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Early life stress lastingly alters the function and AMPA-receptor composition of glutamatergic synapses in the hippocampus of male mice

Niek Brosens | Carla Simon | Helmut W. Kessels | Paul J. Lucassen | Harm J. Krugers

Abstract
Early postnatal life is a sensitive period of development that shapes brain structure and function later in life. Exposure to stress during this critical time window can alter brain development and may enhance the susceptibility to psychopathology and neurodegenerative disorders later in life. The developmental effects of early life stress (ELS) on synaptic function are not fully understood, but could provide mechanistic insights into how ELS modifies later brain function and disease risk. We here assessed the effects of ELS on synaptic function and composition in the hippocampus of male mice. Mice were subjected to ELS by housing dams and pups with limited bedding and nesting material from postnatal days (P) 2–9. Synaptic strength was measured in terms of miniature excitatory postsynaptic currents (mEPSCs) in the hippocampal dentate gyrus at three different developmental stages: the early postnatal phase (P9), preadolescence (P21, at weaning) and adulthood at 3 months of age (3MO). Hippocampal synaptosome fractions were isolated from P9 and 3MO tissue and analyzed for protein content to assess postsynaptic composition. Finally, dendritic spine density was assessed in the DG at 3MO. At P9, ELS increased mEPSC frequency and amplitude. In parallel, synaptic composition was altered as PSD-95, GluA3 and GluN2B content were significantly decreased. The increased mEPSC frequency was sustained up to 3MO, at which age, GluA3 content was significantly increased. No differences were found in dendritic spine density. These findings highlight how ELS affects the development of hippocampal synapses, which could provide valuable insight into mechanisms how ELS alters brain function later in life.

KEYWORDS
development, early life stress, glutamate, hippocampus, synapses

1 | INTRODUCTION

The early life period is a particularly sensitive time window of experience-induced plasticity, modifying and programing synaptic development and homeostasis, dendritic remodeling and axon growth, ultimately contributing to the refinement of network functions. While this period of rapid change shapes later cognitive and emotional development, it also renders the developing brain vulnerable to adverse experiences. Accordingly, exposure to stress during early life (ELS) can disrupt neural development and increase the susceptibility to develop psychopathology and neurodegenerative disorders, and accelerate cognitive impairments and cognitive aging. Despite the
wide range of ELS-induced effects reported by animal studies, the exact mechanisms as to how ELS exerts its enduring effects remain unclear.

A commonly used animal model to study effects of ELS is the limited bedding and nesting model (LBN), in which newborn mice are subjected to unpredictable and fragmented maternal care.⁵¹⁻¹⁴ LBN impairs learning and memory in adulthood, which is paralleled by impaired synaptic function in the hippocampus.¹⁵⁻²³ Thus, LBN lastingly impacts synaptic function in the hippocampus, which possibly contributes to the cognitive impairments. Specifically, the dentate gyrus (DG) of the hippocampus is of interest, as this subregion represents the start of the trisynaptic hippocampal circuit and predominantly develops during the first postnatal weeks. So far, the DG has been reported to be lastingly affected by a variety of models for ELS, but the effects of LBN on synaptic function in early development and adulthood are not fully understood. ELS is suggested to accelerate synaptic development by altering NMDA receptor (NMDAR) composition²⁶,³⁰ and enhancing α-aminoo-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (AMPAR) function early in life, thereby prematurely "unsilencing" otherwise silent synapses. While this is well-characterized in the CA1 area of the hippocampus using the LBN model, the developmental effects of LBN on synaptic function in the DG remain to be investigated.

We therefore set out to assess the effects of ELS at different developmental time points on synaptic function in the DG and glutamate receptor composition in the hippocampus of male mice. First, we assessed synaptic strength in the DG of male mice that were exposed to ELS at the early postnatal phase (postnatal day (P) 9), preadolescence (P21, at weaning) and in early adulthood (i.e., at 3 months of age). Second, we assessed whether the composition of synapses was altered using hippocampal synaptosomal fractions, between mice at P9 and at 3 months of age. We focused on postsynaptic proteins that are known to play a central role in mediating synaptic strength and plasticity. In the adult brain, excitatory neurons predominantly contain GluA1/2 and GluA2/3 AMPARs, and GluN1/2A and GluN1/2B NMDA receptors (NMDARs).³³ Considering that GluA2 and GluN1 are constant factors in their respective receptors, and that GluA1, GluA3, GluN2A and GluN2B are known to be functionally distinct and play a critical role in synaptic development, synaptosomal protein content was determined of the latter subunits only. Finally, we assessed whether as a result of ELS from P2-9, the dendritic spine density was altered in the DG at 3 months of age. A detailed overview of the progressive ELS effects on developing hippocampal synapses could help provide mechanistic insights in the enduring effects of ELS and elucidate potential therapeutic targets.

2 | MATERIALS AND METHODS

2.1 | Mice and breeding

All animal experiments were conducted under Dutch national law and in compliance with the European Union Directive 2010/63/EU. The study design was evaluated and approved by the animal welfare committee of the University of Amsterdam. To standardize the perinatal environment, all mice were bred in a house at a room temperature of 20–22°C and a humidity of 40%–60% with ad libitum food (standard chow; 801 722 CRM [P], Special Diets Services, Essex, UK) and water. Mice were kept on a 12:12 h light–dark cycle (lights on at 8:00 a.m., lights off at 8:00 p.m.).

As this study was part of a larger overarching project, animal cohorts were established with a transgenic mouse model that has a fluorescent reporter gene under the promoter of the immediate early gene Arc (Arc::dVenus mice, kindly provided by Prof. Dr. Steven Kushner, Erasmus University Rotterdam) and a separate cohort was established for the dendritic spine density analysis, using transgenic Thy1-eYFP mice (B6.Cg-Tg[Thy1-YFP]HJrs/J; Jackson Laboratories, kindly provided by Prof. Dr. Helmut Kessels, University of Amsterdam). Both transgenic mouse lines were backcrossed >6 times into the C57Bl/6J background. For breeding, two 6-week-old C57Bl/6J virgin WT females (Harlan Laboratories B.V., Venray, The Netherlands) and one homozygous Arc::dVenus or heterozygous Thy1-eYFP male of approximately 3 months old were housed together 1 week for mating. Subsequently, females were pair-housed for a week in a clean standard cage with one nestlet (5 × 5 cm cotton nesting material: Tecnilab-BMI, Someren, the Netherlands) and a filter top. Eighteen days after the start of the breeding, pregnant females were inspected daily before 9:00 a.m. for the birth of pups. The previous day was designated as postnatal day 0 (P0) upon observation of a new litter.

2.2 | Early life stress

At P2, the limited bedding and nesting material model was used to induce ELS in the Arc::dVenus and Thy1-eYFP offspring until P9, as described previously.¹³,³⁶,³⁷ Briefly, litters of at least n = 5 mice were included and litters of n > 6 mice were culled to six pups per litter. Litters with only one pup of either sex were not included. Hence, litters contained at least n = 2 male/female pups, counterbalanced by the opposite sex to make a total of n = 5/6 pups per litter. Dams and corresponding litters were weighed and randomly assigned to the ELS or control (Ctrl) condition. Ctrl cages consisted of a standard amount of sawdust bedding material with one nestlet whereas ELS cages consisted of a thin layer of sawdust bedding material covered with a fine-gauge stainless steel mesh 1 cm above the cage floor, and half a nestlet (2.5 × 5 cm). Cages of both conditions were covered with a filtertop and were left undisturbed. Litters were split over the three different ages of interest (Figure 1A).

At P9, the dams and litters were weighed and placed in standard cages with standard amounts of bedding and nesting material. At P21, mice were weaned and ear tissue was collected for identification and genotyping. Littermates were housed with 2–4 mice per cage, single housed males were not used for analysis. All animals were left undisturbed (except for weekly cage cleaning) until the experimental
procedures at 3 months of age. As males appear to be particularly vulnerable to the effects of ELS,13,38–41 all experiments were further conducted with male mice.

2.3 | Tissue preparation

Three separate cohorts were established for either electrophysiological, synaptosomal protein or spine density experiments. For all cohorts, mice were sacrificed at the start of the light phase by quick decapitation at the ages of interest. Tissue for electrophysiological experiments was prepared as described in the section on recording mEPSCs. For synaptosomal protein analysis, hippocampi were isolated and stored at −80°C until processing. For spine density analysis, brains were removed and immersion-fixed in 4% paraformaldehyde in phosphate buffer (0.1 M PB, pH 7.4) for 24 h at 4°C and then stored in 0.01% sodium-azide in 0.1 M PB at 4°C until further processing. Prior to tissue slicing, the fixed hemispheres were cryoprotected in 15% sucrose in 0.1 M PB until the brains sunk, followed by an overnight incubation in 30% sucrose in 0.1 M PB. Frozen tissue was cut in 40 μm thick coronal sections in six parallel series using a sliding microtome. The slices were stored in antifreeze solution (30% ethylene glycol, 20% glycerol, 50% 0.05 M PB and 0.9% saline) at −20°C further analysis. Prior to mounting, the slices were washed in 0.9% saline 0.05 M PB and placed in 0.05 M PB. Subsequently, the slices were mounted on slides and cover slipped using 4',6-diamidino-2-phenylindole (DAPI: Vectashield Mounting Medium with DAPI, H-1200, Vector Laboratories Inc.).

2.4 | Plasma corticosterone measurements

At sacrifice, trunk blood was collected in EDTA coated tubes (Sarstedt, the Netherlands), centrifuged and the plasma was stored at −20°C for measures of corticosterone levels. A commercially available ELISA kit (RES2211, IBL International GmbH) was used to measure plasma corticosterone levels, according to the manufacturer’s instructions.

2.5 | Recording miniature excitatory postsynaptic currents (mEPSCs)

Mice were sacrificed by quick decapitation. The brains were rapidly removed and placed in ice-cold, oxygenated (95% O₂/5% CO₂)
artificial cerebrospinal fluid (ACSF: in mM): 118 NaCl, 2.5 KCl, 26 NaHCO₃, 1 NaH₂PO₄, supplemented with 1 MgCl₂, 2 CaCl₂, 22 glucose. Coronal slices (350 μm) were made in ice-cold slicing ACSF (sACSF) using a vibratome (VT1000S, Leica). The sACSF contained (in mM): 139 CsMeSO₃, 3.5 KCl, 6 MgSO₄, 1.25 NaH₂PO₄, 25 NaHCO₃ and 10 D-glucose, saturated with 95% O₂–5% CO₂. For recovery, slices were incubated for 20 min in warm (32°C) oxygenated ACSF followed by a 1-h incubation in oxygenated ACSF at RT. Individual slices containing the dorsal hippocampal area (Bregma −2.0 mm to −3.2 mm) were transferred into a recording chamber submerged in a constant flow of oxygenated ACSF with 1 μM TTX (Tocris) and 100 μM picrotoxin (Sigma). Whole cell recordings in current clamp were performed using a DIC microscope (Axioskop 2 FS Plus, Zeiss) with a water immersion objective (0.8 W), equipped with a CCD Camera (TVCCD 624, Monacor) and a headstage (CV 203BU, Axon Instruments) assembled to a motorized micromanipulator (Scientific). For all the recordings, borosilicate glass pipettes (1.5 mm outer diameter, Hilgerberg, Malsfeld, Germany) were pulled with a Sutter (USA) micropipette puller to establish a pipette resistance of >1 GΩ. Positive pressure, the electrode was directed towards a granular (adjusted with KOH) and the osmolarity was 290.55 mOsm. Under artificial cerebrospinal fluid (ACSF at RT. Individual slices containing the dorsal hippocampal area (Bregma −2.0 mm to −3.2 mm) were transferred into a recording chamber submerged in a constant flow of oxygenated ACSF with 1 μM TTX (Tocris) and 100 μM picrotoxin (Sigma). Whole cell recordings in current clamp were performed using a DIC microscope (Axioskop 2 FS Plus, Zeiss) with a water immersion objective (0.8 W), equipped with a CCD Camera (TVCCD 624, Monacor) and a headstage (CV 203BU, Axon Instruments) assembled to a motorized micromanipulator (Scientific). For all the recordings, borosilicate glass pipettes (1.5 mm outer diameter, Hilgerberg, Malsfeld, Germany) were pulled with a Sutter (USA) micropipette puller to establish a pipette resistance of >1 GΩ. Positive pressure, the electrode was directed towards a granular (adjusted with KOH) and the osmolarity was 290.55 mOsm. Under positive pressure, the electrode was directed towards a granular cell in the suprapyramidal blade of the dorsal DG. Once a seal was established on the cell membrane (resistance >1 GΩ), the membrane patch was ruptured by gentle suction and kept at a holding potential of −65 mV. Neurons with access resistance <30 MΩ were used for whole cell recordings in voltage clamp. Cells were recorded for 10 min and the recordings were amplified using an Axopatch 200B amplifier and digitized with an Axon Digitida 1550A. Data acquisition was performed in pClamp 10.7 and mEPSCs were analyzed with MiniAnalysis (Synaptosoft). Individual events >5 pA were manually included for analysis.

### 2.6 Synaptosomal isolations

Synaptosomal fractions were isolated as described in. Briefly, hippocampi were homogenized in 5 mL of ice-cold iso-osmolar buffer containing 5 mM HEPES pH 7.4, 0.32 M sucrose and protease inhibitor cocktail EDTA free (0469312001, Roche) using a 7 mL glass dounce homogenizer with 20× strokes of the pestle. The homogenate was centrifuged at 1000 × g for 10 min at 4°C. The supernatant was layered on top of a 0.85/1.2 M sucrose density gradient and ultracentrifuged at 100 000 × g for 2 h at 4°C. Synaptosomes were isolated from the 0.85/1.2 M sucrose interface, mixed with sample volume of 5 mM HEPES and iso-osmolar buffer to reach a final volume of 15 mL. The homogenate was centrifuged at 76 000 × g for 30 min at 4°C. The pellet was resuspended in 500 mM HEPES and stored at −80°C until Western blot analysis.

### 2.7 Western blotting

The protein concentration of the samples was determined using a BCA protein assay (23,225, Pierce (Thermo Fisher), The Netherlands). Samples containing 2–10 μg protein in sample buffer were denaturized at 95°C for 5 min. Proteins were separated using an 8% polyacrylamide-SDS gel and transferred to a PVDF membrane (162–0177, Biorad, The Netherlands) using ProSieve EX transfer buffer (Lonzau Bioscience, Allendale, USA). Blots were washed with 0.1 M TB with 0.9% saline (0.1 M TBS) and 0.05% Tween 20 for 5 min followed by a blocking step with 5% milk in 0.1 M TBS and 0.05% Tween 20 for 2 h. The blots were incubated with primary antibodies overnight at 4°C in 0.1 M TBS with 5% BSA and 0.05% Tween 20. Primary antibodies included PSD95 (1:5000, D27E11, Cell Signaling, 95 kDa), GluA1 (1:1000, 1504, Chemicon, 106 kDa), GluA3 (1:1000, 32–0400, Zymed, 99 kDa), GluN2A (1:1000, 5220, GluN2A, 180 kDa), GluN2B (1:1000, 5216, Chemicon, 175 kDa) and GAPDH (1:1000, 21185, Biok, 37 kDa). After washing with 0.1 M TBS with 5% BSA and 0.05% Tween 20, blots were incubated for 2 h at RT with corresponding antibodies (HRP conjugated, Biorad, The Netherlands). Blots were washed with 0.1 M TBS with 5% BSA and 0.05% Tween 20 and the bands were visualized by chemiluminescence using an ECL Prime kit (RPN2232, GE Healthcare, Amersham, GE, The Netherlands) and a Li-COR machine (Odyssey FC; Leusden, the Netherlands). Optical density was determined in Image-J (J1.53f51). Measurements of the proteins of interest were corrected to the loading control (GAPDH band) and normalized to the control group.

### 2.8 Confocal microscopy and analysis of spine density

Fluorescence from the Thy1-eYFP signal was captured using a Nikon A1 confocal microscope. For analysis of dendritic spine density of granular cells in the DG of the hippocampus, pictures were taken of apical dendrites in the molecular layer from granular cells in the suprapyramidal blade of the DG in the dorsal hippocampus (−1.22 mm to −2.18 mm) with a 60× objective. Pictures consisted of a Z-stack with a range of approximately 5 μm and a step size of 0.25 μm. Images were converted to 8-bit grey scale to visualize dendritic spines. Areas with little background and dendritic overlap were selected for analysis, which was done by a researcher blinded to experimental conditions. Approximately six dendrites, with approximately 20 μm per dendrite, were sampled randomly and analyzed per animal. Spine density was expressed as number of spines per 10 μm, averaged per animal.

### 2.9 Statistical analysis

Data were analyzed using Rstudio (March 1, 2023) and data was plotted using Graphpad Prism 9 and expressed as mean ± standard error of the mean (SEM). Data were considered statistically significant when
p < .05 (two-sided testing). Datapoints that were outside the 1.5 x IQR range and had methodological or biological deviations were deemed as outliers and excluded from analysis. Assumptions for parametric analysis were tested using the Shapiro–Wilk normality test and the Levene’s test for homogeneity of variance. Subsequently, appropriate parametric or nonparametric statistical tests were performed. The general approach was as follows: for condition comparisons (Ctrl × ELS), independent t-tests (or nonparametric Wilcoxon rank-sum tests) were performed and differences in distributions were assessed with a Kolmogorov–Smirnov test. For condition and age comparisons, a two-way analysis of variance (ANOVA) was performed. Some of the data was nested within mice or litter (3 – 5 cells per animal for electrophysiological data and 2 – 3 animals per litter for western blot and spine density data). Since this may contribute to effects of animal or litter for the outcome variable we tested, the contributing factor of the “nested” variable was tested using the nlme package in R

and we corrected for this where applicable using a mixed model analysis with mouse/litter as a random factor.

3 | RESULTS

3.1 | Early life stress decreased bodyweight gain during early development but did not affect plasma corticosterone levels throughout development

Newborn litters were subjected to the well-established ELS paradigm, in which the dam and pups are housed with limited bedding and nesting material from P2–9 (Figure 1A). As shown previously, this model leads to a significant lower gain in bodyweight from P2–9 compared with the control pups that were housed under normal conditions (T[57] = 7.99, p < .001) (Figure 1B). Bodyweight gain normalized hereafter at P21 (T[43] = 0.56, p = .58) (Figure 1C) and at 3 months of age (3MO) (T[18] = 0.48, p = .64) (Figure 1D). To assess whether ELS results in altered stress levels, we measured basal plasma corticosterone levels at P9, P21 and at 3MO. In line with the characterization of the first postnatal days of rodent life as a “stress hypersensitive period”, at P9 plasma corticosterone levels were low and not affected by ELS (Figure 1E). At P21 and 3MO plasma corticosterone levels had normalized and were unaffected by ELS (Figure 1F,G, respectively). These findings indicate that ELS does not have immediate and lasting effects on basal corticosterone levels.

3.2 | Early life stress increased mEPSC frequency and amplitude in the dentate gyrus at P9

To investigate the effects of ELS on synaptic strength of granular cells in the DG of the hippocampus during the early postnatal phase, miniature excitatory postsynaptic currents (mEPSCs) were recorded from excitatory neurons in the suprapyramidal blade of the DG at P9. Overall, a significantly higher mean mEPSC frequency was detected (Z = 3.41, p < .001) (Figure 2B inset) and a significant difference was found in the interevent interval (IEI) cumulative probability (D[80] = 0.24, p = .02) (Figure 2B), as ELS had shifted distribution towards the lower IEI range in accordance with a higher mEPSC frequency.

ELS also increased the mean mEPSC amplitude (Z = 2.91, p = .004) (Figure 2C inset). Plotting the cumulative distribution of mEPSC amplitudes (D[80] = 0.29, p = .049) (Figure 2C) as well as their normalized frequency (D[80] = 0.49, p < .001) (Figure 2D), shows that mEPSCs shift towards a higher range across all amplitudes, indicating both weak and strong synapses increased in synaptic transmission. The mEPSC decay time remained unaffected by ELS at P9 (Figure 2E). These results show that ELS increases synaptic strength, both in terms of mEPSC frequency and amplitude, of DG granular cells at P9.

3.3 | Early life stress increased mEPSC frequency but not amplitude at P21

The effects of ELS on synaptic strength of the granular cells at a pre-adolescent stage was assessed by recording mEPSCs in the DG at P21. A significantly higher mean mEPSC frequency was detected (Z = 2.52, p = .01) (Figure 3B inset) as well as a significant shift towards the lower IEI range for the cumulative probability (D[80] = 0.5, p < .001) (Figure 3B). No significant effects of ELS were found on average mEPSC amplitude (Figure 3C inset), nor on cumulative and normalized distribution of mEPSC amplitudes (Figure 3C,D). Finally, a trend towards an increase in the mEPSC decay time was present as a result of ELS (Z = 1.92, p = .055) (Figure 3E). These results show that ELS increases the synaptic strength in terms of mEPSC frequency only, whereas the mEPSC amplitude remains unaffected at P21.

3.4 | Early life stress increased mEPSC frequency but not amplitude at 3MO

To assess the effects of ELS on synaptic strength of granular cells in adulthood, mEPSCs were recorded in the DG at 3MO. ELS resulted in a significantly higher mean mEPSC frequency (Z = 2.9, p = .004) (Figure 4B inset) but did not affect the cumulative distribution of the IEI (Figure 4B). No effects of ELS on the average mEPSC amplitude, amplitude distribution and decay time were observed (Figure 4C–E). These results show that ELS lastingly affects synaptic function of DG granular cells by increasing mEPSC frequency up to 3 months of age, while average mEPSC amplitude and decay time are normalized at this age.

Finally, the progressive effects of ELS over the three different ages depict a persistent increase of ELS on mEPSC frequency (Fcondition [1128] = 17.97, p < .001) (Figure 4F). In terms of mEPSC amplitude, there was a significant condition effect (Fcondition [1128] = 7.52, p = .007) and a significant age effect (Fage[2128] = 5.53, p = .005) with a slight trend towards an interaction between condition and age (Fcondition×age[2128] = 3.03, p = .052) (Figure 4G). Finally, there was a main age effect for the
mEPSC decay time ($F_{agr} = 18.36, p < .001$) (Figure 4H). In summary, these findings show that ELS leads to a persistent increase in mEPSC frequency from the early postnatal phase into adulthood, while mEPSC amplitude is increased only during the early postnatal phase as a result of ELS.

3.5 Early life stress lastingly altered synaptosome composition in the hippocampus

As described, we established that similarly for the CA1, ELS also causes persistent effects on synaptic strength in the DG. To assess whether ELS lastingly changes the postsynaptic protein composition in the hippocampus, we performed western blots for postsynaptic scaffolding protein PSD-95, AMPAR subunits GluA1 and GluA3 and NMDAR subunits GluN2A and GluN2B in synaptosomal fractions isolated from hippocampi at P9 and 3MO. At P9, ELS resulted in a significantly lower synaptic content of PSD-95 ($Z = -2.61, p = .031$) (Figure 5A), GluA3 ($T[10] = 2.35, p = .049$) (Figure 5C) and GluN2B ($T[10] = 4.45, p = .001$) (Figure 5E). Trends towards a decrease were present for GluA1 ($T[10] = -2.16, p = .06$) (Figure 5B) and GluN2A ($T[10] = 1.94, p = .08$) (Figure 5D). For the 3MO timepoint, a significant increase in the synaptic content of GluA3 was present ($T[10] = -2.59, p = .04$) (Figure 5H) but no effects were present for PSD-95, GluA1, GluN2A and GluN2B (Figure 5F–J). These findings show that ELS drastically alters the protein content of synaptosomal fractions from the hippocampus at P9, as postsynaptic glutamatergic proteins decreased. ELS lastingly affects the composition of synaptosomal fractions as GluA3 content is significantly increased.
3.6 Early life stress does not affect dendritic spine density in the DG at 3 months of age

Up until P9, synapses onto hippocampal neurons are mainly present at the dendritic shaft, and only in the third and fourth week after birth spine numbers increase exponentially. We next examined whether ELS lastingly affects spine synapses by analyzing spine density of DG granular cells in the suprapyramidal blade of 3MO Thy1-EYFP. The level of dendritic spine density was on average similar between control and ELS mice (Figure 5M), suggesting that the effects of ELS on mEPSCs at 3MO did not result from altered synapse numbers.

4 DISCUSSION

Several studies suggest a critical role for the glutamatergic synapse in mediating long lasting effects of ELS. To elucidate the developmental effects of ELS on hippocampal synapses, we therefore here assessed immediate (P9 pups), short-term (P21 juveniles) and long-term (3MO mature mice) effects of ELS on synaptic function and composition.

For P9 juveniles, we found that ELS significantly increased mEPSC frequency recorded from DG granular cells, which may result from either an increase in the number of functional synapses (above the 5 pA detection threshold), or an increase in presynaptic release probability. The significantly higher mEPSC amplitude we found as a result of ELS, reflects increased postsynaptic strength in the DG, which could be due to an increased AMPAR density or enhanced AMPAR channel conductance. A previous study reported similar effects of ELS on synapses in the hippocampal CA1 area at P11 following the LBN paradigm, demonstrating that ELS prematurely “unsilences” CA1 synapses by enhancing the recruitment of AMPARs at silent synapses. ELS may similarly induce premature synapse unsilencing at DG synapses.

FIGURE 3 Early life stress increases miniature excitatory postsynaptic currents (mEPSC) frequency in the dentate gyrus (DG) at P21. (A) Representative traces of mEPSCs recorded in DG granular cells. (B) Cumulative probability distribution of the interevent interval (IEI) shows a decrease resulting from early life stress (ELS) (D(80) = 0.5, p < .001). The inset shows an increase in the mean mEPSC frequency by ELS (Z = 2.52, p = .01), tested with a Wilcoxon test. (C) Cumulative probability distributions of the mEPSC amplitude were similar between the conditions. The inset shows similar means of the mEPSC frequency between conditions. (D) Normalized frequency distributions of mEPSC amplitude were similar between the conditions. (E) ELS results in trend towards an increase in the mEPSC decay time (Z = 1.92, p = .055), tested with a Wilcoxon test. Data is presented as mean ± SEM. Ctrl n = 29 cells from seven animals, ELS n = 24 cells from seven animals. Wilcoxon rank sum test p < .05.
FIGURE 4 Early life stress increases miniature excitatory postsynaptic currents (mEPSC) frequency in the dentate gyrus (DG) at 3MO.
(A) Representative traces of mEPSCs recorded in DG granular cells. (B) Cumulative probability distributions of the interevent interval (IEI) were similar between the conditions. The inset shows an increase in the mean mEPSC frequency by ELS ($Z = 2.9, p = .004$), tested with a Wilcoxon test. (C) Cumulative probability distributions of the mEPSC amplitude were similar between the conditions. The inset shows no effects of ELS on the mean mEPSC amplitude. (D) Normalized frequency distributions of the mEPSC amplitude were similar between the conditions. (E) No effects of ELS on the mEPSC decay time were present. (F) ELS persistently increases in the mEPSC is present over the three different ages ($F_{condition}[1,1128] = 17.97, p < .001$). (G) The mEPSC amplitude shows a significant condition effect over age ($F_{condition}[1,1128] = 7.52, p = .007$) and a significant age effect ($F_{age}[2,1128] = 5.53, p = .005$) with a slight trend towards an interaction between condition and age ($F_{condition \times age}[2,1128] = 3.03, p = .052$). (H) An age effect is present for the mEPSC decay time over the three different ages ($F_{age}[2,1128] = 18.36, p < .001$). Data is presented as mean ± SEM. Ctrl $n = 23$ cells from six animals, ELS $n = 19$ cells from six animals. ** Wilcoxon rank sum test $p < .01$; ***Two-way analysis of variance (ANOVA) condition effect $p < .01$; **** Two-way ANOVA condition effect $p < .001$; ## Two-way ANOVA age effect $p < .01$; ### Two-way ANOVA age effect $p < .001$. 
Interestingly, we found an overall decrease in synaptic protein levels in synaptosomal fractions of P9 pups that were exposed to ELS with a significant decrease for PSD-95, GluA3 and GluN2B. The decrease in these postsynaptic proteins seems contradictory to the observed increase in synaptic strength. For instance, a decrease in PSD-95 would lead to an overall decrease in functional synapses.

Although synaptosomal fractions are enriched for postsynaptic proteins, these fractions also contain perisynaptic proteins such as scaffolding and compartmentalization proteins that regulate extrasynaptic positioning of glutamatergic receptors. Thus, synaptosomal fractions do not necessarily reflect functional receptors in the postsynaptic density only. Moreover, the majority of synapses in the early postnatal

**FIGURE 5** Early life stress (ELS) lastingly alters synaptic composition but does not affect dendritic spine density at 3MO. At P9, ELS significantly decreases the synaptic content of PSD-95 ($Z = -2.61, p = .031$) (A), GluA3 ($T [10] = 2.35, p = .04$) (C) and GluN2B ($T [10] = 4.45, p = .001$) (E). ELS resulted in trends towards a decrease for GluA1 ($T [10] = 2.16, p = .06$) (B) and GluN2A ($T [10] = 1.94, p = .08$) (D) at P9. At the 3MO timepoint, ELS does not affect the synaptic content for PSD-95 (F), GluA1 (G), GluN2A (I) and GluN2B (J). ELS significantly increases the synaptic content of GluA3 at 3MO ($T [10] = -2.59, p = .04$) (H). (K) Representative bands from western blots on hippocampal synaptosomal fractions. (L) Representative images of apical dendrites in the molecular layer from granular cells in the dorsal DG. (M) ELS does not affect the dendritic spine density compared to Ctrl (Ctrl $n = 6$, ELS $n = 6$). Data is presented as mean ± SEM. Ctrl $n = 6$, ELS $n = 6$ cells. ^ Wilcoxon rank sum test $p < .05$; * $t$-test $p < .05$; ** $t$-test $p < .01$. Scale bar = 2 μm.
brain are so-called shaft synapses, which do not have a fully developed spine and have a two-fold larger postsynaptic density on average compared to spine synapses. This may affect the isolation of the synaptosomal fraction, as the postsynaptic membrane is not structurally separated from the presynaptic membrane. Therefore, while ELS may lead to a net decrease of glutamatergic receptors in the synaptosomal fraction, there may still be an increase in AMPAR density or AMPAR conductance at active zones in synapses.

Alternatively, the decrease in GluN2B may accelerate synapse unsilencing, since the GluN2B subunit regulates synaptic development by preventing the incorporation of AMPARs and thereby delaying synapse “unsilencing.” These findings are in line with an ELS-induced increase in the hippocampal GluN2A/GluN2B ratio measured as protein expression. Accelerated synaptic maturation following this ELS model has also been reported in the infralimbic cortex, as it decreased the mEPSC frequency and accelerated the age-dependent shift in excitation/inhibition balance. Interestingly, early life stress has been shown to alleviate infantile amnesia. As synapse “unsilencing” and maturation is thought to be required for creating infantile memories, our findings provide a possible synaptic mechanism in the DG by which ELS would accelerate the transition to adult-like memory retention.

We here show that ELS has long-lasting effects on DG synapses. This contrasts a previous study that reported no effects of the LBN model on the synaptic strength of DG granular cells in 4 month old mice, suggesting that the synaptic effects of ELS are more pronounced in younger mice. At both the P21 and 3MO timepoints, the increase in mEPSC frequency following ELS remained significant whereas the amplitude was on average no longer affected. As we did not find an increase in the overall dendritic spine density in 3 month old animals, the effect on the frequency is most likely due to either a higher presynaptic release probability or an increase in the number of functional synapses. However, since we did not measure ELS effects on spine morphology, we cannot exclude that this may have contributed to the effects that we find on mEPSC frequency. A more detailed analysis on the effects of ELS on spine morphology of DG granular cells and dendritic complexity is required to determine whether the effects of ELS on mEPSC is solely due to changes in synaptic function or whether structural effects also play a role. ELS was indeed previously shown to long lasting increase presynaptic release probability at CA1 synapses and in the DG. Interestingly, synaptosomal protein content was similar between the conditions for all proteins of interest at 3MO, except for GluA3, an AMPAR subunit that can exert distinctive modulatory function of synaptic plasticity by opening ion channels of GluA3-containing AMPARs at the postsynaptic membrane. This GluA3-mediated synaptic potentiation is predominantly reflected as an increase in mEPSC frequency at CA1 synapses, similarly as we observed for DG synapses as a consequence of ELS. GluA3 channels are activated via cAMP signaling, which can be induced by beta-adrenergic signaling. Other neuromodulators that stimulate cAMP, such as corticosterone, could potentially contribute to an increase in the number of functional synapses by activating GluA3-containing AMPARs.

In our study, we could not replicate the findings on increased plasma corticosterone levels following ELS exposure that have been reported before. However, several other studies have also reported no ELS effects on basal plasma corticosterone levels, or even found a blunted response following acute stress-exposure. This suggests that ELS effects on plasma corticosterone may differ between experiments and experimental conditions. For a full understanding of how ELS alters HPA-axis activity, it will be important to further characterize the dynamics of plasma corticosterone after stress-exposure, as well as corticosteroid receptor expression. Additionally, since other neuromodulators such as norepinephrine, as well as their respective receptor systems and the receptor configurations (e.g., Gs or Gi coupling), are relevant for stress-induced synaptic alterations, characterizing the effects of ELS on other neuromodulatory systems may provide insights into the mechanisms that underlie the enduring effects of ELS on hippocampal synapses. Moreover, it is important to note that the synaptosomal protein content analysis was performed on the whole hippocampus, whereas the mEPSC measurements were performed in the DG only. Hence, potential subregion-specific differences in ELS-effects on synaptosomal protein content is lost with this approach, and it will be important to address this in future studies.

Our findings concerning the effects of ELS on synaptic strength in the DG outlast the reported effects of ELS on synaptic strength in the infralimbic cortex, which then normalized to Ctrl levels after P21. In line with other studies, this suggests that the hippocampus is particularly sensitive to enduring effects of ELS. In fact, previous studies have shown even longer-lasting effects of ELS on CA1 synaptic plasticity at 6 months of age. Although the experiments in this study are performed in male mice only, accelerated synaptic maturation following ELS exposure is suggested to be independent of sex.

Together with our current findings, dynamic and enduring developmental effects of ELS on hippocampal synapses emerge, which are initially characterized by accelerated synaptic maturation of DG granular cells, observed as an increase in mEPSC frequency and amplitude, and altered synaptosomal AMPAR and NMDAR content. With age, enhanced synaptic activity sustains and synaptic GluA3 content increases. Hippocampal GluA3 expression is increased in old (12 months old) compared to young adult mice (3 months old). Thus, the increase in synaptosomal GluA3 content as a result of ELS in the 3MO group may also reflect accelerated synaptic senescence. Moreover, synaptic hyperactivity may disrupt plasticity mechanisms, contributing to an enhanced susceptibility to stress-related psychopathology and neurodegenerative disorders following ELS. GluA3 is suggested to play a role in a variety of neurodevelopmental and neurodegenerative disorders, including Alzheimer’s disease (AD) pathology. Accordingly, an increase in synaptic GluA3 may explain the increased susceptibility to Alzheimer-related pathology following ELS in a mouse model for AD, and possibly even the correlation between early life experiences and AD prevalence. As accelerated synaptic maturation following ELS exposure is reported to occur independent of sex, our findings in male mice may be similar in female mice during early development, although enduring effects of
ELS are reported to be sex-dependent. Therefore, researching sex-specific consequences of ELS on synaptic function and receptor composition from development into adulthood will be important in future studies.

Normalizing glutamatergic synapse function could prevent stress-related psycho- and neurodegenerative pathology in animal models of ELS. As aberrant postnatal brain development is suggested to enhance disease susceptibility later in life, it would be of interest to assess whether the ELS-induced acceleration of synaptic maturation plays a causal role in the lasting effects of ELS. In agreement, enhancing GluN2B content by modulating protein–protein interactions in the hippocampus could potentially prevent the accelerated unsilencing of synapses, delaying the accelerated maturation after ELS. Another option would be to mitigate the ELS-induced increase in synaptic strength by enhancing glutamate uptake and preventing glutamate release. Whether this may also prevent the accelerated maturation is yet to be determined.

In summary, our study provides novel mechanistic insights into the developmental and enduring effects of ELS on hippocampal synapses. We show that ELS enhances hippocampal synaptic strength by altering the function and composition of synapses from the early postnatal period up to adulthood. These developmental alterations could contribute to the increased susceptibility to stress-induced psychopathology and neurodegenerative disorders following ELS.

AUTHOR CONTRIBUTIONS
Niek Broens: Conceptualization; data curation; formal analysis; investigation; writing – original draft. Carla Simon: Formal analysis. Helmut W. Kessels: Supervision; writing – review and editing. Paul J. Lucassen: Supervision; writing – review and editing. Harm Krugers: Conceptualization; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT
The authors have no conflict of interest to declare.

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DATA AVAILABILITY STATEMENT
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT
All animal experiments were conducted under Dutch national law and in compliance with the European Union directive 2010/63/EU. The study design was evaluated and approved by the animal welfare committee of the University of Amsterdam.

ORCID
Niek Broens https://orcid.org/0000-0002-8347-8522
Harm J. Krugers https://orcid.org/0000-0002-7926-7001

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