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# Early life stress determines the effects of glucocorticoids and stress on hippocampal function: electrophysiological and behavioral evidence respectively

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**ABSTRACT**

Exposure to early-life adversity may program brain function to prepare individuals for adaptation to matching environmental contexts. In this study we tested this hypothesis in more detail by examining the effects of early-life stress – induced by raising offspring with limited nesting and bedding material from postnatal days 2-9 – in various behavioral tasks and on synaptic function in adult mice. Early-life stress impaired adult performance in the hippocampal dependent low-arousing object-in-context recognition memory task. This effect was absent when animals were exposed to a single stressor before training. Early-life stress did not alter high-arousing context and auditory fear conditioning. Early-life stress-induced behavioral modifications were not associated with alterations in the dendritic architecture of hippocampal CA1 pyramidal neurons or principal neurons of the basolateral amygdala. However, early-life stress reduced the ratio of NMDA to AMPA receptor-mediated excitatory postsynaptic currents and glutamate release probability specifically in hippocampal CA1 neurons, but not in the basolateral amygdala. These ex vivo effects in the hippocampus were abolished by acute glucocorticoid treatment. Our findings support that early-life stress can hamper object-in-context learning via pre- and postsynaptic mechanisms that affect hippocampal function but these effects are counteracted by acute stress or elevated glucocorticoid levels.

Keywords: Early life adversity, synapses, stress, AMPA, NMDA, Memory

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**Highlights**

- An acute stressor overcomes effects of Early Life Stress on object-in-context memory
- Brief glucocorticoid treatment in vitro prevents effects of Early Life Stress on hippocampal excitatory synaptic transmission
- Limited nesting and bedding material does not impact hippocampal and amygdala dendritic morphology

## 1. Introduction

Parental care is an important component of the gene-environment interactions in early-life that shape cognitive-emotional development, which is reflected throughout life (Bowlby, 1958). Lack of adequate parental care arising from parental neglect or lack of sensitivity constitute a dominant form of early-life stress in humans (Nelson et al., 2011) that hampers cognitive function, enhances emotionality and increases the risk to develop psychopathology later in life in vulnerable individuals (Caspi et al., 2003).

Animal models of stress developed over the last decades have been beneficial in identifying causal relationships between early-life adversity and later-life cognitive and emotional disabilities and the underlying mechanisms (de Kloet et al., 2005; Meaney, 2001). In rodents, maternal sensory stimulation comes from a set of stereotyped behaviors such as licking/grooming and arched back nursing. Low levels of maternal care have been reported to hamper spatial learning and memory (Liu et al., 2000; Oomen et al., 2010) but enhance fear learning (Champagne et al., 2008; Oomen et al., 2010). Similar effects have been reported in rodents that were raised with fragmented levels of maternal care - achieved by limiting the nesting and bedding material during the early postnatal period (Arp et al., 2016; Brunson et al., 2005; Ivy et al., 2010; Naninck et al., 2015; Rice et al., 2008; Wang et al., 2013).

Indices of glutamatergic functionality in the rat dentate gyrus, such as synaptic responses via NMDA and AMPA receptors, which are critical for synaptic plasticity and memory formation (Kessels and Malinow, 2009), were recently reported to be sensitive to the level of maternal care (Bagot et al., 2012). However, several studies indicate that early-life conditions affect synaptic transmission and plasticity in adulthood differently depending on the experimental conditions during testing. Thus, in adult offspring from low (compared to high) caring mothers, hippocampal LTP was reduced and NMDAR-mediated transmission was enhanced under non-stressful conditions associated with low corticosterone concentrations (Bagot et al., 2009, 2012; Champagne et al., 2008). However, when corticosteroid levels were experimentally raised, LTP was increased in offspring of low caring mothers, while in the offspring of high-caring mothers LTP was reduced and NMDAR mediated transmission enhanced. This may suggest that synaptic transmission and plasticity are optimal and

facilitate behavioral adaptation when early and later-life conditions match, i.e. animals raised under stressful conditions perform well in arousing learning contexts (Champagne et al., 2008; Nederhof and Schmidt, 2012; Santarelli et al., 2017).

In order to examine this in more detail we here addressed three questions. We first examined the impact of early-life stress, induced by raising offspring with limited nesting and bedding material from postnatal days 2-9 (Rice et al., 2008) on memory in low-arousing (object-in-context) learning paradigms as well as its modulation by a brief exposure to stress. Next, we studied whether the lasting behavioral changes induced by early-life stress are associated with alterations in hippocampal dendritic complexity and/or glutamate-mediated synaptic transmission with corticosterone levels that are relevant for either non-stressful or stressful conditions. Finally, regional specificity was determined by investigating behavioral performance, dendritic complexity and glutamatergic transmission related to the basolateral amygdala.

## 2. Materials and Methods

### 2.1. Experimental animals and breeding

The study used adult (10-14 weeks) male C57BL/6 mice bred in-house at the institutional animal facility. Mice were reared in standard conditions (21°C ± 1 and 55% ± 15 humidity on a 12 h light/dark cycle with lights on at 08:00 hr) and had unlimited access to food and water at all times. Experiments were performed in compliance with the European Union Directive on the protection of animals used for from the scientific purposes (2010/63/EU). All experiments were approved (DED192/DED256) by the local animal ethics and welfare committee (University of Amsterdam / Utrecht University). Efforts were made to reduce the number of animals used and their discomfort throughout the duration of the study.

For breeding, 10 weeks old C57Bl/6J male and female mice were purchased from Harlan Laboratories, The Netherlands. One male was housed with two females for one week. Subsequently the male was removed and the females were left undisturbed until being individually housed approximately one week before giving birth. Day of birth was termed postnatal day zero (P0).

2.2. Early-life Stress

Two days after birth (P2), the litter size per dam was adjusted. Litters contained 6 pups with at least one female pup and litters were randomly assigned to either the early-life stress (Arp et al., 2016; Rice et al., 2008; Wang et al., 2011) or control group. Dams and litters in the early-life stress group were shifted to cages having a fine-gauge aluminum mesh floor with limited bedding and nesting material (half-square piece of cotton nesting material (Technilab-BMI, The Netherlands) and little sawdust bedding). Control dams were transferred to standard housing cages with one full square (5 x 5 cm) nesting material and plenty of sawdust bedding. At P9, all animals (dams with litters) were transferred back to standard cages with sufficient nesting and bedding material. Between P22 and P24, the offspring was weaned and socially housed (2-4 animals per cage). Experimental groups for electrophysiological experiments rarely contained animals from the same litters.

2.3. Adult stress

Adult animals were stressed by transferring them to a clean and unfamiliar home cage (without food/water) for 20 min. From previous reports we know that this 'novel-cage paradigm' activates the HPA-axis resulting in moderately raised plasma corticosteroid levels (Pardon et al., 2004), and induces acute glucocorticoid receptor-mediated responses in the hippocampus (Sarabdjitsingh et al., 2014).

2.4. Quantification of corticosterone levels

Radio immuno-assay was performed using a $^{125}$I-labeled corticosterone double-antibody kit (MP Biomedicals) on stored (-80°C) plasma isolated (centrifuged at 14000 rpm for 15 min at 4°C) from fresh trunk blood collected immediately after decapitation of adult mice on the day of the experiment.

2.5. Object-in-context recognition memory test

The object-in-context memory test assesses the recognition of a familiar object in a context that is different from where it was presented previously (Dix and Aggleton, 1999). Performance of this

behavior task requires associating a particular object with a specific context and is heavily dependent on the functional integrity of the hippocampus (Barker and Warburton, 2011; Mumby et al., 2002; Ramsaran et al., 2016). The experiment was conducted over a period of 3 days (Fig. 1A). On day 1, the animals were habituated for 10 min in a rectangular blue box (L x W x H; 54 x 33 x 37 in cm) with no visual cues (context A) or objects apart from sawdust bedding. On day 2, the training day, each mouse was placed for 10 min in context A with two identical objects (Lego blocks) at diagonally opposite corners (15 cm from the closest vertex). Next, the animals were exposed to context B (striped walls) for 10 min with two identical objects (small bottles) before being returned back to their home cage. On day 3, the testing day, animals were placed for 10 min in context B with two familiar objects (one each from context A and B) of which one object (out-of-context object) was never encountered in context B. In a separate experiment, animals were exposed to 20 minutes of novelty stress (see section 2.3) one hour before training on day 2. Between individual sessions, all objects were thoroughly cleaned with 5% ethanol and fresh bedding material was added to the box. All sessions were videotaped and an experimenter blind to the groups scored and analyzed the number of visits and time spent with the out-of-context object.

2.6 Fear conditioning

Mice were tested for contextual fear acquisition as follows (Fig. 2A). On day 1, the conditioning day, each mouse was habituated to the conditioning box (context A, L x W x H: 25 x 25 x 30 in cm) fitted with a stainless steel grid floor connected to a shock generator. After 3 minutes of exploration, one footshock (2 sec, 0.4 mA) was delivered and 30 sec later the mouse was returned back to its home cage. On day 2, the testing day, the mouse was placed back into the same conditioning chamber for 3 minutes. The sessions were videotaped and the freezing behavior (no body movements other than those related to breathing) was scored every 2 sec by an experimenter blind to the experimental groups.

A different cohort of animals was tested on auditory fear conditioning that measured the amygdala-dependent form of associative fear learning (Phillips and LeDoux, 1992; Schafe and LeDoux, 2000). On day 1, mice were first habituated to the conditioning chamber (context A) similar

to the contextual fear conditioning described above (Fig. 2C). At the end of the habituation period, the mouse was presented with a pure tone (100 dB, 2.8 kHz, 30 sec duration) that co-terminated with a footshock (2 sec, 0.4 mA) and was returned back to the home cage after 30 sec. On day 2, the testing day, the mouse was placed in a box with striped walls and smooth floor (context B) and 3 min later was presented with the conditioning tone lasting for 30 sec. The mouse remained in the chamber for another 30 sec before being returned back to its home cage. At the end of the experiment, Context A and B were cleaned with 70% ethanol and 1% acetic acid, respectively. The sessions were videotaped and the freezing behavior was scored every 2 sec by an experimenter who was blind to the experimental groups.

2.7 Golgi-Cox Staining

In order to stain and compare dendritic architecture of individual neurons in the hippocampus and amygdala, the Golgi-Cox staining protocol was followed as reported before (Boekhoorn et al., 2006). Mice were decapitated when the circulating glucocorticoid levels were low (between 0800 and 0900) and the brain was quickly removed and immersed in the Golgi-Cox solution (5% $K_2CrO_4$, 5% HgCl, and 5% $K_2Cr_2O_7$). The brains, after being left undisturbed for 30 days, were thoroughly rinsed in deionized water, dehydrated in ethanol, embedded in celloidine and kept immersed in chloroform for the next 16 hours. Subsequently, the brains were cut into thin (200 μm) coronal slices using a vibratome, stained in 16% ammonia and mounted on glass slides for microscopic analysis. Undamaged and isolated cells with good dye impregnation and clear from precipitations were randomly imaged from both hemispheres of dorsal hippocampus and basolateral amygdala. Dendritic tracks were hand drawn using Neurodraw and Image-Pro softwares by an experimenter who was blind to the treatment groups. Dendritic parameters from 4-5 neurons per animal were averaged for statistical comparisons and plotting.

2.8. Brain slice preparation and electrophysiological recordings

Mice were quickly transferred from their home cage and decapitated without anesthesia before 10:30 am on the day of the experiment. This was necessary to keep the circulating corticosteroid levels low

and uniform across experiments, as both the circadian cycle and anesthesia rapidly alter stress hormones levels in the plasma and brain (Jacobsen et al., 2012; Zardooz et al., 2010). Immediately after decapitation, the brain was quickly removed from the skull and chilled in oxygenated (95% $O_2$ / 5% $CO_2$) ice-cold artificial cerebrospinal fluid (aCSF) of the following composition, in mM: 125 NaCl, 26 $NaHCO_3$, 1.2 $NaH_2PO_4$, 10 Glucose, 3 KCl, 1.3 $MgSO_4$ and 2 $CaCl_2$ at pH ~ 7.35. Subsequently, coronal sections of the brain, containing one or more of the regions of interest (hippocampus and/or amygdala), were cut at a thickness of 300-350 μm using a vibrating blade microtome (VT 1000S, Leica Biosystems, Germany). After a recovery period (10-15 min in aCSF at 25 $^oC$), slices were randomly assigned for acute treatment (20 min) with either corticosterone (100 nM) or vehicle (0.01% ethanol) in an incubation chamber filled with oxygenated aCSF (30 $^oC$). After treatment, all slices were transferred back to the holding chamber containing normal aCSF at room temperature (~ 25 $^oC$) until the commencement of recording.

For recording, one slice at a time was placed in the recording chamber of a patch-clamp setup with a running supply of warm oxygenated aCSF (30$^oC$, ~ 2.5 ml/min) using a peristaltic pump (TC-324B, Warner Instrument Corp., USA). Neurons were visualized using a 40x objective (NA: 0.75, with Nomarsky optics IR-DIC) coupled to a b/w high resolution CCD camera and monitor (TCCCD-624 & CDM-1702, Monacor International, Bremen, Germany) that was attached to an AX10-Examiner microscope (Zeiss, Germany). Whole-cell patch-clamp recording was carried out using an AxoPatch 200B amplifier (Axon Instruments, USA). The signals were sampled (at 50 kHz using Digidata 1322A 16 bit ADC, Axon Instruments, USA), amplified (10 mV/pA) and filtered at 2 kHz without series resistance and capacitance compensations, using pClamp 9.2 software. All recording were analyzed off-line with custom programs written in MATLAB. Excitatory postsynaptic currents (EPSCs) were evoked in CA1 pyramidal neurons by stimulating the Shaffer collateral axons using a concentric bipolar stimulation electrode (FHC, USA) placed ~ 200-300 μm from the patched neuron (Figure 4A). Whereas, in the amygdala, we recorded EPSCs elicited in the pyramidal-like principal neurons of the basolateral amygdala by electrical stimulation of the lateral amygdala (Figure 6A). The stimulations were performed at 0.04 Hz via a stimulus isolator (NeuroLog, Digitimer, UK) that delivered short current pulses. For each cell we adjusted the stimulus duration (100-150 μs) and

intensity (200-700 μA), to obtain monosynaptic EPSCs with an optimal amplitude (~ 100 pA) before commencing baseline recording. EPSCs mediated by α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPARs) were recorded at -70 mV in the presence of a $GABA_A$R-blocker bicuculline methobromide (BIC, 20 μM) for 10-15 min, following which N-methyl-D-aspartate receptors (NMDARs) mediated EPSCs were measured at -70 mV in $Mg^{2+}$-free aCSF that also contained a competitive AMPA/kinate receptor antagonist (CNQX, 20 μM). In a subset of experiments we also blocked NMDAR-EPSCs (using APV, 20 μM) at the end of the recording session. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (The Netherlands).

Patch electrodes were made from thick-walled borosilicate glass capillaries (inner/outer diameter in mm: 1.5/0.86; Harvard Apparatus, UK) pulled on a P-97 Flaming/Brown micropipette puller (Sutter Instruments, USA) to yield a tip of ~ 2 μm (4-6 MΩ with internal solution). The patch pipettes were filled with an intracellular solution that was composed in mM: 145 $CH_3CsO_3S$, 20 CsCl, 10 HEPES, 3.3 BAPTA tetracesium salt, 4 ATP-$Mg^{2+}$ and 0.4 GTP-$Na_2$ at pH 7.36 (adjusted with CsOH).

Baseline EPSC recording typically commenced within 12 min after entering the whole-cell configuration, taking into account the time required for (1) dialysis of the cell by the cesium-containing internal solution and (2) adjusting the stimulation (location and intensity) to obtain stable mono-synaptic EPSCs. After 5-10 minutes of baseline recording of AMPAR-mediated EPSCs, the extracellular perfusion medium (standard aCSF) was exchanged with $Mg^{2+}$-free aCSF that also contained CNQX. Pure NMDAR-EPSCs were visible ~ 20 min after the start of the solution exchange, as confirmed by their relatively large rise and decay times as well as by its blockade by APV. We computed the NMDA / AMPA EPSC ratio from the averaged peak amplitudes of pure AMPAR- and NMDAR-EPSCs. Our computed values for rise time , decay time constants and NMDA / AMPA ratio from vehicle treated control CA1 pyramidal neurons (Table 1) and basolateral amygdala principal neurons (Table 2) largely agree with earlier reported values largely agree with earlier reported values (Spruston et al., 1995; Lack et. al., 2007, Kröner et. al. 2005). Presynaptic changes were accessed using paired-pulse ratios of peak AMPAR-mediated EPSCs measured from

two consecutive stimulations with a 50 ms inter-pulse interval.

The following EPSC parameters were measured off-line using custom MATLAB routines: Peak amplitude: EPSC amplitude averaged within a 100 μs window centered around the actual peak; Rise time: time between 20-80% of the peak amplitude; EPSC decay tau: based on a single exponential fit from peak to tail.

### 2.9 Statistical Analysis

All statistical tests and plotting were done with the 'R' statistical software (R Development Core Team, 2016). The data were examined using either one-way or two-way ANOVA with early-life treatment (early-life stress or control) and later-life treatment (with or without stress/corticosterone exposure) as fixed-factors. A two-way ANOVA with repeated measures (distance from soma) was used to test distance-dependent effects on dendritic length between early-life stress and control groups. Posthoc comparisons between individual groups were done using Welch's t-test (Saville, 1990). The assumptions underlying ANOVA were tested using Shapiro-Wilks and Levene's test of normality and homogeneity of variance, respectively. In plots, the error bars represent SEM, and number of animals or cells is given.

## 3. Results

### 3.1. Plasma corticosterone levels and body weight

At P9, body weight of all animals in the ELS group (3.7 g ± 0.1, N = 15), including female mice which were subsequently not used in this study, was lower than in control animals (4.9 g ± 0.1, N = 16), $p < 0.001$, as reported earlier (Rice et al., 2008). Basal plasma corticosteroid level was examined in both control and early-life stressed adult mice as readout for persistent alterations in HPA axis activity (Figure 1B). In adulthood, control and early-life stressed mice had comparable levels of basal plasma corticosterone levels (control: 7.9 ± 1.6 ng/ml; els: 6.5 ± 1.5 ng/ml; $t(36) = 0.67$, $p = 0.51$) as reported previously (Naninck et al., 2015). Also, we have no evidence that stress-induced plasma corticosterone levels are altered after ELS (Lesuis et al., 2017).

3.2. Exposure to stress prevents early-life stress-induced impairment in object-in-context recognition memory

The impact of stress in adulthood on putative early-life stress mediated impairment in hippocampus-dependent memory was examined using the object-in-context memory task. To test this, we exposed animals in both the early-life stress and control group to a brief episode of novel-cage stress (or control treatment) one hour before training on day 2 (Figure 1A). Two-way ANOVA revealed a significant interaction between early-life stress and later-life stress on exploration time, $F(3, 26) = 4.69$, $p = 0.04$, and frequency of visits to the out-of-context object, $F(3, 26) = 6.74$, $p = 0.02$. Follow-up analysis showed that control mice spent a considerable amount of time and visits to the out-of-context object, whereas adult animals that were exposed to early-life stress were impaired in recognizing the new object in a familiar context (Figure 1C & D). This is evident from the significant difference between groups in mean values for both number of visits (control: 65 % ± 2; els: 55 % ± 2), $t(12) = 4.5$, $p = 0.001$, and time spent (control: 62 % ± 2; els: 52 % ± 2), with the out-of-context object, $t(12) = 3.2$, $p = 0.007$.

This effect was not seen when mice were exposed to a brief stressor prior to testing. Thus, when assessed for memory on day 3, control and early-life stressed mice that were exposed to brief stress showed similar recognition of the out-of-context object (Figure 1C & D). We found no significant difference between early-life stress and control groups in both exploration time (control + stress: 59 % ± 3; els + stress: 62 % ± 4; $t(13) = -0.6$, $p = 0.5$) and visits to the out-of-context object (control + stress: 61 % ± 3; els + stress: 64 % ± 3; $t(14) = -0.6$, $p = 0.5$).

3.3. Early-life stress does not affect context and auditory fear memory in adult mice

In the previous experiment we showed that mice exposed to early-life stress are cognitively impaired in a non-aversive, non-stressful task but perform normally when primed with a brief stress before training. This may suggest that mice that were exposed to early-life adversity perform well when trained under stressful learning conditions later in life. When tested in a contextual fear conditioning paradigm (Figure 2A), mice that were exposed to early-life stress showed slightly enhanced freezing

behavior in response to the footshock (Figure 2B, $t(9) = -2.05$, $p = 0.07$) while freezing behavior of control and early-life stressed mice during the pre-shock period was identical for the groups, $t(8) = -1$, $p = 0.35$, indicating that basal behavioral activity was similar between groups. On the next day, when animals were tested for contextual fear in the same context as in the training day but without shock, both early-life stressed and control mice showed similar levels of freezing behavior, $t(16) = 0.85$, $p = 0.4$. Thus, apart from the enhanced sensitivity immediately following shock, contextual fear memory was unaffected in mice subjected to early-life stress.

Subsequently, we tested for auditory fear memory in a different cohort of animals (Figure 2C). Two-way ANOVA showed significant main effect of training sessions (pre-tone, tone and post-tone), $F(2, 22) = 25.9$, $p < 0.001$, but no significant effect for group ($p > 0.8$) or interactions ($p > 0.8$). These results were also consistent with the observations on the testing day (Figure 2D). There was a significant effect of testing session in both groups, $F(2, 22) = 61.3$, $p < 0.001$, indicating that 24 hr after pairing all animals exhibited significant fear learning. However, this learning was not modified by early-life stress ($p > 0.5$). These results together demonstrate that learning of both context and auditory fear memories were not altered by early-life stress.

### 3.4. Early-life stress does not lead to lasting alterations in the dendritic architecture of pyramidal neurons in the CA1 hippocampal area or basolateral amygdala

We quantified the distribution of dendritic material between concentric spheres placed at equidistance and centered at the soma (Sholl, 1953), Figure 3A. Segmental distributions of apical and basal dendrites of CA1 pyramidal neurons from adult early-life stress and control mice were similar (Figure 3B). This was revealed by a two-way ANOVA which indicated no significance for main effect of group, $F(1, 13) = 0.68$, $p = 0.425$, or its interaction with radial distance, $F(24, 312) = 1.182$, $p = 0.256$, for the apical side. Similar results were obtained for the basal region for main effect of group, $F(1, 12) = 0.11$, $p = 0.75$, and interaction, $F(13, 156) = 0.98$, $p = 0.47$. In agreement with this, total dendritic length were also identical between groups, $t(13) = 0.83$, $p = 0.4$ and $t(11) = 0.32$, $p = 0.75$, for apical and basal sides respectively (Figure 3C).

We next examined dendritic complexity of pyramidal cells in the basolateral amygdala (Fig.

3D). Although there was a trend towards longer dendrites in the early-life stress group, it did not reach statistical significance (Figure 3E & F). A two-way ANOVA indicated no significant main effect, $F(1, 10) = 3.37$, $p = 0.1$, or interaction, $F(19, 190) = 0.81$, $p = 0.7$, of early-life stress on dendritic morphology at increasing distance from soma. This was also evident from total dendritic length wherein the early-life stress group was not significantly different from the control group, $t(10) = -1.8$, $p = 0.1$.

3.5. Effects of early-life stress on glutamate receptor currents and synaptic transmission in the hippocampus are normalized by acute corticosterone treatment

We have shown that early-life stress impairs memory in a hippocampus-dependent object-in-context learning test that is normalized by acute stress in adult animals. However, no major structural alterations in the hippocampal CA1 area of adult early-life stress mice were evident. We next asked whether the mechanisms for the observed effects of early-life stress might putatively be mediated by modifications in excitatory synaptic transmission in the hippocampus. To address this question, we recorded excitatory post-synaptic currents (EPSCs) evoked by stimulation of Schaffer collaterals inputs to CA1 pyramidal neurons (Figure 4A). In order to examine the delayed and presumably gene-mediated effects of glucocorticoids on synaptic transmission, we adopted the protocol from a previous study that reported corticosterone-mediated slow genomic effects on glutamate-activated synaptic currents in the hippocampal CA1 area (Karst and Joëls, 2005). For better between the behavioral effects (particularly during the consolidation phase) and the glucocorticoid effects in vitro, synaptic currents were recorded, on average, fours hours after treatment with either corticosterone or vehicle.

The peak amplitudes of recorded AMPAR-EPSCs were not different among the four groups (Figure 4B & C), as confirmed by two-way ANOVA (Table 1). There was a main effect of corticosterone treatment on the decay, but not rise time of EPSCs. A similar effect was also evident on the membrane capacitance of the recorded cells while input resistance remained unaffected (Table 1). We next measured NMDAR-EPSCs by exchanging the extracellular solution with one that had no $Mg^{2+}$ ions and contained the competitive AMPAR antagonist CNQX. Unlike what we had observed with AMPAR-EPSCs, the peak NMDAR-EPSCs were significantly reduced in vehicle-treated early-

life stress animals when compared to control mice (Figure 4B&D). This was confirmed by we observed a significant interaction between early-stress and control treatment ($F(3, 30) = 2.26$, $p = 0.017$) regarding the peak NMDAR-EPSCs. Post-hoc analysis revealed that in vehicle-treated neurons, early-life stress was associated with a significant decrease in NMDAR-EPSC amplitude (Table 1, Figure 4). However, this reduction completely disappeared after corticosterone treatment. Importantly, corticosterone treatment itself increased in NMDAR-EPSCs in early-life stress animals (Table 1, Figure 4D). Moreover, similar to the effect on AMPAR-EPSCs, there was also a decrease in the NMDAR-EPSC decay constant with corticosterone treatment (Table 1).

The NMDAR-EPSCs recorded from individual cells apart from being modulated by treatments (corticosterone, early-life stress or both) are also highly dependent on the amount of synaptic glutamate as inferred from the strength of AMPAR-EPSCs. We therefore, scaled averaged NMDAR-EPSC from each cell with its corresponding AMPAR-EPSC amplitude. Early-life stress, similar to its previously described effect on NMDA-EPSC amplitude, significantly reduced NMDA/AMPA ratio when compared to control animals (Figure 4E). The significant interaction between early-stress and corticosterone treatment as revealed by two-way ANOVA was further confirmed by post-hoc tests (Table 1). Interestingly, this reduction in the NMDA/AMPA ratio after early-life stress was completely absent after corticosterone treatment, $t(14) = -1.1$, $p = 0.30$. Similar to the earlier result, in the early-life stress group, but not in control, we found a significant increase in NMDA/AMPA ratio after glucocorticoid treatment (Table 1).

We next examined the effect of early-life stress and later-life glucocorticoid-treatment on paired-pulse facilitation (PPF), which is readout for presynaptic release probability (Zucker and Regehr, 2002). PPF was computed from the peak amplitude ratio of the second to the first of a pair of (AMPA receptor mediated) EPSCs separated by a 50 ms interval evoked by stimulating axon terminals arriving at the stratum radiatum layer of CA1 pyramidal neurons (Figure 5A). Two-way ANOVA revealed a significant interaction between early-life stress and corticosterone-treatment, $F(3, 35) = -2.4$, $p = 0.023$. Not only was the PPF ratio significantly increased after early-life stress ($t(19) = -2.47$, $p = 0.023$), but this change was absent after corticosterone treatment (Table 1, Figure. 5B).

3.6. Glutamate receptor currents in the basolateral amygdala are not affected by early-life stress or glucocorticoid treatment.

AMPA and NMDA-mediated EPSCs were recorded from BLA pyramidal neurons similar to the procedure described above for CA1 neurons (see Materials and Methods, Figure 6A & B). Similar to effects of early-life stress and corticosterone treatment described for the hippocampus, there was no significant change in AMPA-EPSC amplitude between groups (Figure 6C). However, unlike hippocampal CA1 pyramidal neurons, BLA neurons also showed no change in peak NMDA-EPSC amplitudes, either with early-life stress or corticosterone treatment (Table 2, Figure 6D). Importantly, there was also no effect on the ratio of NMDA/AMPA responses between control and early-life stress groups there were treated with either vehicle or corticosterone (Figure 6E). Additionally, the kinetic characteristics of AMPAR- and NMDAR-EPSCs in BLA principal neurons were similar across groups (Table 2).

## 4. Discussion

In this study we investigated whether early-life stress, induced by fragmented and erratic levels of maternal care (Arp et al., 2016; Brunson et al., 2005; Rice et al., 2008), alters later cognitive performance and synaptic function in the hippocampus and BLA and whether these effects could be modulated by acute exposure to stress or glucocorticoid hormones respectively. Our results demonstrate that early-life stress-induced impairment on object-in-context memory was absent after a single brief exposure to stress in adult animals. This effect of early-life stress was associated with a reduced ratio of NMDA/AMPA EPSCs and increased paired pulse ratio in the hippocampal CA1 area (but not in the BLA) but without persistent changes in the dendritic architecture of hippocampal CA1 and basolateral amygdala neurons. Interestingly, synaptic changes (NMDA/AMPA EPSC ratio and paired pulse ratio) were normalized by stress levels of corticosterone in adult animals. The behavioral findings indicate that stress-exposure in *early-life* alters the way *later-life* stressors affect hippocampal function and behavior. This provides evidence in favor of the match/mismatch hypothesis that early-life adverse events tune neuronal mechanisms to function optimally under stressful conditions

(Champagne et al., 2008; Daskalakis et al., 2013; Nederhof and Schmidt, 2012; Schmidt, 2011).

There is strong evidence from several studies that early-life stress in rodents induced by low levels of maternal care, maternal deprivation or fragmented care, affects hippocampus-dependent learning and memory processes (Brunson et al., 2005; Liu et al., 2000; Naninck et al., 2015; Oomen et al., 2010; Rice et al., 2008). However, there is also considerable variation in the direction and magnitude of early-life stress-induced cognitive effects across studies. One reason for the observed variability in results could be the differences in stress levels of the behavioral task itself. Interestingly, studies have used behavioral paradigms ranging from low stress learning protocols to high stress fear-conditioning tasks. In order to clearly separate the influence of stress we therefore assessed the impact of early-life stress on cognitive performance using the object-in-context memory test that is low-arousing and also heavily dependent on hippocampal integrity (Barker and Warburton, 2011; Dix and Aggleton, 1999; Mumby et al., 2002; Ramsaran et al., 2016). In line with earlier findings (Rice et al., 2008; Naninck et al., 2015; Kanatsou et al., 2017) we report here that early-life stress impairs the ability of mice to explore a novel object in a familiar context. In stark contrast, several studies have also indicated that the ability to form fear memories are preserved or may even be enhanced by early-life adversity, albeit with variable experimental protocols. Offspring from low-licking grooming mothers exhibited enhanced context fear memories (Champagne et al., 2008) and rodents that were exposed to maternal deprivation displayed enhanced context and auditory fear conditioned responses (Oomen et al., 2010). Erratic and fragmented levels of maternal care did not alter auditory fear conditioning (Arp et al., 2016) but enhanced freezing in-between repeated exposure to auditory cues. In agreement to the above, the current study also showed that conditioned fear was remembered well (though not significantly better) in animals that were raised with fragmented levels of maternal care.

Overall, findings from previous studies as well as the current suggest a maintenance or improvement in cognitive performance in tasks that are moderate to highly stressful in animals that were subjected to stress in early-life. This led us to hypothesize that the impairment in object-in-context memory in mice with an early-life stress history should disappear when animals are exposed to a brief stress. To verify this hypothesis, we subjected animals to a novelty stress protocol (Pardon et al., 2004) one hour before training on the object-in-context paradigm. The observed lack of cognitive

impairment in adult animals that had experienced early-life stress is entirely in agreement with the match/mismatch hypothesis (Daskalakis et al., 2013; Schmidt, 2011) that proposes enhanced ability to cope with stress in animals subjected to early-life adversities.

Various studies have reported that maternal care impacts dendritic morphology in adult hippocampal subfields (Bagot et al., 2009; Liao et al., 2014) including CA1 pyramidal neurons (Champagne et al., 2008; van Hasselt et al., 2012). However, the effects of fragmented care on hippocampal dendritic morphology may be age-dependent. Accordingly, dendritic atrophy in the hippocampal CA1 area of mice exposed to limited nesting and bedding material was only observed in middle-aged, and not in young adult animals (Brunson et al., 2005; Ivy et al., 2010). In agreement with the latter finding, we did not observe effects of early-life stress in the CA1 dendritic architecture of young adult mice. Similar results (unpublished observations) have been obtained when mice were tested at three weeks of age, indicating the possibility that morphological effects on hippocampal dendritic structure are slow in onset and may follow rather than precede changes in synaptic function. This of course does not exclude the possibility that other regions of the hippocampus, especially the CA3 field, which was found to be more susceptible to stressful events, are affected. Indeed, at 6 months of age, animals subjected to fragmented maternal care displayed significant loss of spines in area CA3 but not in CA1 (Wang et al., 2011). The rodent amygdala appears to be resilient to early life stress. Indeed, in young male and female rodents (3 months of age), a 24 h severe maternal deprivation failed to induce any lasting effects on dendritic morphology of amygdala neurons (Krugers et al., 2012). The present study also suggests that dendritic structure in the basolateral amygdala is not strongly affected by early-life stress at this age.

Glutamate and NMDA receptors have central roles in the cellular, functional and behavioral changes induced by stress and glucocorticoids (Krugers et al., 2010; Popoli et al., 2012). Among the hippocampal subregions, the CA1 subfield is strongly implicated in contextual memory formation (Suthana et al., 2009), specifically for novel stimuli (Leutgeb et al., 2004) and CA1 NMDA receptors are crucial for the encoding of recognition information (Place et al., 2012). We therefore probed CA1 pyramidal neurons to understand the mechanisms behind the persistent effects of early-life stress in object-in-context memory. Our results demonstrate that the lasting impact of early-life stress as

evidenced by reduced hippocampal-dependent memory in adult animals is also associated with a reduction in NMDA currents as well as a decrease in NMDA/AMPA ratio. In contrast with our results, another study that used the same early life stress model but a different technique to measure NMDARs failed to observe changes in NMDA receptor contribution (Brunson et al., 2005). Likely, differences in the manner in which NMDAR EPSCs were measured, such as a direct ($Mg^{2+}$-free aCSF in the presence of AMPAR blocker, as employed by us) versus indirect (tail current measurement without blocking AMPARs) approach as well as our use of cesium based internal solution to improve voltage-clamp fidelity might have contributed to the differences in the results.

Extending our observation that exposure to stress prevents the effect of early-life stress on object-in-context memory, we examined whether the important stress hormone corticosterone can also differently regulate synaptic transmission in the hippocampal CA1 area between control and early-life stress animals. It is well established that corticosterone by acting on the glucocorticoid receptors can mediate both fast and slow modulation of glutamate-mediated excitatory postsynaptic currents in the adult brain (Karst and Joëls, 2005; Krugers et al., 2010; Karst et al., 2010). Exactly how corticosterone normalizes NMDA/AMPA receptor current, needs to be examined in detail, but preliminary data suggests that corticosterone can increase NMDA currents (Tse et al., 2011), and corticosterone can via activation of mineralocorticoid receptors within minutes increase synaptic GluN2B content and NMDA currents (Mikasova et al., 2017). Several studies have established that the NMDA to AMPA receptor ratio is a good index for probing synaptic modifications as this ratio is fairly conserved across brain regions (Myme, 2003) and a good indicator for experience-dependent plasticity, in particular with stress (Kole et al., 2002; Suvrathan et al., 2014). Together, the effects of early life stress on NMDAR- mediated currents, NMDA/AMPA ratio, glutamate release and its modification by corticosterone provides a mechanism that may explain reduced synaptic plasticity after early life adversity (Champagne et al., 2008; Bagot et al., 2009; Bagot et al., 2012). The normalization by high levels of corticosterone in vitro further strengthens the role of synaptic mechanisms in the behavioral effects of both early and later-life stress episodes on cognitive function.

It remains to be investigated whether glucocorticoids mediate the effects of stress on enhancing contextual memory in ELS mice, e.g. by measuring hippocampal synaptic function after

stress-exposure or conversely (and more directly) whether GR-antagonists are able to modify the effect of stress on behavior in ELS mice. This clearly is a limitation of the current design and future studies will need to resolve this.

Additionally, it interesting to speculate what potential mechanisms might mediate the early-life stress-induced effects on synaptic function. A decrease in NMDAR function alongside enhanced synaptic facilitation in ELS animals, as we observed, might be a result of reduced glutamate availability at CA1 synapses. Several reports indicate that stress and glucocorticoids modulate both NMDAR and AMPAR trafficking as well as the activation of $Ca^{2+}$-dependent kinases to alter vesicular glutamate release (Popoli et al., 2011). Apart from the direct release from axon terminals, synaptic glutamate levels are also critically dependent on its clearance by the surrounding astrocytes. Stressful insults can modulate the glutamate profile by altering the astrocytic coverage and thereby affect synaptic transmission (Roque et al., 2016; Saur et. al., 2016; Zhang et. al., 2015). Future experiments may provide more insight in the underlying mechanism by e.g. investigating mEPSC properties.

### 4.1. Conclusion

This study, apart from providing cellular and behavioral level evidence for the lasting impact of early-life stress, also reveals glutamate-mediated synaptic transmission as a candidate mechanism through which cognitive performance might be enhanced in stressful situations. Lastly, our results provide direct support for the match/mismatch hypothesis that proposes that early-life adversity may program behavior and synapses to function well under stressful conditions (Santarelli et al., 2017).

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**Table 1:** Cellular and synaptic properties of hippocampal CA1 pyramidal neurons

| | Mean ± SEM | | | | P-values Two-factor ANOVA | | | P-values Between group comparisons | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | Ctrl + Veh | ELS + Veh | Ctrl + Cort | ELS + Cort | Main effect: ELS | Main effect: Cort | Interaction: ELS Vs Cort | Ctrl + Veh Vs ELS + Veh | Ctrl + Cort Vs ELS + Cort | Ctrl + Veh Vs Ctrl + Cort | ELS + Veh Vs ELS + Cort |
| Input resistance (MΩ) | 164.4 ±18 | 160.6 ±21 | 155.3 ±8 | 173.0±17 | 0.68 | 0.94 | 0.52 | 0.89 | 0.39 | 0.66 | 0.68 |
| Membrane capacitance (pF) | 165.6 ±17 | 189.7 ±25 | 135.5 ±10 | 144.0±20 | 0.36 | **0.04** | 0.66 | 0.45 | 0.71 | 0.15 | 0.19 |
| AMPA-EPSC Peak (-pA) | 133.8 ±15 | 166.5 ±47 | 101.4 ±8 | 126.8±27 | 0.22 | 0.13 | 0.88 | 0.53 | 0.39 | 0.08 | 0.48 |
| AMPAR-EPSC Rise time (ms) | 2.2 ±0.1 | 3.0 ±0.7 | 2.3 ±0.2 | 2.5 ±0.1 | 0.08 | 0.50 | 0.30 | 0.31 | 0.28 | 0.61 | 0.52 |
| AMPAR-EPSC Decay constant (ms) | 14.8 ±1.2 | 21.5 ±6.4 | 12.7 ±1.7 | 12.4 ±0.9 | 0.23 | **0.045** | 0.20 | 0.35 | 0.89 | 0.34 | 0.22 |
| NMDAR-EPSC Peak (-pA) | 33.8 ±4 | 17.7 ±2 | 29.8 ±4 | 49.4 ±13 | 0.81 | **0.06** | **0.02** | **0.005** | 0.17 | 0.50 | **0.04** |
| NMDAR-EPSC Rise time (ms) | 7.3 ±0.8 | 10.4 ±1.6 | 7.1 ±0.7 | 6.8 ±0.8 | 0.13 | **0.05** | 0.08 | 0.13 | 0.82 | 0.83 | 0.09 |
| NMDAR-EPSC Decay constant (ms) | 59.8 ±9.1 | 64.4 ±14.5 | 39.7 ±4.9 | 36.9 ±4.1 | 0.91 | **0.006** | 0.65 | 0.79 | 0.66 | **0.07** | 0.12 |
| EPSC ratio (NMDA/AMPA) | 0.26 ±0.03 | 0.14 ±0.03 | 0.31 ±0.05 | 0.37 ±0.02 | 0.44 | **0.001** | **0.03** | **0.017** | 0.30 | 0.37 | **0.0002** |
| Paired-pulse ratio | 1.68 ±0.10 | 2.04 ±0.10 | 1.77 ±0.13 | 1.65 ±0.07 | 0.23 | 0.13 | **0.02** | **0.02** | 0.45 | 0.63 | **0.005** |
| No. of cells (animals) | 9 (8) | 6 (5) | 11 (8) | 8 (5) | | | | | | | |

**Table 2:** Cellular and synaptic properties of basolateral amygdala pyramidal neurons

| | Mean ± SEM | | | | P-values Two-factor ANOVA | | | P-values Between group comparisons | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | Ctrl + Veh | ELS + Veh | Ctrl + Cort | ELS + Cort | Main effect: ELS | Main effect: Cort | Interaction: ELS Vs Cort | Ctrl + Veh Vs ELS + Veh | Ctrl + Cort Vs ELS + Cort | Ctrl + Veh Vs Ctrl + Cort | ELS + Veh Vs ELS + Cort |
| Input resistance (MΩ) | 145.0 ±30 | 126.9 ±19 | 264.2 ±83 | 180.4 ±31 | 0.32 | 0.10 | 0.52 | 0.63 | 0.37 | 0.21 | 0.17 |
| Membrane capacitance (pF) | 280.4 ±24 | 277.4 ±22 | 245.2 ±24 | 219.4 ±16 | 0.52 | **0.046** | 0.61 | 0.93 | 0.39 | 0.32 | **0.057** |
| AMPA-EPSC Peak (-pA) | 142.3 ±28 | 151.0 ±24 | 132.5 ±29 | 161.2 ±32 | 0.52 | 0.99 | 0.72 | 0.82 | 0.52 | 0.81 | 0.80 |
| AMPAR-EPSC Rise time (ms) | 4.7 ±1.4 | 4.3 ±1.0 | 2.9 ±0.5 | 3.6 ±1.0 | 0.87 | 0.21 | 0.61 | 0.85 | 0.56 | 0.27 | 0.59 |
| AMPAR-EPSC Decay constant (ms) | 13.9 ±1.4 | 18.2 ±5.3 | 12.2 ±0.9 | 20.9 ±5.3 | 0.10 | 0.9 | 0.59 | 0.45 | 0.16 | 0.36 | 0.73 |
| NMDAR-EPSC Peak (-pA) | 23.4 ±6 | 18.6 ±3 | 16.9 ±3 | 19.5 ±6 | 0.81 | 0.53 | 0.41 | 0.48 | 0.71 | 0.34 | 0.90 |
| NMDAR-EPSC Rise time (ms) | 10.2 ±1.3 | 10.4 ±2.1 | 11.3 ±1.5 | 9.5 ±2.1 | 0.67 | 0.97 | 0.59 | 0.93 | 0.51 | 0.61 | 0.76 |
| NMDAR-EPSC Decay constant (ms) | 93.1 ±21.9 | 92.2 ±40.8 | 79.7 ±15.4 | 79.2 ±24.6 | 0.98 | 0.65 | 0.99 | 0.98 | 0.98 | 0.63 | 0.79 |
| EPSC ratio (NMDA/AMPA) | 0.20 ±0.05 | 0.14 ±0.03 | 0.16 ±0.03 | 0.12 ±0.02 | 0.20 | 0.37 | 0.75 | 0.39 | 0.33 | 0.51 | 0.55 |
| No. of cells (animals) | 7 (7) | 8 (8) | 8 (6) | 7 (7) | | | | | | | |

**Figure legends**

**Fig. 1.** Early-life adversity impairs object-in-context memory only under non-stressful conditions. (A) Schematic of the experimental setup for object-in-context memory test. Mice were habituated on day 1 and were trained on the subsequent day 1 hr post novel-cage stress or control treatment. On day 3, mice were tested for recognition of the context-mismatched familiar object (arrow). (B) Basal plasma corticosterone levels were not altered by early-life stress. (C) Normalized (% of total) number of visits to the out-of-context object. (D) Normalized time spent with the out-of-context object. Numbers indicate the number of animals/group.

**Fig. 2.** Early-life stress does not impair contextual and auditory cued fear memory. (A) The procedure for contextual fear conditioning consisted of habituation followed by conditioning with the shock on day 1 and testing on day 2. (B) Early-life mice froze more immediately after shock on the conditioning day but did not differ from controls on contextual fear memory tested 24 hours later. (C) Auditory fear conditioning on day 1 was carried out similar to contextual fear conditioning, except that it also included a tone that co-terminated with a shock and on day 2 the animals were tested in a different context in the presence of the same tone. (D) Both early-life stressed mice and controls exhibited similar levels of freezing during conditioning and testing, indicating that auditory fear memory was not altered by early-life stress. Numbers indicate the number of animals/group.

**Fig. 3.** Early-life stress does not alter dendritic morphology of principal neurons in the hippocampal CA1 region and basolateral amygdala. **(**A) Representative CA1 pyramidal neuron impregnated using the Golgi-Cox procedure is overlaid on hand-drawn trace of its dendrites. (B) Averaged segmented dendritic length of the apical and basal sides of the CA1 pyramidal neurons at increasing distance from cell soma are not different between groups. (C) No difference in total dendritic length at the apical and basal regions between adult early-life stress and control animals. (D) Representative basolateral amygdala principal neuron stained using the Golgi-Cox procedure is overlaid over hand-drawn trace of its dendritic tree. (E) Mean segmented dendritic length between equidistant concentric

spheres. (F) total dendritic length of basolateral amygdala neurons were not significantly altered by early-life stress. Numbers indicate the number of animals/group.

**Fig. 4.** Early-life stress modulates glutamate currents in the hippocampus. (A) Schematic of the experimental configuration for slice electrophysiology with the recording (R) and stimulation (S) electrode positions. (B) Representative traces of averaged and individual AMPAR- and NMDAR-mediated EPSCs from each experimental group. (C) Peak amplitude of AMPAR-EPSCs are similar across groups. (D) Early-life stress reduces peak amplitude of NMDAR-EPSCs and is normalized after acute glucocorticoid treatment in adulthood. (E) Early-life stress reduces NMDA/AMPA EPSC ratio and is normalized after acute glucocorticoid treatment in adulthood. Numbers indicate cells (animals in parentheses) per group.

**Fig. 5.** Acute glucocorticoid treatment normalizes early-life stress-induced increase in paired-pulse facilitation in hippocampal CA1 pyramidal neurons. (A) Representative traces of averaged and individual paired AMPAR-EPSCs evoked at 50 ms interval. (B) Paired-pulse ratio is significantly enhanced in adult animals subjected to early-life stress and is normalized by acute glucocorticoid treatment. Numbers indicate cells (animals in parentheses) per group.

**Fig. 6.** Glutamatergic currents in the basolateral amygdala are unaffected by early-life stress or acute glucocorticoid treatment. (A) Schematic of the experimental configuration for slice electrophysiology with the recording (R) and stimulation (S) electrode positions. (B) Representative traces of averaged and individual AMPAR- and NMDAR-mediated EPSCs from all the experimental groups. (C) Peak amplitude of AMPAR-EPSCs are similar across groups. (D) Peak amplitude of NMDAR-EPSCs are similar across groups. (E) Ratio of NMDA/AMPA EPSCs remains unchanged after after early-life stress or acute glucocorticoid treatment in adulthood. Numbers indicate cells (animals in parentheses) per group.

**Figure 1**

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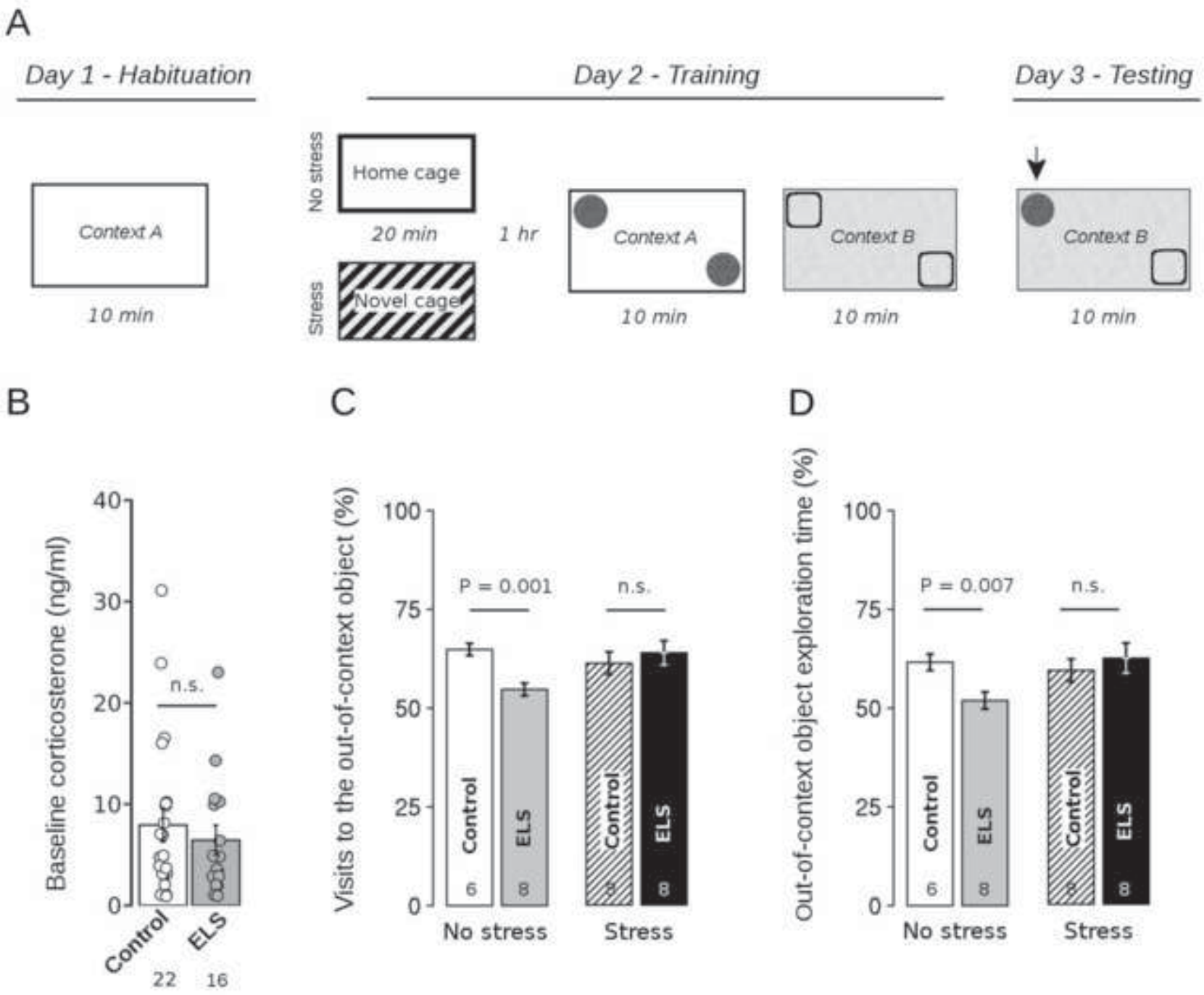

**Figure 2**
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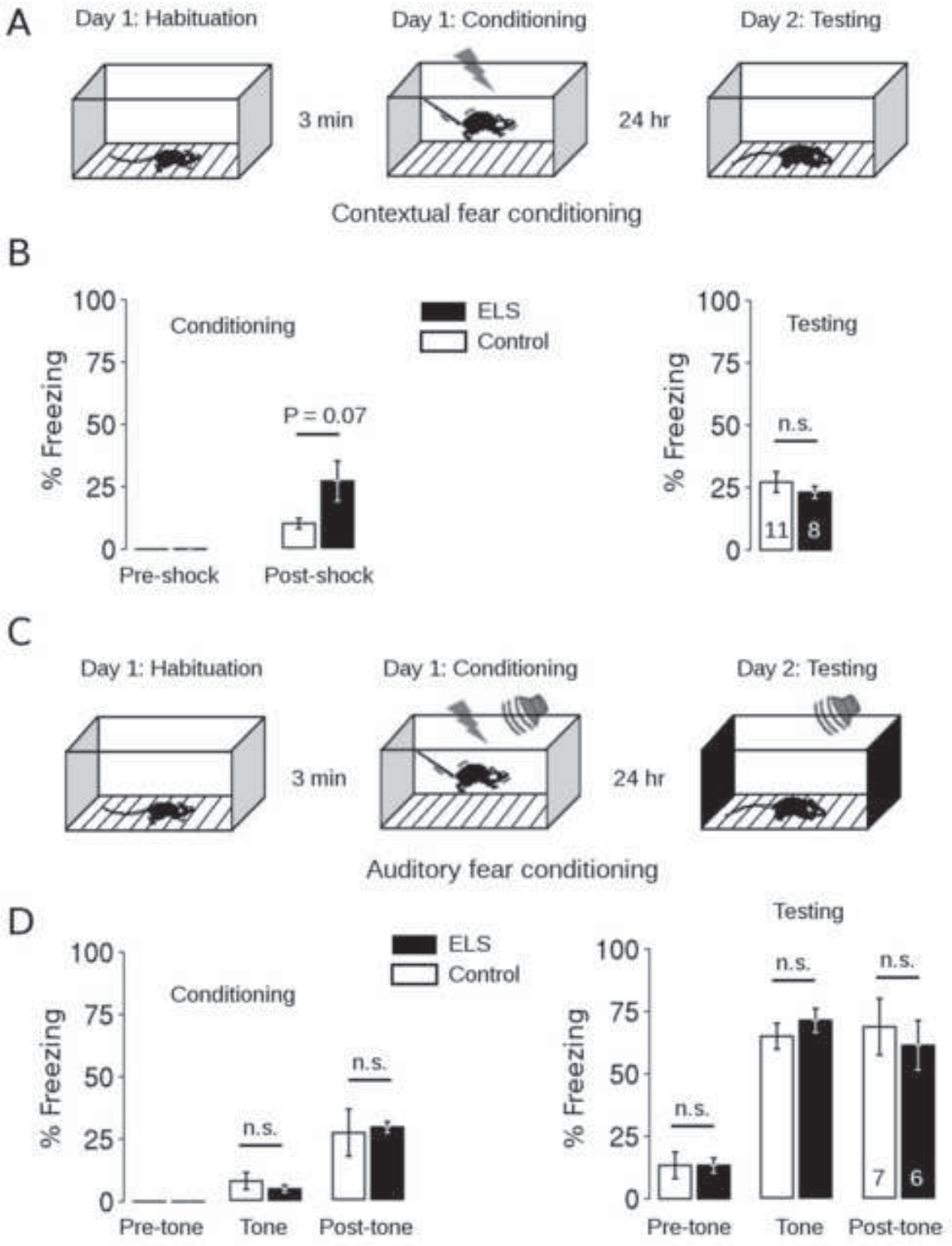

**Figure 3**

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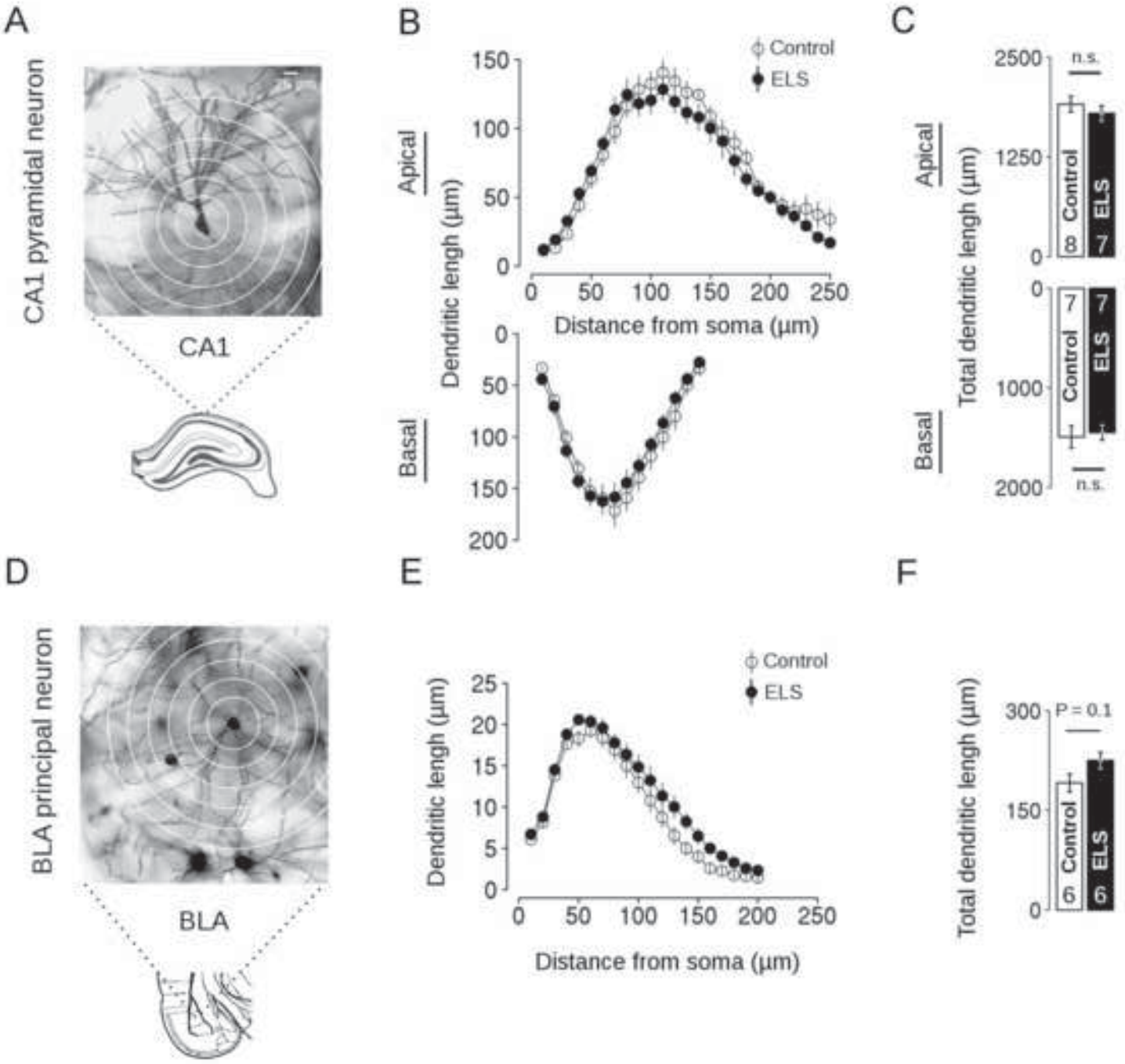

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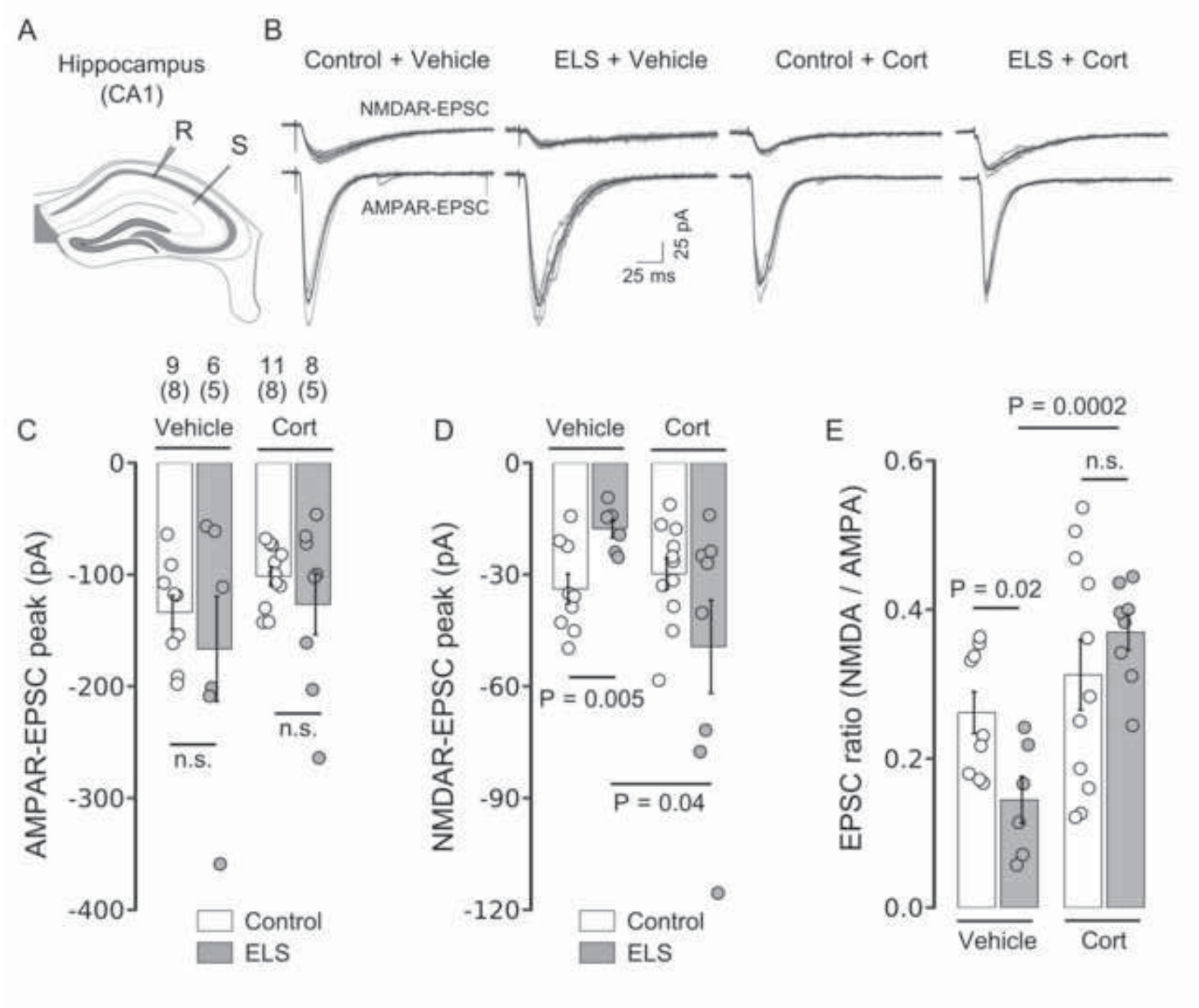

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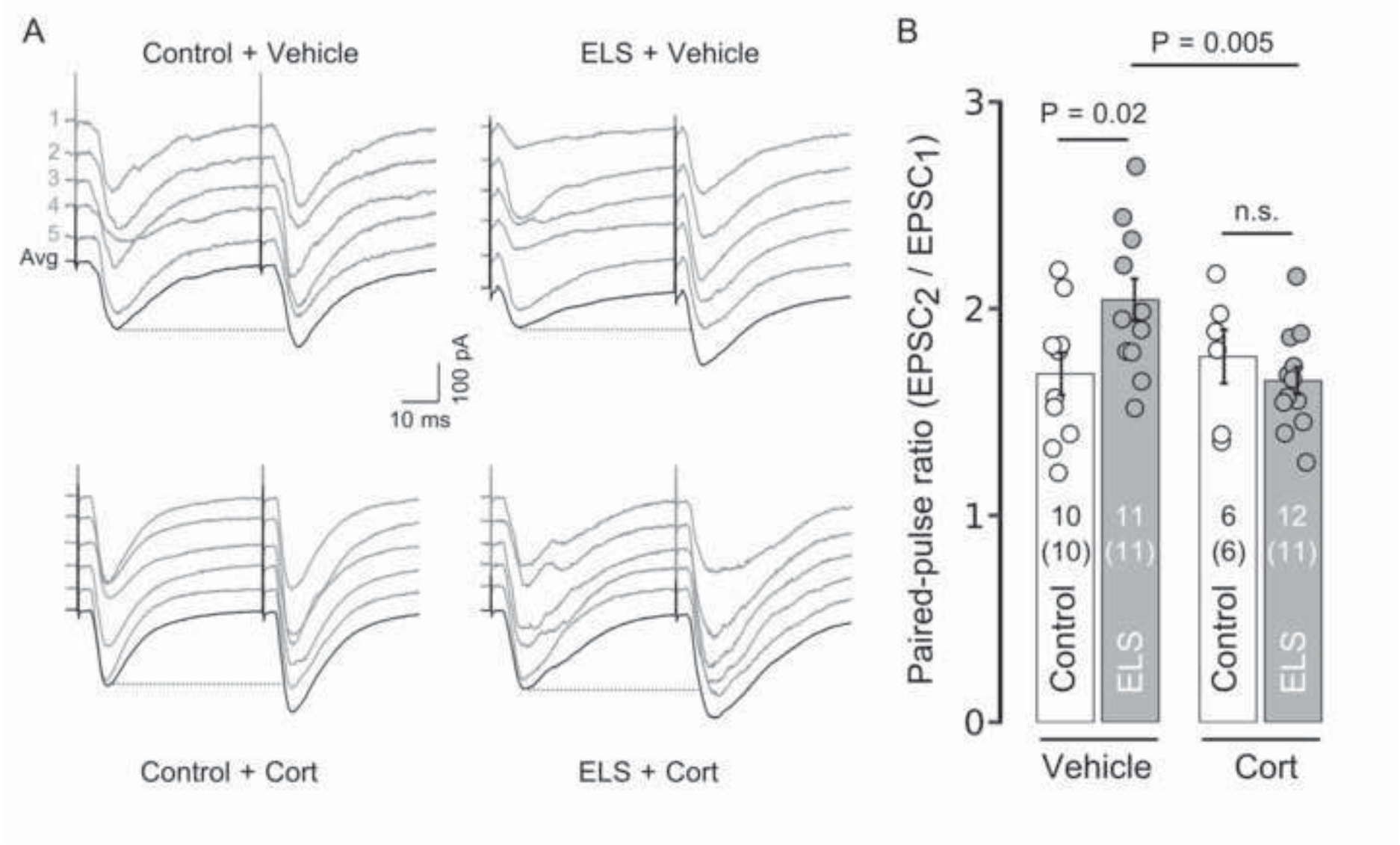

**Figure 6**

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