Stress and memory in health and disease

*Impact on Alzheimer's disease and memory mechanisms*

Lesuis, S.L.

**Publication date**
2019

**Document Version**
Other version

**License**
Other

**Citation for published version (APA):**

**General rights**
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

**Disclaimer/Complaints regulations**
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Early life stress impairs fear conditioning memory and synaptic plasticity; a potential role for GluN2B

Sylvie L. Lesuis, Paul J. Lucassen, Harm J. Krugers

Submitted
Abstract

Programming of the brain by early stress has been associated with alterations in structure and function of the hippocampus. Yet, the underlying molecular mechanisms remain largely elusive. In this study, we examined the effects of early life stress (ELS) – by housing mouse dams with limited nesting and bedding material from postnatal days 2-9 and examined in 6 month old offspring; 1) auditory fear conditioning, 2) expression of the hippocampal N-methyl-D-aspartate receptor (NMDA-R) subunits 2A and 2B (GluN2A, GluN2B), and expression of PSD-95 and synaptophysin, and 3) short- and long-term (LTP) synaptic plasticity. Given its critical role in NMDA receptor function and synaptic plasticity, we further examined the role of GluN2B in effects of ELS on synaptic plasticity and fear memory formation. We demonstrate that ELS impaired fear memory in 6 month old mice and decreased hippocampal LTP as well as the paired-pulse ratio (PPR). ELS also reduced hippocampal GluN2B expression. Interestingly, pharmacological blockade of GluN2B with the selective antagonist Ro25 6981 was less effective to reduce synaptic plasticity in ELS mice, and was also ineffective to impair memory retrieval in ELS mice. These studies suggest that ELS reduces hippocampal synaptic plasticity and fear memory formation and hampers GluN2B receptor function. As such, GluN2B may provide an important target for future strategies to prevent lasting ELS effects on cognition.

Keywords: memory, LTP, early life stress, NMDA, fear conditioning, HPA axis, paired-pulse ratio
1. Introduction

The “Developmental Origins of Health and Disease” (DOHaD) hypothesis postulates that perinatal environmental factors play an important role in determining the risk to develop pathology later in life\(^1\). Indeed, adversity early in life, such as experiencing emotional neglect, physical abuse or traumatic events, increases the risk for developing psychopathologies such as anxiety disorders and depression\(^2-5\) and cognitive dysfunction in later life\(^6-8\). However, the exact mechanisms that underlie the consequences of early life adversity for later brain function remain poorly understood.

Increasing evidence suggests that changes in hippocampal structure, neuronal networks and their functions may contribute to the early life stress-induced cognitive deficits. The hippocampus may be particularly sensitive since this area continues to develop into the postnatal period\(^9,10\). Indeed, human studies have shown that early life adversity is associated with a reduced hippocampal volume in adults\(^11-17\). In agreement, animal studies have confirmed that early life stress induces long-lasting structural and functional alterations in hippocampal neurons, such as a decreased hippocampal dendritic complexity and reduced synaptic density\(^18-24\). In addition, rat pups that received low amounts of maternal care early in life showed decreased dendritic complexity as well as lower expression of synaptic markers in the hippocampal CA1 and dentate gyrus at adulthood\(^20,25,26\). Also the number of hippocampal spines was found to be reduced in pups that received low compared to high amounts of maternal care\(^20,25\), and in mice that were exposed to chronic early life stress\(^27\).

Synaptic plasticity, which is an important substrate for memory formation\(^28-33\) is altered following reduced levels in maternal care and exposure to early life adversity\(^20,22,34\). Understanding how early life stress impacts synaptic plasticity could help to explain effects of early life stress on later-life cognitive impairments. As the composition of N-methyl-D-aspartate receptors (NMDARs) is crucial for the induction of LTP, and their properties may be altered by early life experiences\(^25,35,36\), NMDAR modifications could potentially be a link between effects of early life stress and cognitive impairment later in life. In this study we therefore examined whether early life stress (ELS) – induced by housing mice with limited nesting and bedding material from postnatal days 2-9\(^37-40\) – affects memory and long- as well as hippocampal short-term plasticity later in life. In addition, we tested whether GluN2B is involved in effects of ELS, given its role in synaptic plasticity, and learning and memory processes.
2. Materials and Methods

2.1. Animals

All mice were kept under standard housing conditions (a 12/12h light schedule (lights on at 8 a.m.), temperature 20-22 °C, 40-60% humidity, standard chow and water were available ad libitum, and a radio provided background noise\textsuperscript{37,39,41}). Experimental procedures were conducted under the national Dutch law and European Union directives on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam. To obtain experimental mice, two 10-week-old C57Bl/6J virgin females and one male mouse (Harlan Laboratories B.V., Venray, The Netherlands) were housed together for one week to allow mating. After another week of paired-housing, pregnant females were housed individually in a standard cage covered with a filter top and monitored daily for the birth of pups. When a litter was born before 10.00 a.m., the previous day was considered as the day of birth (postnatal day 0; PND 0), after which the early life paradigm was initiated at PND 2. At PND 21, mice were weaned and were housed with 2-5 same sex littermates per cage. All experimental mice were left undisturbed (except for cage cleaning once a week) until start of experimental procedures at 6 months.

2.2. Early life stress

At PND 2, litters were culled to 6 pups per litter, and dams and their litters were weighed and randomly assigned to the early life stress (ELS) or control condition, as described before\textsuperscript{37–40}. Control dams were provided with a standard amount of sawdust bedding and nesting material (one square piece of cotton nesting material (5 x 5 cm; Tecnilab-BMI, Someren, the Netherlands)). The ELS dams were provided with a strongly reduced amount of sawdust bedding and half the nesting material (1/2 square piece of cotton nesting material (2.5 x 5 cm)), and a fine-gauge stainless steel mesh was placed 1 cm above the cage floor. Both control and ELS cages were left undisturbed until PND 9, after which all litters were weighed and placed in standard cages, with standard amounts of bedding and nesting material until weaning at PND 21.

2.3. Behavioural testing

In the present study we used male mice. At 5 months of age, i.e. one month prior to behavioural testing, mice were moved to a reversed day-night rhythm (lights on at 8.00 p.m.). One week prior to testing, mice were moved into the
testing room, where they were single housed and handled daily. Tests were conducted by an experimenter blind to the condition of the animals, in the dark, active phase of the animals between 1 and 5 p.m. in a testing room lit by two red spots (EGB, 25 Watt). During testing, mice were recorded by a camera connected to a computer with Ethovision software (version 13, Noldus, The Netherlands) and automatically scored by the software.

2.3.1. Fear conditioning
Mice were tested in a contextual and auditory fear-conditioning paradigm, a classic paradigm to assess fear memory. On day 1, mice were placed in a chamber which had a stainless steel grid floor connected to a shock generator\(^2\). The chamber was cleaned with 1% acidic acid to create a recognisable odour trace and remove previous odours. Mice were allowed to explore the context for three minutes, after which a 30 seconds tone (76 dB, 2.8 kHz) was used. During the last 2 seconds of the tone, the mice received a single mild foot shock (0.4 mA). After this shock, the mice remained in the chamber for another 30 seconds. Twenty-four hours later, mice were reintroduced into the shock context for 3 minutes. One hour later, mice were placed in a novel context (round Plexiglas chamber cleaned with 20% EtOH), and after 3 minutes they were exposed to 30 second tone for 6 times, with 60 second intervals. Freezing behaviour of the animals was scored during every trial by an observer who was unaware of the experimental condition. Freezing was being defined as “no body movements except those related to breathing”\(^3\) and was expressed as % of total observation time.

2.3.2. Forced swim test
Seven days after fear conditioning, a forced swim test (FST) was conducted to elicit a stress response. Between 8 and 9 p.m. (start of the light phase) mice were placed in a cylinder with 1500 ml water from which they could not escape. After 6 minutes, mice were removed from the water and dried before being placed back in their home cage. Although the FST was merely used as a stressor, floating time and latency to float as percentage of total time in the cylinder were scored. However, no behavioural differences were observed, and these data are therefore not further presented/discussed.

2.4. Stress response
Blood samples were obtained by tail cut at 30 (“response CORT”) and 90 minutes (“recovery CORT”) after exposure to the FST. Blood was collected in EDTA coated tubes and analysed as described below (“Plasma corticosterone measurements”).

---


2.5. Sacrifice and organ collection

Mice were sacrificed one week after the last behavioural test by quick decapitation at the beginning of the light phase (8 p.m.). Blood samples were collected for basal CORT measurements, brains were dissected and the hippocampus was isolated from the right hemispheres and kept at -80 °C. Upon sacrifice, adrenal glands were removed and cleaned by removing fat tissue and weighted. Weights were presented as a percentage of body weight.

2.6. Plasma corticosterone measurements

Blood samples were collected in ice cold, EDTA-coated tubes (Sarstedt, Etten-leur, The Netherlands), placed on ice and centrifuged at 14,000 rpm for 15 minutes after which plasma was stored at -20 °C. Plasma CORT levels were measured using a commercially available radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands).

2.7. Western blot

To compare hippocampal protein levels between the groups, 6 month old mice were decapitated and hippocampi were dissected in saline on ice. Tissue was stored at -80 °C. For protein extraction, hippocampi were homogenised in RIPA buffer (150 mM NaCl, 1% Triton X100, 0.5% Sodium deoxycholate, 0.1% SDS at pH 7.6) using a small syringe. The samples were incubated on ice for 30 minutes and then centrifuged for 20 minutes at 16,000 rpm at 4 °C. Protein lysate was stored at -20 °C. For each sample the protein concentration was measured using a BCA Protein Assay (23225, Pierce (Thermo Fischer) The Netherlands). Samples containing between 10-30 μg protein in sample buffer were denaturised at 95 °C for 5 minutes. A polyacrylamide-SDS gel (Biorad, The Netherlands) was used for protein separation by electrophoresis. The proteins were transferred to a PVDF membrane (162-0177, Biorad, The Netherlands) in a Tris-glycine buffer. The membranes were cut for incubation with different antibodies. The membrane strips were then blocked in TBST containing 5% BSA for 1 hour. After blocking, blots were washed with TBST and incubated with primary antibodies at 4 °C overnight. Primary antibodies included MR (1:500, mouse; 1-18-1D5 Gomez-Sanchez), GR (1:500, rabbit, H-300, Santa Cruz), GluN2A (1:1000, mouse, MAB5216, EMD Millipore), GluN2B (1:1000, mouse, MAB5220, EMD Millipore), PSD-95 (1:3000, rabbit, D27E11, Cell Signalling), synaptophysin (1:3000, mouse, SY38, Abcam), α-tubulin (1:1000, mouse, 10D8, Santa Cruz) and GAPDH (1:3000, rabbit; 2118S, Cell Signaling). After washing with TBS, blots were incubated with secondary antibodies for 2 hours at room
temperature (HRP conjugate, Biorad, The Netherlands). Blots were washed again and bands were visualised by chemiluminescence using an ECL Prime kit (RPN2232, Amersham, (GE Healthcare) The Netherlands). A Li-COR machine was used to measure the chemiluminescence. Optical density was determined in ImageJ. Measurements of the proteins of interest were corrected for total protein (GAPDH or α-tubulin band). Protein levels were calculated as the mean of three independent replicates.

2.8. Synaptic plasticity

2.8.1. Long-Term Potentiation (LTP)
Mice were sacrificed between 9 and 10 a.m. through quick decapitation for slice preparation. Immediately after decapitation, the brain was rapidly removed, and collected in ice-cold oxygenated (95% O₂/5% CO₂) solution containing (in mM): Cholinechloride (120), glucose (10), NaHCO₃ (25), MgSO₄ (6), KCl (3.5), NaH₂PO₄ (1.25), CaCl₂ (0.5). 350 µm thick coronal slices were cut using a microtome (Leica VT1000S). For recovery, slices were then incubated for 20 minutes in warm (32 °C) oxygenated standard artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (120), KCl (3.5), MgSO₄ (1.3), NaH₂PO₄ (1.25), CaCl₂ (2.5), glucose (10), NaHCO₃ (25), after which the sections were maintained at room temperature. Sections containing the hippocampal CA1 area (bregma -2.0 mm to -3.2 mm) were placed in a recording chamber with a constant flow of oxygenated aCSF. Field excitatory postsynaptic potentials (fEPSPs) were recorded as described previously in the absence and presence of the GluN2B-NMDAR subunit antagonist Ro25 6981 (3 µM, Sigma) to assess a possible selective role of this particular subunit in these changes.

Field excitatory potentials (fEPSPs) were evoked using a stainless steel bipolar stimulation electrode (60 µm diameter, insulated except for the tip) positioned on the Schaffer collaterals and recorded through a glass electrode (2-5 MΩ impedance, filled with aCSF) positioned in the CA1 stratum radiatum. A stimulus-response curve was generated by gradually increasing the stimulus intensity to define a level that generated the half-maximal response that was used for the remainder of the experiment. Once the input-output curve for each recording was established, baseline synaptic transmission was monitored (0.03 Hz) during 10 minutes. When recordings were stable, a high frequency stimulation (10 Hz, 90 seconds) was applied. After the tetanus, the degree of potentiation was determined by recording the fEPSP every 30 seconds during 1 hour (0.03 Hz). The fEPSP magnitude was assessed by analysing the slope of the signal. The average baseline value was normalised to 100% and all values of the experiment were normalised to this baseline average.
2.8.2. Paired-pulse ratio
Paired-pulse facilitation was measured by determining the ratio of the evoked response to two subsequent stimuli at half maximal stimulus intensity. The second stimulus was compared to the first one, with varying inter-stimulus intervals between 20 and 500 ms (two trials at each interval) (adapted from47).

2.9. Drug treatment
The GluN2B antagonist Ro25 6981 maleate (Bioconnect, The Netherlands) was dissolved in 0.9% saline and injected i.p., 30 minutes prior to context retrieval, at a dose of 0 mg/kg, 3 mg/kg or 10 mg/kg (injection volume: 0.5 µl/gram body weight).

2.10. Statistical analysis
Data were analysed using SPSS 22.0 (IBM software). All data are expressed as mean ± standard error of the mean (SEM). Data were considered statistically significant when p<0.05. Outliers were determined using a Grubb’s test. Independent-samples t-tests were performed to compare between control and ELS groups. Appropriate corrections were applied when assumption of homogeneity of variance was not met. When the assumption of normality was not met, a Mann-Whitney test was conducted. A repeated measure ANOVA was performed to assess freezing behaviour during the different tones. Greenhouse-Geisser correction was applied when the assumption of sphericity was violated. To determine the effects of ELS on the degree of LTP, a repeated-measures ANOVA was performed using condition (control vs. ELS) as between-subject factor and slope of the pre- and post-stimulation fEPSP as the within-subject factor. We compared the baseline (-10 to 0 minutes) with total LTP (0 – 60 minutes after 10 Hz stimulation) and late LTP (50 – 60 minutes after 10 Hz stimulation). When significant, a post hoc Tukey test was performed to compare groups.

Table 1. Effects of ELS on body weight.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>ELS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain PND 2-9 (g)</td>
<td>3.32 ± 0.17 (12)</td>
<td>2.78 ± 0.15 (11)*</td>
</tr>
<tr>
<td>Body weight PND 21 (g)</td>
<td>8.54 ± 0.27 (12)</td>
<td>8.45 ± 0.17 (11)</td>
</tr>
<tr>
<td>Body weight 6 months (g)</td>
<td>29.4 ± 0.89 (12)</td>
<td>28.9 ± 0.55 (11)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M (n). * p<0.05.
3. Results

3.1. Early life stress paradigm

Housing litters in a cage with limited nesting and bedding material from PND 2-9 reduced body weight gain in the male early life stress (ELS) offspring when compared to control litters (t(21)=2.4, p=0.030) (Table 1). These findings are consistent with previous reports of the model\textsuperscript{37–39}, validating the effective application of the early stress paradigm. From PND 21 onwards, differences in body weight were absent between the experimental groups (PND 21: t(21)=0.27, p=0.784; 6 months: t(21)=0.47, p=0.64).

3.2. Fear conditioning

To assess emotional fear memory, mice were trained in an auditory fear-conditioning paradigm. Freezing behaviour was comparable between the

Figure 1. Fear memory following ELS at adulthood. A. No difference in freezing levels was present immediately after the foot shock. B. When placed back in the training context, ELS mice freeze less than Ctrl mice. C. In response to the tone, Ctrl mice freeze more than ELS mice. D. Also the average freezing percentage over the tones in lower in ELS mice. *: significant difference.
groups prior to the tone-foot shock combination (data not shown) and during the 30 seconds after the shock was received \( (t(7)=1.81, p=0.11) \) (Figure 1A). When mice were placed back in the training context 24 hours later, ELS mice froze significantly less than Ctrl mice \( (t(18)=2.33, p=0.03) \) (Figure 1B). When introduced in a novel, “safe” context one hour later, mice showed comparable low levels of freezing (“habituation”) (Figure 1C). In response to the tone, ELS animals overall froze less than Ctrl mice \( (F(1,18)=8.59, p=0.01) \). Post hoc tests revealed that this was significant on tone 3, 4, and 6 (Figure 1C), and also the average freezing levels comparing all tones was lower in ELS mice relative to controls \( (t(19)=2.3, p=0.03) \) (Figure 1D).

### 3.3. HPA axis signalling

To assess HPA axis responsiveness, corticosterone (CORT) levels were determined under basal conditions, and at 30 (“response levels”) and 90 minutes (“recovery levels”) after an acute stressor, i.e. after exposure to a forced swim test (Figure 2A). ELS did not affect basal CORT levels, nor the response or recovery of CORT levels following the stressor \( (F(1,20)=0.47, p=0.50) \). However, the relative weight of the adrenal glands, which produce CORT, was significantly increased following ELS \( (t(7)=3.42, p=0.01) \) (Figure 2B). Whereas hippocampal GR expression was not affected \( (t(11)=1.49, p=0.17) \) (Figure 2C; see Supplementary Figure 1 for raw images of Western blots), MR levels were significantly reduced after ELS \( (t(6)=8.25, p<0.001) \) (Figure 2D).

### 3.4. Synaptic proteins

Hippocampal GluN2A expression was not significantly affected by ELS \( (t(8)=1.56, p=0.15) \), but GluN2B expression was lower after ELS \( (t(16)=4.4, p=0.0004) \) (Figure 2E,F). No differences in PSD-95 and synaptophysin were found (PSD-95: \( t(6)=0.03, p=0.9 \); synaptophysin: \( t(6)=1.2, p=0.27 \)) (Figure 2G,H).

### 3.5. LTP and paired pulse facilitation

We first assessed the effect of ELS on baseline properties of the CA1 fEPSPs, based on input-output curves fitted with a Boltzmann equation (Table 2). ELS had no effect on the maximal slope of the fEPSP \( (t(68)=0.87, p=0.39) \), nor on the half-maximum stimulus intensity \( (t(68)=0.95, p=0.35) \) or the slope of the input-output curve \( (t(68)=0; p=1.0) \). Using a 10 Hz, 90 seconds stimulation paradigm, LTP was found to be significantly reduced in ELS mice \( (F(1,22)=6.68, p=0.017) \) (Figure 3A). Also during the last 10 minutes of recordings, when the signal had
Figure 2. HPA axis activity after ELS measured at 6 months of age. A. HPA axis activity is not affected by ELS under basal condition, and 30 and 90 minutes after a stressor. B. Adrenal gland weight relative to total body weight increased following ELS. C. Hippocampal GR levels were comparable between Ctrl and ELS mice. D. MR expression was reduced following ELS exposure. E. GluN2A expression was not affected by ELS, but GluN2B was lower in ELS mice (F). G, H. ELS did not affect the levels of PSD-95 (G) and synaptophysin (H). *: significant t-test.
Chapter 7

Table 2. Basal field potential characteristics for hippocampal CA1 area.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>ELS</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_{\text{max}} ) (mV/ms)</td>
<td>-0.31 ± 0.05</td>
<td>-0.26 ± 0.02</td>
</tr>
<tr>
<td>( I_h ) (µA)</td>
<td>2.24 ± 0.06</td>
<td>2.32 ± 0.06</td>
</tr>
<tr>
<td>( S )</td>
<td>0.16 ± 0.03</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>N (mice (slices))</td>
<td>9 (38)</td>
<td>6 (32)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M (n). Abbreviations used: maximal slope of the fEPSP \( (R_{\text{max}}) \), half-maximum stimulus intensity \( (I_h) \), and the slope of the input-output curve (slope factor \( S \)) in the CA1 area.

stabilised, ELS resulted in a reduction of LTP \((t(22)=2.32, p=0.03)\) (Figure 3B). While we were able to induce significant LTP in control animals of 19.9% (mean slope in the interval 50-60 minutes post stimulation compared to baseline (100%): Ctrl: \(t(12)=3.70, p=0.003\)), LTP could not be elicited to the same extent in ELS mice (4.6%, compared to baseline (100%): ELS: \(t(10)=1.38, p=0.20\)) (Figure 3A,B).

Figure 3. Synaptic plasticity in the CA1 of the hippocampus. A. After a 10 Hz, 90 sec stimulation, LTP was significantly decreased following ELS, and B. also the average potentiation during the last 10 minutes was decreased. C. The paired-pulse ratio was lower in ELS mice than in Ctrl mice, with significant post hoc tests in the intervals from 50-200 ms. D. After 10 minutes of Ro25 6981 application, the 10 Hz 90 sec stimulation did not induce synaptic potentiation in either Ctrl or ELS mice, E. which was also reflected by the similar fEPSP during the last 10 minutes of recording. F. The relative change in the slope fEPSP following Ro25 6981 application was significantly bigger in Ctrl mice when compared to ELS mice.
In addition, we examined the paired-pulse ratio (PPR), a measure for short-term presynaptic plasticity, at different intervals ranging from 20 to 500 ms. Over the entire range between 20 to 500 ms, ELS mice displayed a significantly lower PPR than control animals (F(1,15)=5.44, p=0.03), and post hoc analysis showed that at all intervals until 100 ms ELS mice displayed a significantly lower PPR than control animals (20 ms: p=0.007; 40 ms: p=0.01; 60 ms: p=0.02; 80 ms: p=0.03; 100 ms: p=0.02; 150-500 ms: p>0.05) (Figure 3C).

3.6. GluN2B and synaptic plasticity

We next applied the GluN2B antagonist Ro25 6981 to the slices prior to the stimulation, in order to assess a potential role for GluN2B in effects of ELS on synaptic potentiation. Ro25 6981 had no effect on the properties of the slope fEPSP measured during baseline recording (F(1,22)=0.014, p=0.907) (Figure 3D). Following the 10 Hz, 90 seconds stimulation, both experimental groups showed no synaptic potentiation (F(1,22)=0.11, p=0.74) (Figure 3E). The relative difference in synaptic potentiation with and without Ro25 6981 was calculated by measuring the slope fEPSP (as % of baseline) with Ro25 6981 minus the slope without Ro25 6981 during the last 10 minutes of recording. This difference was significantly smaller in ELS mice compared to Ctrl mice (t(22)=2.42, p=0.02) (Figure 3F).

To extend our findings in vivo, we treated mice prior to the retrieval in a fear conditioning paradigm with 3 mg/kg or 10 mg/kg Ro25 6981 (or vehicle). There was a significant interaction effect between condition and treatment on freezing behaviour to the context (F(2,27)=6.59, p=0.005) (Figure 4A). Post hoc
Chapter 7

analysis revealed that in Ctrl mice, 3 mg/kg and 10 mg/kg reduced freezing levels to the context, whereas no effects of Ro25 6981 treatment were observed in ELS mice (Figure 4A). In response to the cue, Ro25 6981 also resulted in an interaction effect (F(2,27)=4.57, p=0.02), whereby 10 mg/kg Ro25 6981 treatment resulted in reduced freezing in Ctrl, but not ELS mice (Figure 4B).

4. Discussion

In this study we investigated the effects of early life stress (ELS), induced by exposing dams and pups to limited nesting and bedding material from PND 2-9, on fear conditioning, hippocampal protein expression and hippocampal synaptic plasticity at 6 months of age. We demonstrate that fear memory was significantly impaired following ELS exposure. In parallel, both long term potentiation (LTP) and paired-pulse ratio were decreased in ELS animals. The expression of the GluN2B subunit, known to be important for LTP, was reduced after ELS. Interestingly, the application of the selective GluN2B antagonist Ro25 6981 revealed that ELS mice were also functionally less sensitive to modification of GluN2B function.

4.1. Effects of chronic early life stress

Previous studies have reported that exposing the dams and pups to limited nesting and bedding materials results in increased exits of the dam from the nest (i.e. fragmented maternal care) and a reduction in body weight gain of the pups between PND 2-938–41. In line with this, we report a similar reduction in body weight, confirming the effective application of stress in this model. At later ages, body weight was comparable between ELS and control mice as also reported by these studies. Previous studies have suggested that early life stress results in increased HPA axis reactivity and impaired negative feedback in response to stress48–51. As a consequence, life-long, cumulative glucocorticoid exposure may be increased, providing a mechanism through which ELS-associated cognitive impairments could be mediated. Interestingly, in the current study, no differences were present in basal CORT levels, nor in the CORT response or in its recovery from stress-exposure. The absence of effects on corticosterone levels occurred despite the significant increase in adrenal gland weight following early life stress, which suggests the adrenal glands have been activated before for a prolonged period of time. Possibly, differences in CORT release at an earlier age may underlie this, or there are differences in CORT levels present at other time points than we currently investigated (e.g. differences in circadian rhythm that are revealed during the dark phase).
These possibilities may be supported by the decrease in hippocampal MR levels following ELS, which is in line with reports from other models of early life adversity (e.g.\textsuperscript{20,52}). In contrast to other studies using the presently applied model\textsuperscript{52,53} or other paradigms of early life adversity\textsuperscript{51,54,55}, we did not observe differences in hippocampal GR level expression at 6 months of age. This may point to strain or model-specific effects of early life stress on GR levels, but is consistent with our findings that also the recovery of the CORT levels in the same animals was comparable.

ELS reduced fear expression in a mild auditory fear-conditioning paradigm. In response to the context, Ctrl mice displayed higher levels of freezing than ELS mice, suggesting a reduced memory for the context in ELS mice. In addition, ELS mice also froze less in response to the tone in a neutral context. This was not observed following the first two tones, which may be caused by a ceiling effect, as freezing levels were high overall already. The finding that auditory fear conditioning was affected by ELS suggests that, in addition to the hippocampus, also the amygdala may be affected by ELS\textsuperscript{56}. Earlier studies have reported that ELS enhances freezing responses between the tones (i.e. in a potentially safe context) while the response to the tone itself was unaffected\textsuperscript{37}. Possibly, the fact that the animals were exposed to a fearful context just prior to exposure to the tones in our present study. This may have resulted in enhanced corticosterone levels already before tone exposure and could potentially affect subsequent freezing responses to the tone, which needs confirmation. The presently found effects are most likely not related to basolateral amygdala structure or basal synaptic transmission which was unaffected after ELS\textsuperscript{57,58} although effects of ELS on synaptic plasticity in the amygdala requires investigation. The presently applied 10 Hz stimulation paradigm induced LTP in the hippocampal CA1 area of control animals, while such synaptic potentiation was absent following ELS. Between PND 2-9, when ELS was applied, both glutamatergic and GABA-ergic transmission are still developing\textsuperscript{59–63}, and shape network activity. Exposure to early life adversity during this period may therefore lead to long lasting changes in synaptic plasticity and LTP\textsuperscript{19,35,64} that could underlie the deficits in learning and memory observed following ELS. For instance, chronic early life stress, introduced using a different stimulation paradigm, induced LTP deficits in the hippocampal CA3\textsuperscript{21,27} and CA1 subregion\textsuperscript{19, 21} and offspring that received low amounts of maternal care did not show potentiation following a stimulation in the dorsal dentate gyrus\textsuperscript{25,65} or hippocampal CA1\textsuperscript{20,66}. Furthermore, maternal separation also impaired LTP in the prefrontal cortex\textsuperscript{67}, while maternal deprivation impaired LTP in the dentate gyrus\textsuperscript{22} and CA1\textsuperscript{64}. Together, these findings indicate that the early postnatal period is highly sensitive to disruptions like early stress that can have long-lasting consequences for the ability to induce LTP in adulthood.
We found that not only LTP was affected, but also that short-term synaptic plasticity as measured by the paired pulse ratio (PPR) was affected by ELS. Several studies suggest that short-term plasticity, such as the PPR, plays an integral role in cognitive processing and memory. Although the effects of early life stress on the PPR have received relatively little attention, an impaired PPR in the CA1 of the hippocampus has been reported following exposure to an acute stressor, following exposure to inescapable foot shocks, or in vivo after CORT application for minutes or hours. Our data suggest a strong reduction in the PPR following ELS. PPR is believed to depend on presynaptic mechanisms, such as an increased probability of neurotransmitter release and presynaptic Ca\textsuperscript{2+} mobilisation. Changes in these processes, possibly induced by ELS, may thus underlie the observed impairment in PPR. Although still debated, changes in presynaptic neurotransmitter release have been suggested to contribute to LTP induction and could thus possibly also contribute to the current ELS-induced impairment in LTP.

4.2. Role of the GluN2B

At present we found that ELS did not alter the expression of the synaptic proteins PSD-95 or synaptophysin in hippocampal homogenates. This is in line with data showing that ELS also failed to alter spine density in CA1, although a slight reduction was found in the CA3 subregion. In contrast, we found that the expression of the GluN2B subunit was decreased following ELS, while GluN2A expression was unaffected. NMDA receptors are heteromeric assemblies consisting of an GluN1 subunit and various GluN2 subunits, of which GluN2A and GluN2B are the major components in the hippocampus. During development, there is a switch in NMDAR composition. GluN2B is predominantly present in the early postnatal brain (notably coinciding with the time at which we applied ELS), and GluN2A increases during postnatal development. This process can be disturbed by early life stress, as maternal deprivation prevented the switch to a mature, GluN2A dominated NMDAR phenotype at PND 28-31. In addition, adult offspring from mothers who gave low levels of maternal care also showed higher GluN2B levels as well as higher GluN2A levels. Interestingly, by 8 weeks of age, the effects of maternal prenatal stress on GluN2B were found to be opposite, and hippocampal GluN2B-NMDAR subunit expression was reported to be reduced. This is in line with our current observation that chronic early life stress reduced GluN2B expression in 6 month old mice. The functional consequences of these GluN2B reductions are emphasised by the effects of blocking this subunit prior to LTP induction. Application of Ro25 6981, a selective GluN2B antagonist reduced LTP in Ctrl mice, but was relatively less effective in ELS mice. To further validate a role for
the GluN2B subunit after ELS, we examined whether Ro25 6981 differentially affected fear conditioning in ELS mice. Ctrl mice showed a dose-dependent impairment in memory retrieval following Ro25 6981 exposure. Interestingly, ELS mice were less sensitive to either an intermediate or high dose of Ro25 6981, and maintained the ability to retrieve the fear memory comparably to vehicle treated mice. This is, to the best of our knowledge, the first time that it has been demonstrated that ELS exposure results in reduced contribution of the GluN2B subunit to synaptic plasticity as well as memory formation. These findings thus support the hypothesis that the function of GluN2B is affected in adult mice that were previously exposed to ELS.

5. Conclusion

We report that ELS from PND 2-9 leads to persistent changes in fear conditioning and impairs short- and long-term synaptic plasticity at 6 months of age. This is associated with reduced hippocampal GluN2B expression and GluN2B function after ELS. A better understanding of the lasting consequences of early life adversity on behavioural and neurobiological parameters is crucial for understanding the sensitivity to develop psychopathology later in life. The present studies point to a potentially relevant role of GluN2B.

6. Acknowledgements

This study was supported by a grant from Internationale Stichting Alzheimer Onderzoek (ISAO)/Alzheimer Nederland (grant: #12534 to HJK)
7. References


22. Oomen CA, Soeters H, Audureau N, Vermunt L, van Hasselt FN, Manders EMM et al. Severe early life stress hampers spatial learning and


44 Pu Z, Krugers HJ, Joëls M. Corticosterone time-dependently modulates beta-adrenergic effects on long-term potentiation in the
47 Cazakoff BN, Howland JG. Acute stress disrupts paired pulse facilitation and long-term potentiation in rat dorsal hippocampus through activation of glucocorticoid receptors. Hippocampus 2010; 20, 1327–1331.
GluN2B and early life stress


78 Citri A, Malenka RC. Synaptic plasticity: multiple forms, functions and mechanisms. *Neuropsychopharmacology* 2008; **33**:18–41.


80 Ahmed MS, Siegelbaum SA. Recruitment of N-type Ca2+ channels during LTP enhances low release efficacy of hippocampal CA1 perforant path synapses. *Neuron* 2009; **63**:372–385.


8. Supplementary figures

Supplementary Figure 1. Typical examples of Western blots. Each lane represents a different animal. C: control mice; E: ELS mice; GR: glucocorticoid receptor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MR: mineralocorticoid receptor; NR2A: NMDA receptor subunit A; NR2B: NMDA receptor subunit B; PSD-95: post-synaptic density 95; a-tub: alpha-tubulin.

*Figure continues on next page*
Supplementary Figure 1 (continued).