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Lesuis, S.L.; Lucassen, P.J.; Krugers, H.J.

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Early life stress impairs fear memory and synaptic plasticity; a potential role for GluN2B

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

Brain Plasticity Group, Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, Science Park 904, 1098 XH, Amsterdam, the Netherlands

HIGHLIGHTS

• ELS impaired fear memory, and decreased hippocampal LTP and paired-pulse ratio.
• ELS also reduced hippocampal GluN2B expression.
• The GluN2B antagonist Ro25 6981 was less effective to reduce synaptic plasticity in ELS mice.
• Ro25 6981 was also ineffective to impair memory retrieval in ELS mice.
• GluN2B may thus provide an important target for future strategies to prevent lasting ELS effects on cognition.

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Keywords: Memory LTP Early life stress NMDA Fear conditioning HPA axis Paired-pulse ratio

ABSTRACT

Programming of the brain by early life stress has been associated with alterations in structure and function of the dorsal 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.

1. Introduction

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 (Heim and Nemeroff, 2001; McEwen and Magarinos, 2001; Mills et al., 2013; Norman et al., 2012), and cognitive dysfunction later in life (Amerman et al., 1986; Kaplan et al., 2001; Meaney et al., 1988). 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 early life stress-induced cognitive deficits. The hippocampus may be particularly sensitive since this area continues to develop into the postnatal period (Eckenhoff and Rakic, 1984; Pleasure et al., 2000). Indeed, human studies have shown that early life adversity is associated with a reduced hippocampal volume in adults (Andersen and Teicher, 2008; Bremner et al., 1997; Frodl et al., 2010; Gatt et al., 2009; Gross et al., 2012; Teicher et al., 2012; Vythilingam et al., 2002). In agreement, animal studies have confirmed that early life stress (ELS) induces long-lasting structural and functional alterations in hippocampal neurons, such as a decreased hippocampal dendritic complexity and reduced synaptic
density (Brunson et al., 2005; Champagne et al., 2008; Huot et al., 2002; Ivy et al., 2010; Leventopoulos et al., 2007; Oomen et al., 2011, 2010). 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 (Bago et al., 2009; Champagne et al., 2008; Liu et al., 2000). Also the number of hippocampal spines was found to be reduced in pups that received low compared to high amounts of maternal care (Bago et al., 2009; Champagne et al., 2008), and in mice that were exposed to chronic early life stress (Wang et al., 2011).

Synaptic plasticity, which is an important substrate for memory formation (Kessels and Malinow, 2009; Malinow and Malenka, 2002; Mitsushima et al., 2011; Nabavi et al., 2014; Rumpel et al., 2005; Whitlock et al., 2006), is altered following reduced levels of maternal care or exposure to early life adversity (Champagne et al., 2008; Fenoglio et al., 2005; Oomen et al., 2010). Understanding how ELS 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 (Bago et al., 2009; Rodenas-Ruano et al., 2012; Son et al., 2006), NMDAR modifications could potentially be a link between effects of ELS and cognitive impairment later in life. In this study we therefore examined whether ELS – induced by housing mice with limited nesting and bedding material from postnatal days 2–9 (Arp et al., 2016; Lesuis et al., 2016; Naninck et al., 2015; Rice et al., 2008) – 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 (Arp et al., 2016; Lesuis et al., 2018; Naninck et al., 2015)). 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 (Arp et al., 2016; Lesuis et al., 2016; Naninck et al., 2015; Rice et al., 2008). Control dams were provided with a standard amount of sawdust bedding and nesting material (one square piece of cotton nesting material (5 × 5 cm; Teknilab-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 × 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 (Zhou et al., 2010). The chamber was cleaned with 1% acetic acid to create a recognisable odour trace and remove previous odours. Mice were allowed to explore the context for 3 min, after which a 30 s tone (76 dB, 2.8 kHz) was used. During the last 2 s 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 s. Twenty-four hours later, mice were reintroduced into the shock context for 3 min. One hour later, mice were placed in a novel context (round Plexiglas chamber cleaned with 20% EtOH), and after 3 min they were exposed to 30 s tone for 6 times, with 60 s 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” (Zhou et al., 2009) and was expressed as % of total observation time.

2.3.2. Forced swim test (FST)

In order to elicit a stress response, a forced swim test (FST) was conducted seven days after fear conditioning. The FST consisted of placing the mouse in a cylinder with 1500 ml water (20 °C) from which the animal could not escape. The FST was conducted between 8 and 9 p.m. (start of the light phase), when basal CORT levels are low and the effects of a stressor on circulating CORT levels will be most pronounced. After 6 min, 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 min (“recovery CORT”) after exposure to the FST. Blood was collected in EDTA coated tubes and analysed as described below (“2.6. 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 hemisphere. To obtain the dorsal hippocampus, it was cut at 2/5th of its length from the anterior side and was then kept at ~80 °C until use. 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 min after which plasma was stored at −20 °C. Plasma CORT levels were measured using a commercially available radioimmunooassay 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 min and then centrifuged for 20 min 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 and 30 μg protein in sample buffer were denaturised at 95 °C for 5 min. A polyclonal antibody against GAPDH (1:5000, 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, MAB2280, EMD Millipore), PSD-95 (1:500, 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 Signalling). After washing with TBS, blots were incubated with secondary antibodies for 2 h at room temperature (1:3000, rabbit; 2118S, Cell Signalling). The membranes were cut for incubation with different antibodies. The membranes were then blocked in TBST containing 5% BSA for 1 h. 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, MAB2280, 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 Signalling). After washing with TBS, blots were incubated with secondary antibodies for 2 h at room temperature (HRP conjugate, Biorad, The Netherlands). Blots were washed again and bands were visualised by chemiluminescence using an ECL Prime kit (RP22232, 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

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 min in warm (32 °C) oxygenated standard artificial cerebral spinal 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 dorsal hippocampal CA1 area (bregma −2.0 mm to −3.2 mm) were placed in a recording chamber with a constant flow of oxygenated aCSF.

2.8.1. Long-term potentiation (LTP)

Field excitatory postsynaptic potentials (fEPSPs) were recorded as described previously (Bagot et al., 2009; Pu et al., 2007) 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. Slices were transferred to a recording chamber, where they were submerged in, and continuously perfused with, aCSF at room temperature.

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. An input-output 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, and fEPSP magnitude was assessed by analysing the slope of the signal at 30–70% of the slope, after which it was fitted with a Boltzmann equation. Once the input-output curve for each recording was established, baseline synaptic transmission was monitored (0.03 Hz) during 10 min. When recordings were stable, a 10 Hz, 90 s stimulation was applied (Mayford et al., 1996; Wiegent et al., 2006). After the stimulus, the degree of potentiation was determined by recording the fEPSP every 30 s during 1 h (0.03 Hz). 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 ratio was measured as 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 from Cazakoff and Howland, 2010).

2.9. Drug treatment

The GluN2B antagonist Ro25 6981 maleate (Bioconnect, The Netherlands) was dissolved in 0.9% saline and injected i.p., 30 min prior to context retrieval, at a dose of 0 mg/kg, 3 mg/kg or 10 mg/kg (injection volume: 0.5 μl/g 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 Grubbs’s test. However, no data points were removed based on this criterion. 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 min) to the slope of the pre- and post-stimulation fEPSP (50–60 min after 10 Hz stimulation) and late LTP (50–60 min after 10 Hz stimulation). When significant, a post-hoc Tukey test was performed to compare groups.

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 (Arp et al., 2016; Lesuis et al., 2016; Naninck et al., 2015), validating the effective application of the early life stress paradigm.
3.4. Synaptic proteins

GluN2A expression in the dorsal hippocampus 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) (Fig. 2E and 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) (Fig. 2G and H).

3.5. LTP and paired pulse facilitation

We first assessed the effect of ELS on baseline properties of the dorsal 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) = 0.55, p = 0.58), or 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 s stimulation paradigm, LTP was found to be significantly reduced in ELS mice (F (1,22) = 6.68, p = 0.017) (Fig. 3A). Also during the last 10 min of recordings, when the signal had stabilised, ELS resulted in a reduction of LTP (t(22) = 2.32, p = 0.03) (Fig. 3B). While we were able to induce significant LTP in control animals of 19.9% (mean slope in the interval 50–60 min 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) (Fig. 3A and B).

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 and 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) (Fig. 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) (Fig. 3D). Following the 10 Hz, 90 s stimulation paradigm, LTP was found to be significantly reduced in ELS mice (F (1,22) = 6.68, p = 0.017) (Fig. 3A). Also during the last 10 min of recordings, when the signal had stabilised, ELS resulted in a reduction of LTP (t(22) = 2.32, p = 0.03) (Fig. 3B). While we were able to induce significant LTP in control animals of 19.9% (mean slope in the interval 50–60 min 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) (Fig. 3A and B).

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 and 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) (Fig. 3C).

3.7. GluN2B and fear memory

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) (Fig. 4A). In line with our findings in Fig. 1, post-hoc analysis showed that ELS mice display lower freezing levels to the context. However, 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 (Fig. 4A). In response to the cue, Ro25 6981 also resulted in an interaction effect (F(2,27) = 4.57, p = 0.02), whereby untreated ELS mice again displayed lower freezing levels than

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Table 1

<table>
<thead>
<tr>
<th>Effect</th>
<th>Ctrl</th>
<th>ELS</th>
</tr>
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<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>
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Data are expressed as mean ± S.E.M. *p < 0.05.
Ctrl mice, while 10 mg/kg Ro 25 6981 treatment resulted in reduced freezing in Ctrl, but not ELS mice (Fig. 4B).

### 4. Discussion

In this study we investigated the effects of early life stress (ELS), that were 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 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 min 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). (H) ELS did not affect the levels of PSD-95 (G) and synaptophysin. N_{Ctrl} = 12; N_{ELS} = 10. *: significant t-test.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>ELS</th>
</tr>
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<tbody>
<tr>
<td>R_{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_{max}), half-maximum stimulus intensity (I_{h}), and the slope of the input-output curve (slope factor S) in the CA1 area.
A. Ctrl  
B. ELS  

Fig. 3. Synaptic plasticity in the CA1 of the hippocampus. (A) Typical example of a fEPSP at baseline (black), and 50 min after stimulation (grey). After a 10 Hz, 90 s stimulation, LTP was significantly decreased following ELS. (B) Also the average potentiation during the last 10 min was decreased. (C) Overlay of the paired-pulse ratio at 150 ms, with the first pulse in black, and the second pulse in grey. The paired-pulse ratio was lower in ELS mice than in Ctrl mice, with significant post-hoc tests in the intervals from 50 to 200 ms. (D) Typical example of a fEPSP at baseline (black), and 50 min after stimulation (grey). After 10 min of Ro25 6981 application, the 10Hz 90 s stimulation did not induce synaptic potentiation in either Ctrl or ELS mice. (E) This was also reflected by the similar fEPSP during the last 10 min 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.

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 2–9 (Lesuis et al., 2018, 2016; Naninck et al., 2015; Rice et al., 2008). In line with this, we body weight gain of the pups between PND 2–9 (Lesuis et al., 2018, 2016; Naninck et al., 2015; Rice et al., 2008). In line with this, we found no specific effects of early life stress on GR levels, but is consistent with our findings that also the recovery of the CORT levels was comparable between Ctrl and ELS mice.

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 overall high already. It is unlikely that these effects can be attributed to the enhanced extinction in ELS mice, as previous studies have shown that ELS did not enhance extinction, neither by re-exposure to the tone on day 3 (Arp et al., 2016), nor to context (Kanatsou et al., 2017). 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 (Phillips and LeDoux, 1992). 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 (Arp et al., 2016). Possibly, the fact that in our present study, the animals were exposed to a fearful context just prior to exposure to the tones may have resulted in enhanced corticosterone levels already before tone exposure, which could potentially have affected subsequent freezing responses to the tone. Yet, this option needs further confirmation. The current effects are most likely not related to basolateral amygdala structure or basal synaptic transmission, which was unaffected after maternal deprivation and ELS (Kruegers et al., 2012; Pillai et al., 2018), although effects of ELS on synaptic plasticity in the amygdala could be involved, which requires further study.

The presently applied 10 Hz stimulation paradigm induced LTP in the hippocampal CA1 area of control animals, while such synaptic rhythm that are revealed during the dark phase). These possibilities are supported by the decrease in hippocampal MR levels following ELS, which is consistent with other models of early life adversity (e.g. (Bath et al., 2016; Champagne et al., 2008)). Synaptic plasticity is known to be facilitated under predominant MR activation (Pavlides et al., 1996), while MRs are also involved in the rapid increase in mEPSCs frequency after elevated corticosterone levels (Karst et al., 2005). Thus, reductions in MR expression may contribute to reduced synaptic potentiation, as presently observed.

In contrast to other studies using the presently applied model (Avishai-Eliner et al., 2001; Bath et al., 2016) or other paradigms of early life adversity (Ladd et al., 2004; Vázquez et al., 1996; Weaver et al., 2004), 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 was comparable between Ctrl and ELS mice.

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 overall high already. It is unlikely that these effects can be attributed to the enhanced extinction in ELS mice, as previous studies have shown that ELS did not enhance extinction, neither by re-exposure to the tone on day 3 (Arp et al., 2016), nor to context (Kanatsou et al., 2017). 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 (Phillips and LeDoux, 1992). 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 (Arp et al., 2016). Possibly, the fact that in our present study, the animals were exposed to a fearful context just prior to exposure to the tones may have resulted in enhanced corticosterone levels already before tone exposure, which could potentially have affected subsequent freezing responses to the tone. Yet, this option needs further confirmation. The current effects are most likely not related to basolateral amygdala structure or basal synaptic transmission, which was unaffected after maternal deprivation and ELS (Kruegers et al., 2012; Pillai et al., 2018), although effects of ELS on synaptic plasticity in the amygdala could be involved, which requires further study.

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 (Cellot and Cherubini, 2013; Cherubini et al., 1991; Durand et al., 1996; Hsia et al., 1998; Liao and Malinow, 1996), and shape network activity. Exposure to early life adversity during this period may therefore lead to long-lasting changes in synaptic plasticity and LTP (Brunson et al., 2005; Derks et al., 2016; Rodenas-Ruano et al., 2012) that could underlie the deficits in learning and memory observed following ELS. For instance, chronic early life stress, introduced using a different paradigm, induced LTP deficits in the hippocampal CA3 (Ivy et al., 2010; Wang et al., 2011) and CA1 subregion (Brunson et al., 2005; Ivy et al., 2010) and offspring that received low amounts of maternal care did not show potentiation following a stimulation in the dorsal dentate gyrus (Bagot et al., 2009, 2012) or hippocampal CA1 (Champagne et al., 2008; Nguyen et al., 2015). Furthermore, maternal separation also impaired LTP in the prefrontal cortex (Choczyk et al., 2013), while maternal deprivation impaired LTP in the dentate gyrus (Oomen et al., 2010) and CA1 (Derks et al., 2016). Together, these findings indicate that the early postnatal period is highly sensitive to disruptions like early life stress that can have long-lasting consequences for the ability to induce LTP in adulthood.

We found that not only was LTP affected. Short-term synaptic plasticity, as measured by the paired pulse ratio (PPR), was also affected by ELS. Several studies suggest that short-term plasticity, such as the PPR, plays an integral role in cognitive processing and memory (Cao and Leung, 1991; Dobrunz and Stevens, 1999; Kushner et al., 2005; Silva et al., 1996). Although the effects of early life stress on the PPR have received relatively little attention, impaired PPR in the CA1 of the hippocampus has been reported following exposure to an acute stressor (Cazakoff and Howland, 2010), following exposure to inescapable foot shocks (Gao et al., 2008), or evivo after CORT application for minutes (Karst et al., 2005) or hours (Zhou et al., 2000). 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 Ca2+ mobilisation (Gitri and Malenka, 2008; Debsanne et al., 1996; Hess and Kuhn, 1992; Jiang et al., 2000; Zucker and Regehr, 2002). 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 (Ahmed and Siegelbaum, 2009; Enoki et al., 2009) and could thus possibly contribute to the current ELS-induced impairment in LTP.

4.2. Role of 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 (Wang et al., 2011). 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 (Monyer et al., 1994). 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), while GluN2A increases during postnatal development (Quinlan et al., 1999; Sheng et al., 1994; Williams et al., 1993). This process has been shown to be disturbed by early life stress, as maternal deprivation prevented the switch to a mature, GluN2A dominated NMDAR phenotype at PND 28–31 (Rodenas-Ruano et al., 2012). In addition, adult offspring from mothers who gave low levels of maternal care also showed higher GluN2B levels as well as higher GluN2A levels (Bagot et al., 2012). 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 (Son et al., 2006). 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 consequences of blocking this subunit prior to LTP induction. Application of Ro25 6981, a selective GluN2B antagonist, reduced LTP in control mice, but was relatively less effective in ELS mice. Electrophysiological recordings have shown that GluN2A and GluN2B containing NMDARs have different opening probabilities (GluN2B has a 3–5 times lower opening probability; Wylie et al., 2013) and different activation and deactivation rates (Shipton and Paulsen, 2014), thereby giving rise to different intracellular signalling cascades. With respect to plasticity, both GluN2A- and GluN2B-containing NMDARs mediate calcium influx to support LTP (although LTP is preferentially induced by GluN2A-containing NMDARs (Mourt and Harvey, 2011). However, the currently used relatively mild stimulation paradigm may activate relatively more GluN2B-containing than GluN2A-containing NMDARs compared to high frequency stimulation paradigms (> 100 Hz) (Erreger et al., 2005). This GluN2B activation results in an association with different downstream pathways e.g. through association with CaMKII, and may thus help to maintain CaMKII activation (Shipton and Paulsen, 2014).

Thus, the currently observed reduction in GluN2B may relate to reduced LTP as presently observed, as well as alterations in other secondary pathways.

To further validate a role for the GluN2B subunit after ELS, we examined whether Ro25 6981 differentially affected fear conditioning in ELS mice. Crl 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. This observation suggests a compensatory mechanism through which ELS-exposed mice compensate for the reduction in GluN2B expression.

4.3. 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.

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Appendix A. Supplementary data

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