Different mechanisms mediate hippocampal-dependent memory impairments after social defeat stress at the short and long term

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Abstract
Cognitive functions, such as learning and memory are greatly affected by stress, but the synaptic changes causing these deficiencies are largely unknown. Therefore, we investigated hippocampus-dependent memory function and concomitant expression of synaptic glutamate receptors over time after social defeat stress in rats. Acutely, 24 hours after cessation of social defeat exposure (5 daily social defeat encounters), plasma corticosterone levels were increased 2.5 fold, and performance in a hippocampus-dependent object place recognition task was significantly reduced. Moreover, this impaired spatial memory was associated with decreased synaptic expression of glutamate receptor subunits GluN1, GluN2B and GluA2 in the dorsal hippocampus. In addition, we found that glutamate receptor levels were unaltered 3 months after social defeat, whereas spatial memory performance remained impaired. Therefore, we conclude that different synaptic molecular mechanisms underlie memory impairment at the short and long term after social defeat stress.
Introduction

Cognitive functions such as learning and memory are greatly affected by stress\textsuperscript{128}. In humans, acute stress disrupts declarative memory retrieval for previously learned information\textsuperscript{42,129}. Also, in stress-related psychiatric disorders, such as depression, decreased attention and spatial memory have been found\textsuperscript{130}. In rodents, acute and chronic stress also disrupts spatial memory formation and retrieval\textsuperscript{40,41,131}.

The hippocampus is a medial temporal lobe structure implicated in the consolidation of declarative memory in humans and spatial memory in rodents\textsuperscript{132}. Increased corticosterone after stress has been shown to alter hippocampal synaptic plasticity, and to affect spatial memory performance\textsuperscript{133}. Stress and glucocorticoids have a profound influence on the two most well characterized forms of synaptic plasticity of the hippocampal CA1 region, i.e., long-term potentiation (LTP) and long-term depression (LTD), the proposed cellular substrates for learning and memory\textsuperscript{134}.

Stress hormones have been extensively studied and were shown to have facilitating or impairing effects on hippocampal physiology and memory\textsuperscript{37}. Recent evidence shows that, on the short term, rapid, non-genomic effects of corticosterone are mediated via high-affinity mineralocorticoid receptors (MRs), which act to enhance alpha-amino-3-hydroxy-5-methyl-4 isoazolepropionic acid receptor (AMPAR) mobility\textsuperscript{135} and miniature evoked post-synaptic current (mEPSC) frequency\textsuperscript{136}, and facilitate synaptic potentiation\textsuperscript{137}. However, long-lasting effects (>1 hour) are mediated via genomic glucocorticoid receptors (GRs), which impair N-methyl-D-aspartate receptor (NMDAR)-mediated LTP\textsuperscript{43} and facilitate LTD\textsuperscript{43,133}. This hippocampal LTD has been shown to be necessary and sufficient to cause acute stress-induced impairment of spatial memory retrieval and is dependent on GluN2B-containing NMDARs\textsuperscript{133}. Similarly, acutely (~24 h) after chronic stress (>20 days exposure) spatial recognition memory has been repeatedly demonstrated to be impaired\textsuperscript{128}.

Thus, the degree through which stress alters spatial memory is dependent on many factors among which the severity and duration of the stressor, and the time between stress experience and behavioral assessment\textsuperscript{128,138}. A persistent finding is that the time frame for chronic stress to influence performance is critical; for instance, a wire mesh restraint over a relatively long period (21 days) impaired spatial recognition on the Y-maze, but this restraint over a shorter time frame (5 – 10 days) was ineffective\textsuperscript{139}.

Here, we used a social defeat paradigm to assess short- and long term effects of a natural type of stressor. Social defeat stress was inflicted over a short 5-day period, and tested for its effects immediately thereafter or after a period of 3 months; socially defeated rats were individually housed from the first day of defeat onward. The repeated social defeat
stress as used here has previously been shown to reduce hippocampal LTP, and to increase LTD on the long-term (up to 9 months)\textsuperscript{140}. In this study, we investigated both the direct and long-term effect of social defeat on glutamate receptor synaptic membrane expression in conjunction with the associated spatial memory deficits.

**Materials and methods**

**Animals**

Male Wistar rats (Harlan, Horst, The Netherlands) 8 weeks of age and weighing 180–200 g at the time of arrival were initially socially housed (2 per cage) in Makrolon type IV cages (Tecniplast, Milan, Italy). Long-Evans male rats (Harlan, UK), weighing 300–350 g were used as residents for social defeat\textsuperscript{141}. These animals were pair-housed with age-matched sterilized females in plastic cages (63 x 25 x 33 cm) located in a separate room. All animals were housed in a temperature-controlled room (21±1 °C) under regulated lighting conditions (lights on at 7:00 p.m. and off at 7:00 a.m.). Food and water were available *ad libitum*. All experimental manipulations were conducted during the dark phase (activity period) under a dim red light. All experiments were approved by the Animal Users Care Committee of the VU University Amsterdam.

**Experimental design and social defeat procedure**

Wistar rats (age ≥ 11 weeks) of the social defeat group were subjected to 5 days of social defeat stress and were then housed individually in macrolon class III cages from the first defeat onwards, as described before\textsuperscript{140,142}. Control rats were housed in pairs. The social defeat procedure consisted of daily resident-intruder interaction sessions using dominant male Long-Evans rats. Control animals were daily handled. All behavioral, electrophysiological and molecular analyses were performed either 24 h (acute) or 3 months (long-term) after the last social defeat session.

**Corticosterone assay**

Corticosterone levels were measured as described previously\textsuperscript{142}. In short, trunk blood samples were collected via decapitation between 9:00 am and 11:00 am. Samples were collected into a 7-mL heparin-coated tube (Greiner Bio-One, Monroe, North Carolina) and kept on ice. The samples were spun at 1000x g for 10 min. Plasma was decanted and stored at −80 °C until the assay was used. Levels of plasma corticosterone were assessed using a rat Glucocorticoid (GC) ELISA kit (Cusabio Biotech Co., LTD), according to the manufacturers instructions.
Recognition tests
Recognition memory testing was conducted based on two previously reported tasks relying on spontaneous exploration of objects in an open field\textsuperscript{143,144}. All testing was carried out in a rectangular arena, 79 x 57 surface area with 42 cm tall black walls. The box was surrounded by visual cues: computer light coming from the west side; a white wall (north); a metal rack (east side); and an open space where the experimenter was sitting (south). The arena was always placed inside the room at the same location and in the same orientation. All rats were habituated to the empty arena twice for 30 minutes on the two days preceding behavioral testing, as well as on the test day for 10 min and 1 min prior to the sample phase.

Object-recognition task – Round or square aluminum bars were used as novel or familiar objects and were chosen using a pseudorandom protocol, balanced across treatment groups. In the 4-minute sample phase, rats were exposed to two identical objects (round or square metal bars) followed by a 15 min inter-trial interval (Supplemental Fig. 1). In the test trial, one object (chosen using a pseudorandom protocol, balanced across treatment groups) was replaced with a novel object and rats were allowed to explore both objects for 4 minutes (Supplemental Fig. 1). The familiar object was a third copy of the two objects seen in the sample phase, to prevent possible carry-over of olfactory cues. Each session was recorded by a video camera suspended above the field and interfaced with a computerized tracking system using the ‘Viewer’ software package (BIOBSERVE, GmbH, Bonn, Germany). The nose of the rats was tracked by the ‘Viewer’ software and during both trials, the object exploration (defined as the time of the nose spent within 2 cm from the object) was measured for each object. Only rats that had accumulated at least 15 s of exploration at each object within the sample phase were included in the analysis (all but one tested met criteria). To further analyze object discrimination during the test trial, the discrimination index ((novel-familiar)/(novel + familiar)) was calculated for each rat using the individual object-exploration times recorded.

Object-place task – For the 4-minute sample phase, rats always entered the testing box from the south, and were placed facing the south wall. Rats were exposed to two identical objects (round aluminum bars) followed by a 15 min inter-trial interval. During test trials, rats entered the box from the east or west side and were placed facing the east or west wall, respectively. Thus, for each trial, the entry point on the sample and test phases were different. In the test phase a third and fourth copy of the same object were placed back in the arena, one in a familiar corner and one in a novel corner. The location of the novel object-place configuration was counterbalanced such that on each trial different corners were used as familiar and novel locations. Rats were now only able to discriminate between
objects based on their location with respect to its surrounding. Further analysis of discrimination was the same as for the object recognition task.

Tissue preparation
Following decapitation, brains were removed and rapidly frozen in ice-cold isopentane and stored at −80 °C until further use. The dorsal hippocampus (Bregma: −2.56 till −5.0) was removed freehand at −20 °C from 1-mm-thick slices. Synaptic membrane fractions were isolated for every hippocampus (left and right pooled). Samples were homogenized in ice-cold 0.32 M sucrose (5% of homogenate was collected as total cell lysate) and then centrifuged at 1000 g for 10 min. The supernatant was loaded on top of a sucrose gradient consisting of 0.8 and 1.2 M sucrose. After centrifugation at 100,000 g for 2 h, the synaptosome fraction at the interface of 0.85/1.2 M sucrose was collected and then lysed in hypotonic solution. The resulting synaptic membrane fraction was recovered by centrifugation using the sucrose step gradient as stated above. The synaptic membrane fraction was collected from the 0.85/1.2 M interface and protein concentrations were determined using a Bradford assay (Bio-Rad, Hercules, CA, USA).

Immunoblotting
Synaptic membranes of the dorsal hippocampus were isolated from two independent groups of animals (n=6 each) per time point. Samples (3 – 5 µg) were lysed in Laemli lysis buffer, separated by electophoresis on a Criterion 10.5 – 14% Tris-HCl sodium dodecyl sulfate-polyacrylamide precast gel and blotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories) as described before. All antibodies were checked for specificity, i.e. whether they showed a band at the designated height on Western blot. The following antibodies were used: mouse anti- PSD95, (NeuromAB; 1:5000), mouse anti-GluN1 (BD Biosciences; 1:1000), rabbit anti-GluN2A (Abcam, 1:1000), mouse anti-GluN2B (NeuroMab, 1:1000), rabbit anti-GluA1 (GenScript, 1:1000), mouse anti-GluA2 (NeuroMab, 1:500).

Statistics
Determination of corticosterone levels (n=8 acute; n=6 long-term), behavioral assessments (n=12), and molecular analysis (n=6 in both experiments) were performed in independent groups of animals for each of the time points addressed. Physiological parameters were assessed in all experimental animals (n=32 acute, n=24 long-term). Significant differences between stress and control groups was determined by unpaired, two-tailed, Student’s T-tests.
Results

Effect of repeated social defeat on physiological parameters
Social defeat stress resulted in a 2.5 fold increase in plasma corticosterone levels 24 hours after the last social defeat exposure (Fig. 1A, $P=0.0023$). These levels were back to normal at 3 months after social defeat, and there was no difference between treatment groups. The acute effect of social defeat was also evident by a decrease in body weight (Fig. 1B, $P<0.001$), and a decrease in food- (Fig. 1C, $P<0.001$) and water consumption (Fig. 1D)
All of these physiological parameters were back to normal at 3 months after social defeat. Previously described age-related differences in corticosterone levels were observed ($P<0.001$)\(^{46}\), as well as age-related increases in body weight (Fig. 1A,B).

**Object-place memory is reduced short and long-term after social defeat stress**

To test whether repeated social defeat stress affected hippocampus-dependent and/or hippocampus-independent memory, object place recognition and novel object recognition tests were performed, respectively (Langston and Wood 2009). For both tests, during the sample trials, two similar objects were explored equally for the different treatment groups. In the object-place recognition test, control rats showed place recognition by having a preference for the object placed in the novel location. This spatial memory was significantly reduced acutely after social defeat stress (Fig. 2A, $P=0.038$). Interestingly, this hippocampus-dependent cognitive deficit was still present 3 months after social defeat ($P=0.0034$). At this stage, depressive-like phenotypes in the affective domain were also present in this paradigm\(^{33}\), indicative of a multi-layered phenotype. In contrast, in the object recognition test, both the acute and the long-term social defeat groups did not differ from their controls in the test-phase (Fig. 2B), suggesting that this form of hippocampus-independent memory was still intact. Interestingly, hippocampus-dependent processing in the object-place task seemed prone to ageing, although this effect was not significant ($P=0.11$).

**Changes in synaptic expression of glutamate receptor subunits in the dorsal hippocampus short after social defeat stress**

Reduced spatial memory performance is caused by altered synaptic plasticity at excitatory neurons of the dorsal hippocampus\(^{43}\). Therefore, it is of interest to investigate whether glutamate receptors, mediating hippocampal synaptic plasticity, are involved in the observed social defeat stress-induced impairment in spatial memory performance. We compared the expression of ionotropic glutamate receptors in dorsal hippocampal synaptic membrane fractions using quantitative immunoblot analysis (Supplemental Fig. 1). This revealed significantly lower levels of two NMDAR subunits, namely GluN1 ($P<0.001$) and GluN2B ($P=0.048$), acutely after social defeat stress (Fig. 3A). Synaptic expression of GluN2A was unaffected. Also, synaptic expression of the AMPAR receptor subunit GluA2 was lower (Fig. 3B, $P=0.035$), while GluA1 was unaffected. The change in expression of these subunits in synaptic membrane fractions was validated in an independent set of rats. The expression of the synaptic scaffold protein PSD-95 did not change after social defeat (Fig. 3B).
We then measured the expression of synaptic glutamate receptor subunits 3 months after social defeat stress. Despite the reported change in LTP at this moment\textsuperscript{[40]}, we did not find any change in levels of the glutamate receptor subunits, nor in the level of PSD-95 (Fig. 3).

**Discussion**

Here, we show that repeated social defeat stress for 5 consecutive days resulted in a hypothalamic-pituitary-adrenal (HPA) axis response, expressed by an increase in plasma levels of the stress hormone corticosterone 24 hours after cessation of the stressor. This 5-day social defeat exposure also reduced performance in a hippocampus dependent spatial memory task, and this was associated with reduced synaptic expression of the glutamatergic receptor subunits GluN1, GluN2B and GluA2. However, long-term after social defeat the...
spatial memory deficit remained in the absence of elevated corticosterone levels and changed glutamate receptor expression.

Synaptic expression of GluN1, GluN2B and GluA2 is reduced acutely after social defeat stress, and associated with the deficit in spatial memory performance. Since GluA2 endocytosis underlies NMDAR-induced LTD\textsuperscript{124,147}, it is likely that the reduction in synaptic GluA2 expression acutely after repeated social defeat stress induces hippocampal CA1 LTD. This in turn might contribute to the observed memory impairments. The regulation of GluN1 and GluN2B levels acutely after social defeat stress is less straightforward to explain, especially since the number and subunit composition of synaptic NMDARs have long been considered to be quite static\textsuperscript{148}. However, recent evidence shows that neuronal activity drives NMDAR synaptic targeting and incorporation, receptor retrieval, and lateral diffusion between synaptic and extrasynaptic sites\textsuperscript{149}. An emerging concept is that activity-dependent, bidirectional regulation of NMDAR trafficking provides a dynamic and potentially powerful mechanism for the regulation of synaptic efficacy and remodeling. Indeed, it has been shown that alterations in NMDAR number and/or subunit composition contribute to the expression mechanisms of LTP (NMDAR-LTP)\textsuperscript{150} and LTD (NMDAR-LTD)\textsuperscript{151}. Low frequency stimulation and mGluR group I agonists induce NMDAR-LTD at CA1 synapses in hippocampal slices and the internalization of NMDARs in hippocampal neurons\textsuperscript{152}. This might explain the reduced synaptic expression of GluN1 and GluN2B directly after social defeat stress. Alternatively, synaptic over-activation, accelerating the turnover rate of a subset of receptor subunits, such as GluN1 and GluN2B, may change the relative abundance of these subunits and decrease their levels in highly active synapses\textsuperscript{153}.

Stress-induced alterations in NMDAR-mediated synaptic strength are of great interest in that they involve potentiation of the plasticity trigger itself and, as such, would be expected to critically influence metaplasticity. This supports the current view\textsuperscript{37} of stress-induced plasticity in which on the short term stress-induced hormones facilitate strengthening of contacts involved in the formation of memories directly associated to the stressor. As such a mild stressor enables avoidance learning of stressful associations. However, high levels of corticosterone and severe chronic stress suppress hippocampal LTP and promote LTD, with a delay of at least an hour. This would lead to an increased threshold for synaptic strengthening of input from other sources, thereby dampening the aversive learning process. Here, we show that repeated social defeat stress decreased expression of specific NMDA and AMPA receptor subunits. This might potentially underlie the process of metaplasticity and affect hippocampus-dependent learning.
We found that differences in levels of glutamate receptors were absent months after social defeat. However, at this time point hippocampus-dependent spatial memory function was still impaired. Also, previously it was shown that at this time point hippocampal LTP was affected. Thus, despite the changes in hippocampal physiology and hippocampus-dependent task performance, the plasticity of ionotropic glutamate receptors expression at the synaptic membrane is not affected. There are several possible explanations for this outcome. For example, glutamate receptors, or their interacting proteins, may have undergone persistent posttranslational modifications that were not studied here. In particular, more in depth molecular analysis of the hippocampal synaptic proteome of rats 3 months
after social defeat may shed light on this. Alternatively, these effects could be caused by changes in hippocampal circuitry, such as altered neuronal morphology or reduced neurogenesis\textsuperscript{18,36,142}). Taken together, these results show that different synaptic mechanisms are involved in deficits in hippocampal plasticity and spatial memory performance long and short after social defeat stress.