltered short-term memory in the step-down avoidance task and enhanced aggressive traits, as compared to non-stressed mice ($n = 10$). Stressed mice showed increased basal serum corticosterone concentrations, hippocampal mRNA expression for the NR2A subunit of the NMDAR and in the NR2A/NR2B ratio; mRNA expression of NR2B and NR1 was unchanged. Thus, stress-induced aberrations in both hippocampal-dependent performance and emotional abnormalities are associated with alterations in hippocampal mRNA NR2A levels and the NR2A/NR2B ratio and not with mRNA expression of NR2B or NR1.

Introduction

N-Methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission is involved in the etiology of stress-related cognitive deficits and behavioral abnormalities (Cull-Candy et al., 2001). This particularly implicates altered expression of the two NR1 (GluN1) and two NR2 (GluN2) subunits (NR2A and NR2B) of NMDAR tetramers, which are the most abundant in the hippocampal formation, a brain structure that mediates stress-induced aberrations in both learning and

emotional behavior (Li & Ju, 2012). Both NR2A and NR2B were shown to have distinct pharmacology and a role in the regulation of NMDAR, and have been suggested to be differentially involved in the mechanisms of learning and emotionality (Fleischmann et al., 2003; Li & Tsien, 2009). A body of evidence has demonstrated the involvement of NR2A, NR2B and NR1 subunits in the neurobiology of neuropsychiatric conditions such as anxiety, psychosis, impulsivity, Alzheimer's disease and major depression (Davies et al., 2012; Geissler & Lesch, 2011; Tsang et al., 2008).

Most experiments investigating the roles of NMDAR subunits in neuropsychiatric symptoms target selective subunits of this receptor using pharmacological and genetic manipulations that are frequently applied *in vitro* (Boyce-Rustay & Holmes, 2006; Cui et al., 2013; Longordo et al., 2009). However, fewer studies have addressed the changes in NMDAR subunit expression in

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Burrowing behavior, contextual fear memory, hippocampus, N-methyl-D-aspartate receptor, predator stress, social defeat

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disease-modeling conditions. Understanding changes in relative-fold mRNA expression of NMDAR subunits in animal models of cognitive and emotional disturbances related to chronic stress is important for the identification of new pharmacological interventions and therapies that effectively alter glutamatergic transmission (Vignisse et al., 2013). Previous work on gene expression (Strekalova et al., 2011) has highlighted abnormal hippocampal expression of NMDAR subunits in mice subjected to repeated stressors. We have hypothesized that in male C57BL/6J mice, a novel 2-week stress procedure, comprising ethologically valid stressors, exposure to a rat and social defeat, results in concomitant cognitive, emotional and NMDA receptor expression abnormalities in the hippocampus. Thus, the current study specifically investigated the hippocampal relative-fold mRNA expression of NR2A, NR2B and NR1 subunits in the above-mentioned chronic stress procedure in mice that mimics clinically relevant situations of stress-related disturbances in learning and emotionality (Cline et al., 2012; Strekalova & Steinbusch, 2010).

Materials and methods

Animals and housing

Male C57BL/6J and CD1 mice were 3 months old, Wistar rats of 3–5 months age were used for applying predator stress; mice and rats were provided by Harlan, the Netherlands and Charles River, France, respectively. Fourteen days before the behavioral experiments, mice were single housed under a reverse 12h:12h light–dark cycle (lights on: 21:00h) in standard laboratory conditions (22 ± 1 °C, 55% humidity, food and water *ad libitum*). All experiments were carried out in accordance with the European Committees Council Directives and had been approved by the ethics committee of Maastricht University for animal research (CPV, DEC-UM 2009-109).

Experimental conditions and study outline

This study applied only ethological stressors to male C57BL/6J mice, for 14 d, based on previous work (adapted from Cline et al., 2012; Couch et al., 2013). The stress procedure consisted of dark-cycle rat exposure between the hours of 09:00h and 18:00h and light-cycle application of a social defeat paradigm, combined with exposure to an aggressive CD1 mouse. Body weight and parameters of social behavior were determined 1 week before the chronic stress procedure in a social interaction test as described elsewhere (Strekalova et al., 2004). The experimental and control groups were balanced for these parameters. Ethological stressors were applied to a stress group for 14 d, as described below. The control group received daily handling only. At the end of the stress experiment, 12h after the application of the last stressor, short-term memory in the step-down avoidance task (Vignisse et al., 2011) and aggressive behavior in a resident-intruder test (Strekalova et al., 2004) were investigated in stressed and control mice. At this time, body weight was also assessed. The next day (day 1), mice were tested in the elevated O-maze and food displacement tube tests (Strekalova & Steinbusch, 2010). On days 2 and 3, respectively, contextual fear conditioning training and testing for recall (Vignisse et al., 2013) were performed.

The open-field testing was carried out on day 4 (under red light) and on day 5 (under white light). All behavioral tests were recorded on video.

We used a battery of behavioral tests based on previous literature (Calabrese et al., 2012). Although it is possible that the different behavioral tests may affect subsequent tests, previously published test batteries with similarly employed paradigms have revealed an absence of any testing effects in C57Bl/6J mice (Malatynska et al., 2012; Strekalova & Steinbusch, 2009, 2010; Vignisse et al., 2011, 2013). Hence, we considered possible interfering effects of multiple behavioral test to be minimal in the current study.

A separate cohort of mice from both groups was pre-exposed to a mixture of CO₂ and O₂ and euthanized via cervical dislocation (according to Dutch law), for gene expression analysis 24h after the termination of the stress procedure.

Chronic stress procedure

Rat exposure while in a small container

Mice were introduced into cylindrical containers, which were placed into a rat home cage for 15 h (overnight, from 18:00h to 9:00h). Containers (15 cm × Ø 8 cm) were made from customized transparent plastic with holes in the covers (Ø < 0.5 cm). This ensured protection of the mouse from the rat, but allowed visual and odor contact. During the weekends, mice were kept in their home cages, situated on top of the rat cages.

Social defeat stress

Social defeat procedures took place during the dark phase of the light cycle (between 12:00h and 16:00h). To enable visual control by the experimenter over the resident-intruder confrontation, the test was carried out under red light. In a preliminary test, aggressive CD1 mice that were able to attack the counter-partners in less than 60 s, without injuring them, were selected for this procedure; these mice were introduced into the home cages of mice from the stress group during social defeat sessions for 5 min. During social defeat stress, test mice typically showed flight responses, submissive postures and vocalizations. Pairs of mice were carefully observed in order to prevent physical harm. In rare cases, aggressive mice were immediately removed from the cage of resident mice. After a 5-min period of social defeat, C57BL/6J mice were placed into small containers and put inside a CD1 mouse cage, where they stayed for a 3 h-period. After the 3 h period, the 5-min social defeat procedure was repeated. In order to randomize the procedure, the same pairs of C57BL/6J and CD1 mice were never put together.

Behavioral procedures

Step-down passive avoidance model

The step-down passive avoidance test was used as described elsewhere (Strekalova et al., 2001; Vignisse et al., 2011, 2013). The step-down apparatus (Evolocus LLC Tarrytown, NY and Technosmart, Rome, Italy) was a transparent plastic cubicle (25 cm × 25 cm × 48 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter), onto which a square wooden

platform (7 cm × 7 cm × 1.5 cm) was placed. The illumination strength was 25 lux. A shocker was used to deliver an alternating electric current (AC, 50 Hz). In this paradigm, mice are trained to avoid an electric shock by staying on the platform above the grid floor. During the training session, mice were placed on the platform inside a transparent cylinder for 30 s to prevent them from stepping down immediately. After removal of the cylinder, the time until the mouse left the platform, with all four paws, was measured as baseline latency of step-down. Immediately after step-down, mice received a single electric foot shock (0.5 mA, 2 s) and were returned to their home cages. One hour later, during the recall trial session, mice were exposed to the apparatus again by being handled in the same way as in the training session; no foot shock was delivered. Latency of step-down with all four paws was measured until 180 s had elapsed.

Elevated O-maze

Testing on the elevated O-maze was carried out as described elsewhere (Strekalova et al., 2005). The O-maze consisted of a black circular path (runway width 5.5 cm, $\varnothing = 46$ cm) with two opposing compartments protected by walls made of polyvinyl-chloride (height = 10 cm) and two open sectors of equal size. The maze was elevated 20 cm above the ground and illuminated from the top with red light. At the start of the testing session, mice were placed inside one of the two closed compartments. The test was recorded with a web camera. The latency to the first entry into the anxiety-related open arms of the maze, total number of entries into the open arms and total duration of time spent in open arms were scored for 5 min.

Food pellet displacement (burrowing) behavior in a tube test

In order to further assess hippocampal function, all experimental groups were tested for burrowing behavior. Burrowing behavior, a tendency to displace small objects, e.g. small stones or food pellets, from a tube inside the home cage, is species-specific in mice and has been demonstrated to depend on an intact hippocampal formation. Using a paper tube (internal diameter 4 cm, length 10 cm), filled with 20 food pellets and placed in the middle of a mouse home cage, the latency to displacement of the first food pellet, time required to empty the tube, number of pellets removed after 1 h and 1 h 30 min were assessed in stressed and control mice during the dark phase (Strekalova & Steinbusch, 2009, 2010). Time elapsed was 90 min.

Contextual fear-conditioning paradigm

The contextual fear-conditioning test procedure was adapted from previously described protocols (Strekalova et al., 2003; Vignisse et al., 2013). The apparatus consisted of a transparent plastic cubicle (25 cm × 25 cm × 50 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter). A shocker was used to deliver an alternating electric current (AC, 50 Hz; 0.7 mA, 2 s) after a 2-min acclimatization of a mouse to the chamber. After delivery of the current, the mouse was immediately placed back in the home cage. Freezing behavior was scored by visual observation during a test of memory recall that was carried out 24 h later. The occurrence of freezing behavior in the chamber was assessed every 10 s for

180 s; each 10-s score was assigned to a freezing or non-freezing period, and the percentage of time spent in freezing was calculated.

Open field

The open-field apparatus consisted of four square arenas (25 cm × 25 cm × 40 cm), made of wood covered with white resopal. Mice were put in the center of one of the four square open field arenas, and their behavior was video recorded for 10 min. The open field was illuminated with white light (25 lux) or red light. Behavior was analyzed off-line using the Any-maze software (Stoelting Co, Wood Dale, IL). Among other parameters, time spent immobile was analyzed in the central (area 20 × 20 cm) and peripheral (remaining part of the apparatus) zones.

Resident-intruder test

The resident-intruder test procedure was performed as described elsewhere (Strekalova et al., 2004). In this paradigm, the C57BL/6J mice were placed individually in an observation cage (30 cm × 60 cm × 30 cm) for 30 min. Thereafter, a male CD1 mouse, which was group housed before the test, was introduced as an intruder to the same cage and left with the resident mouse for 8 min. During the observation period, resident and intruder mice were scored for aggressive social behaviors. Latency of the first attack and number of attacks were scored.

Brain dissection and blood collection

On the day following the termination of the stress procedure, a cohort of mice from both groups was euthanized as described above, their hippocampi were dissected and trunk blood was collected for corticosterone evaluation.

Corticosterone concentration

To assay serum corticosterone, trunk blood was stored at 4 °C overnight and centrifuged at 10 × g for 10 min; the assay was performed as previously described (Pawluski et al., 2012). Serum was collected and stored at –80 °C until use. All samples were run in duplicate. For total serum corticosterone concentrations, a commercially available radioimmunoassay (RIA) kit for rat corticosterone from MP Biomedicals (corticosterone I25 for rats and mice, MP Biomedicals, LLC, Orangeburg, NY) was used. Average intra- and inter-assay coefficients of variation for all corticosterone assays were below 10%. Assay sensitivity was 7.7 ng/mL.

RNA isolation and RT-PCR

RNA was extracted as previously described (Couch et al., 2013) using the RNeasy RNA extraction kit (Qiagen, Hilden, Germany) and first strand cDNA synthesis was performed using random primers and Superscript III transcriptase (Invitrogen, Darmstadt, Germany); 1 µg total RNA was converted into cDNA. Quantitative RT-PCR (qPCR) for *NR2A*, *NR2B*, *NRI* genes and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was performed using TaqMan probes and the CFX96 Real-time System (BioRad, Hercules, CA). Cycling conditions and

sequences of primers used are indicated in Table 1 of supplementary data. Results were normalized to GAPDH mRNA expression and calculated as relative-fold changes compared to control mice as described elsewhere (Couch et al., 2013). Results of the qPCR measurements were expressed as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product was 0.05% of normalized maximal signal. We used the comparative Ct method and computed the difference between the expression of the gene of interest and GAPDH expression in each cDNA sample ($2^{-\Delta\Delta Ct}$ method). Results are given as expression-folds compared to the mean expression values in non-stressed control mice (Couch et al., 2013, adapted from Livak & Schmittgen, 2001).

Statistical analysis

GraphPad Prism 5.00 (San Diego, CA) was used for analyses. A comparison of normally distributed independent variables was carried out using unpaired two-tailed *t* tests. Independent measurements that were not normally distributed were analyzed via the non-parametric Mann–Whitney test, and repeated measurements were compared by the Wilcoxon non-parametric test. The Pearson test was applied for correlation analysis. The level of confidence was set at 95% ($p < 0.05$).

Results

Stressed mice showed a significant loss of body weight compared to the control mice ($p < 0.0001$, $t = 6.801$, $df = 21$; unpaired *t* test; Figure 1A) and a significant elevation of circulating corticosterone concentration ($p = 0.030$, $U = 39$; Mann–Whitney test, Figure 1B), indicating a profound impact of the 2-week stress procedure. In comparison to control mice, the open-field activity of the stressed mice was significantly higher both in the peripheral ($p = 0.040$, $U = 61.00$, Mann–Whitney test) and even more notably, in the central zones ($p = 0.011$; $U = 73.50$) of the apparatus, when white lighting was employed (Figure 1C), but not when activity was scored under red light ($p = 0.15$, $U = 68.00$ and $p = 0.64$, $U = 97.50$, respectively). There was a significantly lower latency to the first exit in the elevated O-maze ($p = 0.010$, $U = 49.50$, Mann–Whitney test), increased time spent in the open arms ($p = 0.020$, $U = 55.50$) and number of entries into the open arms ($p = 0.028$, $U = 58.50$; Figure 1D) in stressed versus control mice. Together, these data indicate differences in the brightly lit open field induced by stress: behavioral invigoration and an anomalous “anxiolytic-like” profile. In addition, chronically stressed mice displayed a significant increase in the number of attacks and no change in the latency to the first attack in the resident-intruder test ($p = 0.049$, $t = 1.698$, $df = 30$ and $p = 0.12$, $t = 1.78$, $df = 23$; unpaired *t* test; Figure 1E).

Both stressed and control mice showed a significant increase in the latency of step down 1 h after training, in comparison to baseline latencies ($p = 0.033$, $W = -37.00$ and $p = 0.05$, $W = -51.00$, respectively, Wilcoxon test). There was no significant difference between stressed and control mice in the latencies for step-down evaluated at baseline conditions ($p = 1.0$, $U = 71.50$) or 1 h after training ($p = 0.98$, $U = 71.00$; Figure 2A), indicating similar scores of

anxiety-like behavior and short-term memory in these groups. In the contextual fear-conditioning paradigm, stressed mice spent a significantly shorter time freezing during a recall session than the control group ($p = 0.021$, $U = 50.00$, Mann–Whitney test; Figure 2B), which demonstrates impaired hippocampus-dependent contextual memory in this group. Stressed mice showed no significant inhibition of burrowing behavior in comparison to the control group, as assessed by latency for the first pellet displacement ($p = 0.18$, $U = 73.00$; Figure 2C) and number of pellets displaced over time intervals of 0–60 min ($p = 0.14$, $U = 53.50$) and 60–90 min ($p = 0.34$, $U = 47.50$, Mann–Whitney test). Nonetheless, there was a significant correlation between the two latter measures and contextual freezing ($r = 0.36$, $p = 0.046$ and $r = 0.36$, $p = 0.048$, respectively). There was no significant correlation between the duration of freezing and the latency for pellet displacement ($r = -0.16$, $p = 0.39$, Pearson correlation).

Concerning mRNA levels of NMDA receptor subunits in the hippocampus, in comparison to control mice, the stressed group had a significant increase in expression of NR2A ($p = 0.029$, $t = 2.050$, $df = 15$, unpaired *t* test; Figure 3A) and no significant change in the expression of NR2B ($p = 0.27$, $t = 0.6431$, $df = 15$, Figure 3B). The ratio of NR2A/NR2B was significantly increased ($p = 0.015$, $t = 2.452$, $df = 13$; Figure 3C) but the expression of the NR1 subunit was unaltered ($p = 0.64$, $t = 0.4665$, $df = 14$; Figure 3D).

Discussion

In accordance with our hypothesis and in line with the literature (Calabrese et al., 2012; Cull-Candy et al., 2001; Fleischmann et al., 2003; Li & Ju, 2012), the current study implicates altered expression of NMDAR subunits of the hippocampus in stress-induced deficits in both cognitive and emotional traits. The present study showed that in C57BL/6J mice, a 2-week ethological stress paradigm, comprised of exposure to a rat and social defeat, resulted in the impairment of contextual fear conditioning, as well as behavioral disinhibition in the open field test and increased aggressive behavior. These cognitive and emotional abnormalities were accompanied by increases in the hippocampal mRNA expression of the NR2A subunit of the NMDAR and in the NR2A/NR2B ratio, while the mRNA expression of NR2B and NR1 was unchanged.

A significant reduction in body weight and increase in basal serum corticosterone concentration of stressed mice demonstrated the impact of the 2-week stress procedure (Figure 1A, B). Stressed mice displayed behavioral hyperarousal under stressful testing conditions, as shown by a significant decrease in the time spent immobile in the central and peripheral parts of the brightly illuminated open field, but a lack of these changes during stress-free open field testing under red light (Figure 1C). Behavioral alterations of the stressed group in the open field illuminated with white light are in agreement with behavioral changes in these mice in the elevated O-maze. In the O-maze test, stressed mice showed a significant shortening of the latency to enter the open arms, an increase in the time spent and the number of entries into the open arms (Figure 1D). Together, these findings indicate that the stress procedure evoked anomalous “anxiolytic-like”

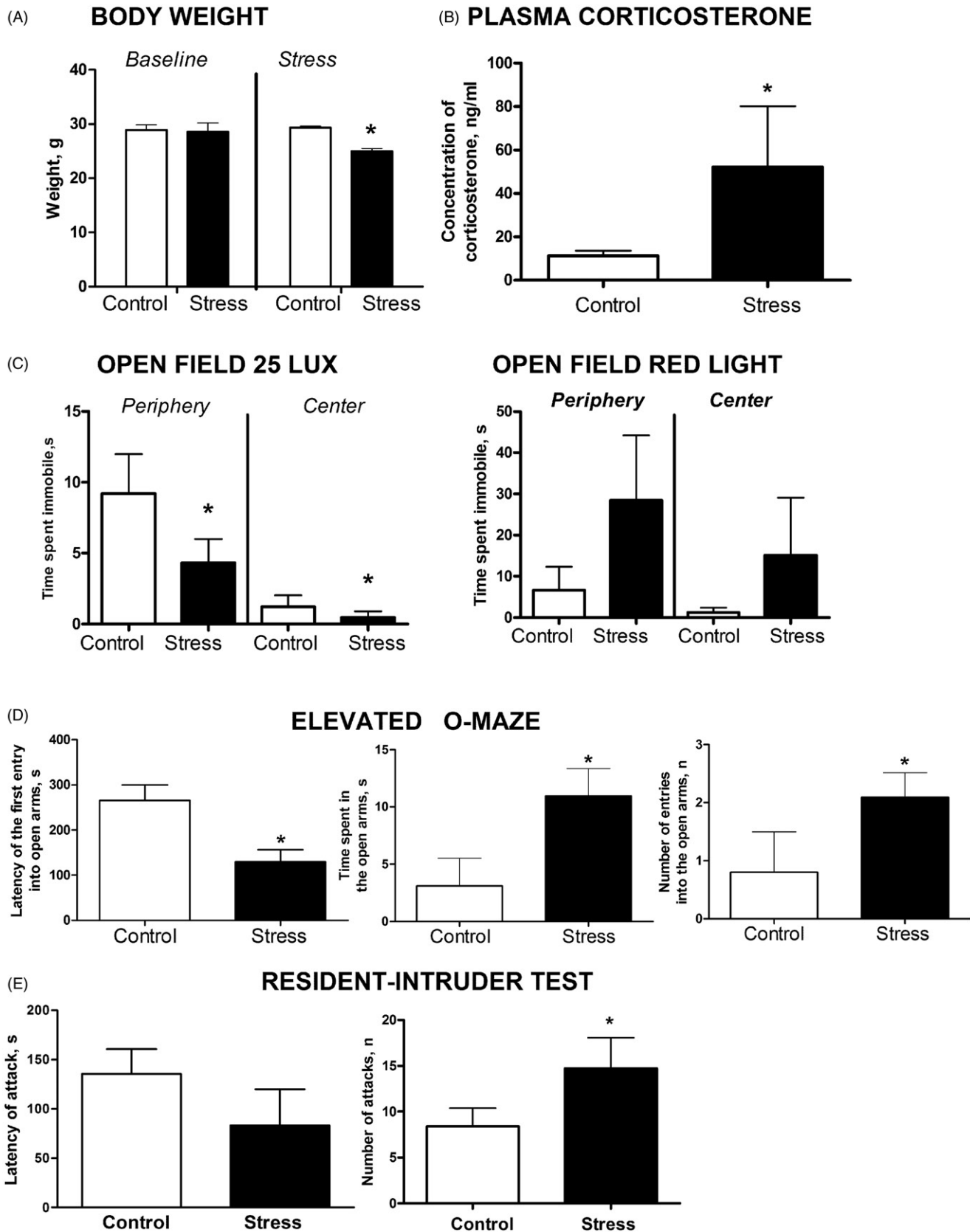


Figure 1. Exposure of mice to stressors for 2 weeks affects body weight, serum corticosterone and parameters of emotionality. (A) Weight loss and (B) increased serum corticosterone concentration in the stress group. (C) Stressed mice showed a reduced total time spent immobile at the periphery and in the center of the open field lit with white light. There were no significant differences in locomotor behavior between groups tested under red light. (D) Stressed mice displayed decreased latency of entries into the open arms, an increased time spent therein and increased number of entries. (E) Elevated aggressive behavior (number of attacks) in stressed mice. * $p < 0.05$ versus control (A, E: unpaired t test, B–D: Mann–Whitney test). Control group, $n = 10$; stress group, $n = 22$. All data are means \pm standard error of the mean (SEM).

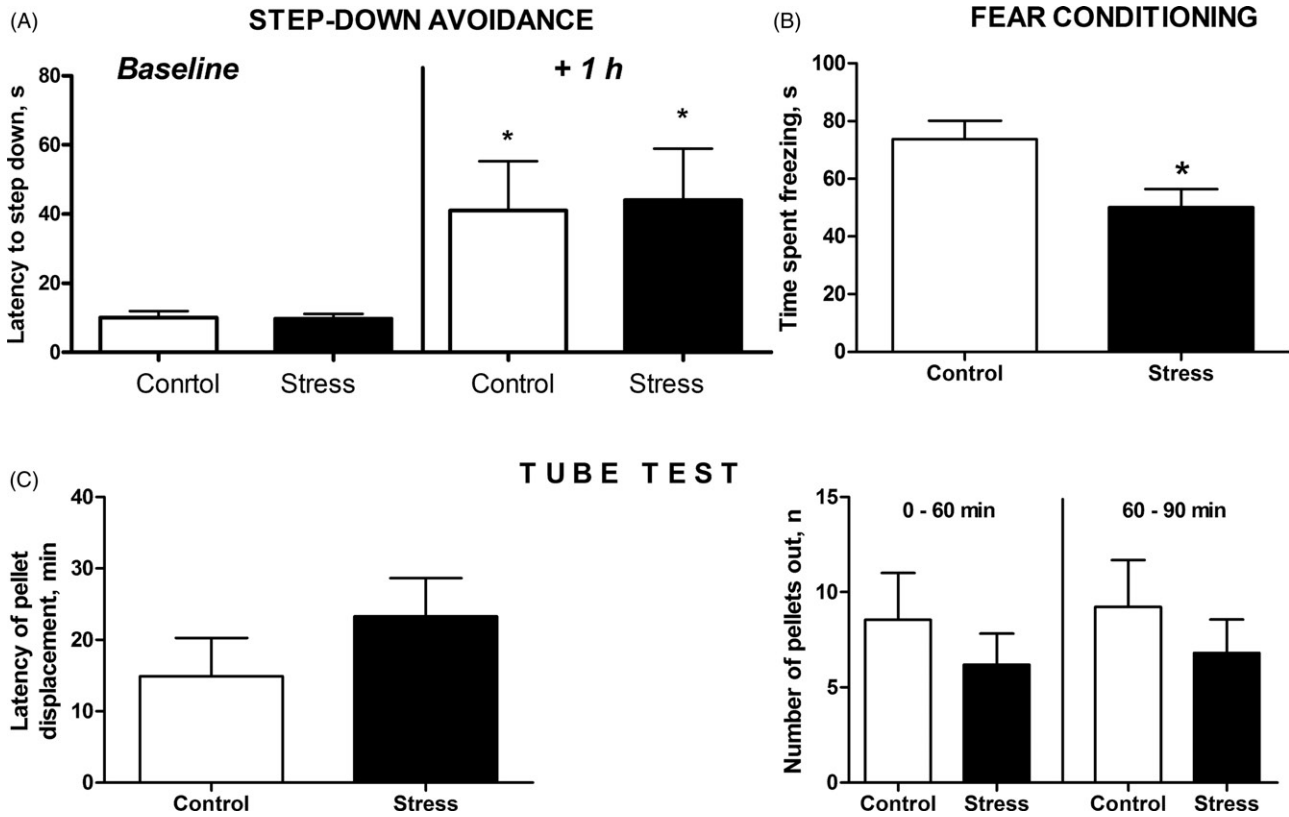


Figure 2. Hippocampus-dependent performance in stressed mice. (A) Stressed and control mice showed a significant increase in the latency for step down 1 h after training, in comparison to baseline latencies; $*p > 0.05$ versus baseline, Wilcoxon test; there were no differences between groups. (B) Stressed mice spent a significantly shorter time freezing, during a recall session in the fear-conditioning paradigm; (C) there were no significant differences for latency to food displacement or number of pellets displaced at 0–60 min and 60–90 min in the tube test. $*p < 0.05$ versus control; Mann–Whitney test. Control group, $n = 10$; stress group, $n = 22$. All data are means \pm the standard error of the mean (SEM).

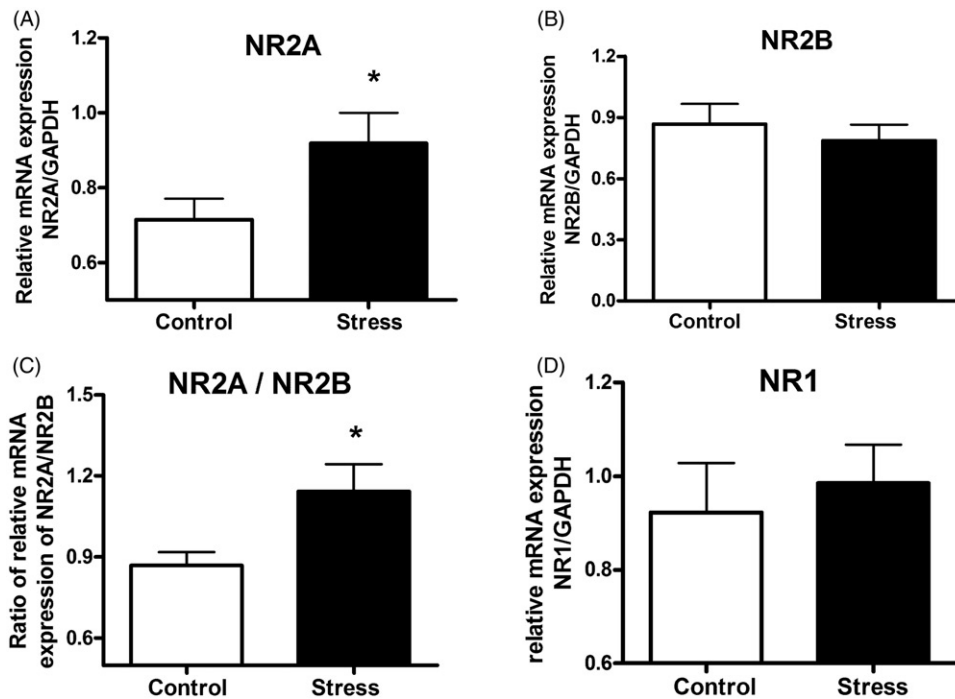


Figure 3. Hippocampal expression of mRNAs for *N*-methyl-D-aspartate receptor (NMDAR) subunits in stressed mice. In the stressed group, relative to controls: (A) mRNA expression of the NR2A subunit of the NMDAR was significantly greater; (B) mRNA expression of NR2B was not altered; (C) the ratio of mRNAs for NR2A/NR2B was significantly increased; (D) mRNA expression of the NR1 subunit of NMDAR was not altered; $*p < 0.05$ versus control; unpaired *t* test. Control group, $n = 9$; stress group, $n = 12$. Data are means \pm the standard error of the mean (SEM).

traits and behavioral invigoration in mice. In our experiments, these changes were accompanied by a significant increase in the number of attacks by stressed mice in the resident-intruder paradigm, which is an indicator of enhanced aggressive traits (Figure 1E). This is in agreement with previous work showing that increased anxiety levels typically correlate with increased scores of aggressive behavior in C57BL/6J male mice (Willner, 2005).

In line with our findings, different chronic stress procedures have been reported to cause an increase in time spent in anxiety-related compartments of the elevated plus maze and the dark/light box in rodents, which was interpreted as a sign of “anxiolytic-like” effects (Cancela et al., 1995; D’Aquila et al., 1994; Hata et al., 2001; Sanchez, 1997). Such phenomena, also considered as manifestations of impulsivity and disinhibition, are well-known consequences of chronic stress in rodents (Belujon & Grace, 2011; Strekalova et al., 2005; Willner, 2005). It is commonly accepted that the limbic system, in general, and the hippocampus, in particular, have a central role in the behavioral inhibition system (Abela & Chudasama, 2013; Geissler & Lesch, 2011; Gray & McNaughton, 1983; McNaughton et al., 1997; McNaughton & Gray, 2000; Rawlins et al., 1985), while the crucial role of the hippocampus for memory was established much earlier (Squire, 1992). Behavioral invigoration, such as during stress, is likely to be associated with alterations of other hippocampal functions, which may include learning processes and molecular changes related to gene expression regulating glutamatergic neurotransmission (Belujon & Grace, 2011; Calabrese et al., 2012; Geissler & Lesch, 2011). Thus, these potential changes were investigated in the present study.

Stressed mice had a normal acquisition of the short-term step-down avoidance task, a form of cortex-dependent learning, which was shown by their unaltered latencies to step-down (Figure 2A). However, a lower percentage of freezing during the recall session of the fear-conditioning test in stressed mice indicates a deficiency in their long-term hippocampus-dependent memory (Figure 2B). The possibility that a hyperactivity of chronically stressed mice in our study interferes with scoring of freezing was largely ruled out by a lack of differences in locomotor activity during a 2-min acclimatization pre-training period between stressed and control mice (Strekalova et al., 2003). Also, both groups had similar values for the baseline latencies of step-down behavior (Figure 2A), indicating similar anxiety-like traits when assessed under the testing conditions that were subsequently used to assess contextual learning. Baseline step-down behavior was previously reported as a highly sensitive measure of subtle changes in anxiety and locomotion in C57BL/6J mice (Strekalova & Steinbusch, 2009, 2010; Vignisse et al., 2011, 2013).

The changes in contextual freezing significantly correlated with decreased burrowing behavior, although burrowing parameters were not altered significantly (Figure 2C). A tendency to displace food pellets is often regarded as not a fully specific indicator of hippocampal dysfunction in rodents (Hart et al., 2012; Kaczmarczyk et al., 2013; Tarr et al., 2012) and its neurobiology is debatable. Hence the finding of a correlation between this behavior and hippocampus-dependent learning in the present study might be

potentially important in relating the burrowing behavior to the dorsal hippocampus, as originally proposed (Deacon et al., 2002).

This study revealed stress-induced increases in the hippocampal expression of NR2A and the NR2A/NR2B ratio (Figure 3A, C), which were previously shown to accompany elevated anxiety (Boyce-Rustay & Holmes, 2006; Calabrese et al., 2012; Gao et al., 2010), impulsivity and aggression (Bortolato et al., 2012; Meyer et al., 2004), home cage hyperactivity and a stress-induced increase in peripheral concentrations of corticosterone (Huang et al., 2010; Longordo et al., 2009) in various conditions. A limitation of our study, however, is the need for confirmation that the mRNA changes result in corresponding changes in subunit protein levels and in altered synaptic function in the hippocampus. In agreement with our data, separate studies have reported that molecular changes such as we found are associated with a disruption of long-term memory, but not short-term learning (Calabrese et al., 2012; Cui et al., 2013; Huang et al., 2010). In the present experiment, mRNA expression of NR2B and the NR1 in the hippocampus was not significantly changed by stress (Figure 3). However, previous work has reported a significant decrease in NR2B expression in several brain areas during stress (Cull-Candy et al., 2001; Huang et al., 2010), aging (Dere et al., 2003) and compromised plasticity (Bortolato et al., 2012). Previous work has also reported a decrease in NR1 mRNA after stress (Cull-Candy et al., 2001; Schenberg et al., 2006). Discrepancies between our findings and others may be due to the different stress paradigms employed. However, previous work we have done in a gene expression profiling Illumina study (Integragen, Evry, France and Northwestern Chicago University, USA and Ingenuity Systems, Redwood city, CA), using hippocampi obtained in a similar chronic stress model (Strekalova et al., 2011), suggests diminished expression of NR1 receptor subunit mRNA in stressed mice in particular, and speaks for systemic differences in the hippocampal glutamatergic receptors expression in these mice in general. In addition to NR1 expression results, these microarray data pointed to statistically significant changes in several elements of the glutamatergic system in the hippocampal formation of chronically stressed mice: NR2B receptor, AMPA receptor, glutamate metabotropic receptor 5 and in NR2A/NR2B ratio, which changes are overall in line with the outcome from mRNA evaluation in the current study. Remarkably, a segregation of stress-susceptible and stress-resilient individuals in the microarray study revealed differential expression of the above genes between the sub-groups that additionally supports functional importance of molecular changes in response to stress, obtained both in the microarray and mRNA experiments

In summary, this study demonstrates that elevated hippocampal expression in stressed mice of mRNAs for NR2A and of the NR2A/NR2B ratio, but not for NR2B and NR1, is associated with concomitant abnormalities in both cognitive and emotional elements that mimic one of the most characteristic consequences of experiencing chronic stress in humans. These data suggest that a paradigm comprising ethological stressors evokes behavioral disinhibition and molecular changes that likely mimic epidemiologically

spread human syndromes associated with stress-related emotional and cognitive deficits. Thus, the stress paradigm employed in the present study can be useful for translational studies in the search for pharmacological compensation of these combined symptoms of stress-related pathologies.

Declaration of interest

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