



Early life stress accelerates behavioral and neural maturation of the hippocampus in male mice

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ABSTRACT

Early life stress (ELS) increases the risk for later cognitive and emotional dysfunction. ELS is known to truncate neural development through effects on suppressing cell birth, increasing cell death, and altering neuronal morphology, effects that have been associated with behavioral profiles indicative of precocious maturation. However, how earlier silencing of growth drives accelerated behavioral maturation has remained puzzling. Here, we test the novel hypothesis that, ELS drives a switch from growth to maturation to accelerate neural and behavioral development. To test this, we used a mouse model of ELS, fragmented maternal care, and a cross-sectional dense sampling approach focusing on hippocampus and measured effects of ELS on the ontogeny of behavioral development and biomarkers of neural maturation. Consistent with previous work, ELS was associated with an earlier developmental decline in expression of markers of cell proliferation (Ki-67) and differentiation (doublecortin). However, ELS also led to a precocious arrival of Parvalbumin-positive cells, led to an earlier switch in NMDA receptor subunit expression (marker of synaptic maturity), and was associated with an earlier rise in myelin basic protein expression (key component of the myelin sheath). In addition, in a contextual fear-conditioning task, ELS accelerated the timed developmental suppression of contextual fear. Together, these data provide support for the hypothesis that ELS serves to switch neurodevelopment from processes of growth to maturation and promotes accelerated development of some forms of emotional learning.

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Introduction

Early life stress (ELS) impacts neural development and significantly increases the lifetime risk for the development of cognitive and affective pathology (Felitti et al., 1998; Anda et al., 2006). The effects of ELS on the development and functioning of the underlying neural circuitry is thought to be a major contributing factor increasing risk for adverse outcomes. Currently, the predominant focus has been on the effect of ELS on the birth, survival, and morphology of cells. In the developing hippocampus and cortex, ELS or stress hormone exposure lead to: diminished cell proliferation and increased cell death (Gould et al., 1991c; Gould et al., 1991b; Tanapat et al., 1998), enhanced turnover and progressive loss of dendritic arbors and spines (Chen et al., 2008; Liston and Gan, 2011; Chen et al., 2013), decreased synaptic density (Teicher et al., 2006), and reduced volume in adolescence and adulthood (Vythilingam et al., 2002; Frodl et al., 2010). These results are consistent with observations in the adult animal, where chronic stress leads to dendritic simplification, spine loss, cell death, and suppressed neurogenesis (Gould et al., 1991a; Radley et al., 2004; Wood et al., 2004; Hajszan et al., 2009).

Based upon these findings, it has been argued that ELS serves to truncate the process of neural development, with adverse behavioral outcomes often being characterized as developmental delays.

This early silencing of growth has also been linked with what appears to be precocious behavioral maturation. For example, in rodents, priming of the developing brain with stress hormones leads to an earlier emergence of defensive behaviors (Takahashi, 1995, 1996). ELS in the form of manipulations of maternal care or stress hormone exposure leads to a precocious switch from appetitive to aversive learning in a fear conditioning paradigm (Sullivan et al., 2000; Moriceau and Sullivan, 2004) as well as adult-like forms of fear extinction in juvenile animals (Callaghan and Richardson, 2014). Recent work indicates that ELS may also alter neurobehavioral development in humans, as individuals exposed to institutionalized rearing were found to have an earlier expression of adult-like functional connectivity between frontal and limbic brain regions compared with age matched controls (Gee et al., 2013). While these studies provide elegant demonstrations that ELS contributes to what appear to be more mature patterns of behavior and neural activity, these studies did not directly assess the effects of ELS on the ontogeny of neural maturation. It is unlikely that the earlier halting of neurogenesis or effects on cell death are solely supporting the accelerated profile of behavioral development observed by others. Here, we sought to test if in addition to truncating processes of cell

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birth, ELS impacts the rates of neural maturation, and if so, what mechanisms might be driving such effects.

To test if ELS impacts rates of maturation, we examined a mouse model of ELS, fragmented maternal care (Rice et al., 2008), and focused on the hippocampus, an area that is highly sensitive to stress, and an area that has reliably been implicated in the development of stress-associated pathology. We used a cross-sectional dense sampling approach throughout early development (4 to 50 days of age) in which we measured developmental changes in hippocampus-dependent contextual fear, gene expression, and immunohistochemical markers of development at short intervals (~4–10 days, Fig. 1). This approach allowed us to directly examine the effects of ELS on the ontogeny of both neural and behavioral measures of development within the same circuit.

Here, ELS led to an earlier emergence of the timed developmental suppression of hippocampus-associated fear behavior in a fear-conditioning paradigm. Using gene expression analyses and immunohistochemistry, we found that ELS led to the earlier onset and expression of biomarkers associated with neural maturation in the hippocampus and an earlier silencing of biomarkers associated with cell proliferation and differentiation. Taken together, these data provide strong support for the hypothesis that, in the hippocampus, ELS leads to a precocious switch from processes of growth to earlier neural and behavioral maturation.

Materials and methods

Subjects

Male C57BL/6N mice were bred in house, had ad libitum access to food and water, and were housed on a 12 h:12 h light:dark cycle. All animal procedures were approved by the Brown University Institutional Animal Care and Use Committee and consistent with the guide for the care and use of animals in research.

Fragmented maternal care

Four days following birth of a litter, the dam and pups were transferred from their standard home cage, to a home cage with a wire mesh floor and a 2 × 4 cm cotton nestlet as their only source of bedding (modification from- (Rice et al., 2008)). Mice continued to have ad libitum access to food and water. Dam and litters remained in these modified housing conditions for seven days, and were then returned to standard housing, containing cob bedding and a 4 × 4 cm nestlet. Control mice were left undisturbed throughout these procedures. Litters were composed of both male and female pups and litters ranges in size from 5 to 8 pups per litter. We observed no effect of litter size on growth restriction or other measures of neurodevelopment (data not shown). All pups were weaned and sex segregated at 21 days of age. For each assay (corticosterone, neurodevelopment, gene expression, and behavior), we took care to insure that animals from a minimum of two separate litters were sampled from. In previous studies of both mice and rats, elegant studies find that this manipulation leads to a fragmentation in

maternal care and elevations in stress hormones in the dam (Molet et al., 2016; Rice et al., 2008; and Avishai-Eliner et al., 2001). Specifically, in those reports, the authors observed an increase in the number of departures from the nest by the dam, but no change in licking and grooming or arched back nursing (Molet et al., 2016). As maternal behavior is difficult to assess mice, in part due to their small size and lack of stereotyped nursing posture, we did not carry out detailed assessment of maternal behavior, and instead relied on our successful replication of other core features of this paradigm, which includes growth restriction of litters.

Developmental assays

To measure early developmental outcomes in control and ELS mice, we collected whole body weight at multiple time points across development. At postnatal day 17, mice were tested on the inverted wire hang task. Mean hang duration over 5 repeated trials was calculated for each animal and used for statistical comparison. On postnatal day 12 and 21 mice were removed from their home cage and individual mice were placed in an open field. Activity of the mouse was digitally recorded for a period of 7 min and distance traveled was measured with the aid of Ethovision XT 9.0 software (Noldus, Leesburg, VA).

Corticosterone ELISA

Mice were transported from the animal colony to the laboratory space and allowed to acclimate for 1 h. Trunk blood was collected by rapid decapitation. To eliminate potential cohort effects, a minimum of at least 2 different litters were used for each developmental time point. Serum corticosterone levels were measured using a competition-based ELISA (AssayMax, Corticosterone ELISA Kit, AssayPro, St. Charles, MO) using the manufacturers protocol. This kit reports a sensitivity of up to 0.3 ng/mL, with a 5–7% intra-assay reliability, and <2% cross reactivity with steroid and stress-related hormones. With this assay, we have observed an intra-assay reliability of 5.5%.

Contextual fear conditioning

Fear conditioning was carried out in Med Associates (St. Albans City, VT) operant chambers using the procedures described in (Pattwell et al., 2011). Briefly, mice received a single session of contextual fear conditioning (3 × 0.7 mA shocks, 1 s in duration) followed 24 h later by a single context test (5 min). Different cohorts of animals, across multiple litters, were used to test mice at the different developmental time points (n = 7 to 18 mice per group/time point). Freezing behavior was scored using the activity tracker module in Noldus Ethovision XT9.0 and verified from video by observers blind to treatment.

Real-time quantitative polymerase chain reaction (RT-qPCR)

For each developmental time point, the hippocampus was collected from animals from at least 2 different litters to eliminate the possibility

Measure	Age								
	P4	P8	P12	P16	P21	P28	P35	P50	P75
Bedding Restriction									
Corticosterone	X	X	X	X					X
Motor Behavior			X	X	X				
Gene Expression	X	X	X	X	X	X	X	X	
IHC				X	X	X			
Conditioning					X	X	X	X	
Freezing Behavior					X	X	X	X	

Fig. 1. Experimental Design. We collected a wide variety of biochemical, genetic, histological, and behavioral measures across early development. Here we plot the timeline of our experimental manipulations, with bedding restriction occurring between P4 and P11. Both during and following this manipulation, we sampled from multiple separate litters of mice to collect basal serum corticosterone levels (Corticosterone), measures of early motor development (Motor Behavior), mRNA to assess gene expression (Gene expression), fixed brain tissue for immunohistochemistry (IHC), and carried out contextual fear conditioning in separate sets of mice (conditioning) and testing for fear expression 24 h following conditioning (Freezing Behavior). For all manipulations, individual animals contributed to only a single measure with unique cohorts of animals being used for the collection of each time point.

of cohort effects on measures of gene expression ($n = 5$ animals per group/per age). Brains were dissected on ice, hippocampus was isolated followed by homogenization in RNazol (Molecular Research Center, Cincinnati, OH) and stored at -80°C until processing. RNA isolation was in accordance with the manufacturers protocol. First strand cDNA synthesis was in accordance with New England Biolabs MmULV protocols (NEB, Ipswich, MA). We used pre-designed and pre-validated Taqman assays from Applied Biosystems (Life Technologies, Norwalk, CT) run in multiplex with housekeeping gene (Beta Actin). For all assays we verified effects using alternate housekeeping genes (GAPDH and 18S rRNA) and found nearly identical effects. For each plate and assay, gene expression was calculated based upon a standard curve included on each plate. We used a CFX384 RT-qPCR system (Biorad, Hercules, CA) and associated software for all gene expression profiling.

Immunohistochemistry

To assess developmental changes in the density of Parvalbumin (PV) or doublecortin (DCX) cells, 16, 21, or 28-day old control or ELS mice were transcardially perfused with saline followed by 4% paraformaldehyde and processed for immunohistochemistry as described in (Bath et al., 2008). Briefly, brains were removed and post-fixed for 1 h, immersed in 30% sucrose and then sectioned on a freezing microtome ($40\ \mu\text{m}$). For immunohistochemical labeling, free-floating sections were blocked with 10% normal goat serum and 0.1% triton-X in phosphate buffered saline. Sections were then incubated with either anti-Parvalbumin (1:1000- Swant, Germany) or anti-doublecortin (1:2000-Millipore, USA) antibody in 4% Normal serum and 0.1% Triton-X for 72 h. Antibody labeling was then revealed by incubation with a species appropriate biotinylated secondary (goat anti-mouse 1:400 or goat anti-guinea pig 1:400- Vector laboratories), followed by ABC (Vector Laboratories; ABC Elite kit), and DAB peroxidase (DAB tablet set; Sigma). Sections were then mounted on glass slides, dehydrated and coverslipped.

To quantify the density of PV and DCX cells in the hippocampus, we used StereoInvestigator and took a systematic random sampling approach. For PV labeling, we sampled a minimum of 6 sections per animal and counted all immuno-positive cells throughout the extent of dentate gyrus (DG). For DCX labeling, we again sampled a minimum of 6 sections per animal and counted all immune-positive cells in the subiculum of the hippocampus. We focused on subiculum, as DCX labeling in other regions of the hippocampus during this developmental period are too diffuse for accurate quantification (Takacs et al., 2007).

Statistics

We used 2-way ANOVA to test for main effects and interactions for age and treatment on measures of gene expression. To test for effects of ELS on the shift in the ratio of NR2a:NR2b, we use a single sample t -test and compared observed ratio's to 1 (equal levels of NR2a:NR2b). For fear conditioning, we used a two-way ANOVA to assess effects of treatment and age on levels of freezing. We used a one-way ANOVA to test for differences in levels of freezing within group, with post-hoc comparisons (Tukey's LSD to correct for multiple tests). For all statistical tests, alpha for significance was set at 0.05. Effect sizes were further estimated by calculating the value of η^2 and Cohen's d for data analyzed by ANOVA and Student's t -test, respectively.

Results

ELS elevates basal stress hormone levels and delays somatic growth

Consistent with previous results in mice (Rice et al., 2008), we found that ELS led to elevated basal serum corticosterone levels compared with control animals (ANOVA, corticosterone, $F_{(1,25)} = 9.67$, $p < 0.01$, $\eta^2 = 0.036$; Fig. 2A). This effect emerged following the completion of

the ELS manipulation, at 16 days of age (post hoc, $p < 0.05$, $d = 2.5$), and was no longer present when mice reached adulthood (post hoc, $p > 0.05$, $d = 0.2$; Fig. 2A). ELS reared pups gained less weight and weighed less than control reared animals across all developmental time points measured (ANOVA, treatment, $F_{(1,413)} = 174.28$, $p < 0.01$, $\eta^2 = 0.015$; Fig. 2B). ELS mice also showed impairments in physical endurance on the wire hang task at 17 days of age (Student's t -test, hang duration, $t_{(1,55)} = 2.26$, $p = 0.03$, $d = 0.6$; Fig. 2C), but no effects on general locomotor activity levels, as measured in the open field test at either 12 days of age (Student's t -test, distance, $t_{(1,74)} = -0.70$, $p = 0.49$, $d = 0.02$) or 21 days of age (Student's t -test, distance, $t_{(1,54)} = 0.64$, $p = 0.53$, $d = 0.18$; Fig. 2D).

ELS accelerates behavioral development in a hippocampus-dependent contextual fear conditioning task

In mice, stable contextual fear (freezing when exposed to a context in which the animal was previously shocked) develops as early as ~15 days of age (Akers et al., 2012). Previously, we have identified a latent period of contextual fear inhibition that rapidly emerges at 28 days of age and persists until 35 days of age when high levels of freezing return (Pattwell et al., 2011). During this period of time, fear responding to the context (freezing) is significantly diminished relative to both earlier and later developmental time points. Here, we tested for changes in this latent period in order to test the effects of ELS on both the timing and rate of behavioral development (e.g. effects of ELS on the onset or length of this window).

Using separate cohorts of control and ELS mice, *independent groups of animals* underwent fear-conditioning training at either P18, P21, P28, P38, or P50 followed by a single test (24 h later) to assess fear of the conditioning context (freezing). We observed a significant overall effect of age on levels of freezing (ANOVA, $F_{(4,110)} = 7.17$, $p < 0.01$, $\eta^2 = 0.179$) and age \times treatment interaction (ANOVA, $F_{(4,110)} = 4.56$, $p = 0.01$, $\eta^2 = 0.114$). Consistent with our previous results, in control mice we found a significant effect of age on levels of freezing (ANOVA, age, $F_{(4,52)} = 5.28$, $p = 0.01$, $\eta^2 = 0.280$) with control mice showing a significant reduction in freezing behavior at 29 days of age compared with all other developmental time points tested (post hoc LSD, $p < 0.05$, Fig. 3A). In ELS mice, we also observed a significant effect of age on levels of freezing (ANOVA, age, $F_{(4,58)} = 6.34$, $p < 0.01$, $\eta^2 = 0.304$). However, for ELS mice, reduced levels of freezing occurred at 22 days of age compared with all other time points (post hoc LSD, $p < 0.05$, Fig. 3B), a full week earlier than control-reared mice (Fig. 3C). For both control and ELS mice, levels of freezing returned to control levels within 1 week following the decrease in freezing behavior.

ELS accelerates neural maturation

Parvalbumin (PV) cells are a late developing class of interneurons in the hippocampus (Wu et al., 2014) with increasing incorporation of PV cells throughout early postnatal development. To assess the impact of ELS on PV cell arrival we used multiple approaches, RT-qPCR to assess developmental changes in gene expression and immunohistochemistry to quantify developmental changes in cell density. For RT-qPCR, we collected mRNA from the hippocampus of mice ($n = 5$ per group) across multiple developmental time points, spanning from pre-stress (4 days of age) to adolescence (50 days of age). We found a significant effect of age on PV expression (ANOVA, age, $F_{(4,56)} = 88.71$, $p = 0.01$, $\eta^2 = 0.614$), and treatment \times age interaction (ANOVA, $F_{(4,56)} = 4.18$, $p = 0.01$, $\eta^2 = 0.029$) with ELS leading to a significant and earlier rise in PV levels. PV levels were higher in ELS mice compared with controls at 12 ($p < 0.01$, $d = 1.93$) and 16 ($p < 0.01$, $d = 2.01$) days of age (Fig. 4A).

In a separate cohort of animals, we used immunohistochemistry and stereological techniques to measure PV cell density across three developmental time-points (P16, P21, and P28). We observed a significant

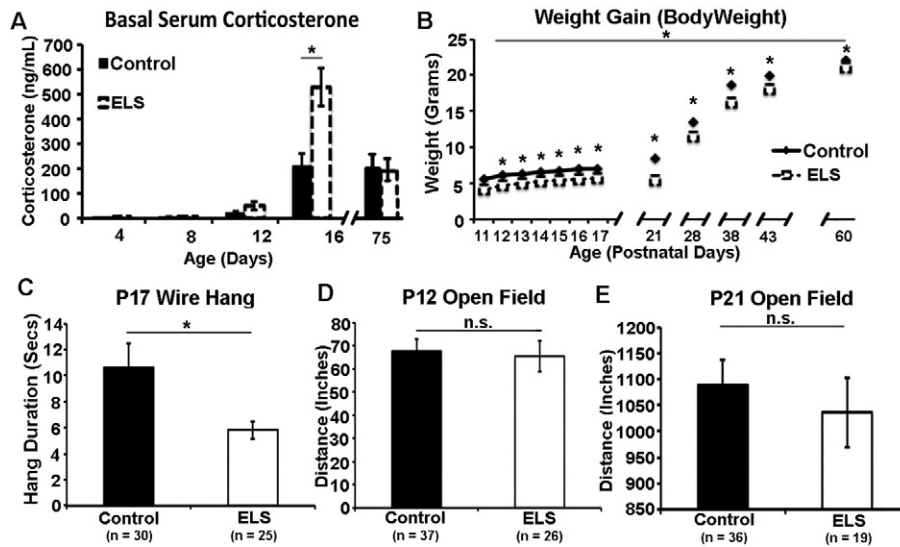


Fig. 2. ELS significantly alters somatic and HPA development. A) Plots of the mean basal serum corticosterone levels sampled across early development. B) Plots of the mean weight for control (closed) and stressed (open) mice. C) Mean hang duration of mice on the inverted wire hang task at 17 days of age (P17). D) Plots of average distance traveled in the open field at 12 days of age (P12) and 21 days of age (P21). For all plots, error depicts the standard error of the mean. * $p < 0.05$.

effect of age (ANOVA, $F_{(2,31)} = 10.47$, $p = 0.01$, $\eta^2 = 0.309$) and significant effect of groups (ANOVA, $F_{(2,31)} = 11.38$, $p = 0.01$, $\eta^2 = 0.168$). At 16 days of age, we observed low levels of labeling and no differences in the density of PV-positive cells between control and ELS animals (Student's t -test, $t = -1.03$, $p = 0.33$, $d = 0.57$ - Fig. 4B). By 21 days of age, we observed an early arrival and increase in PV cell density, and found ELS mice to have a significantly higher PV cell density compared with control mice at this age (Student's t -test, $t = -2.57$, $p < 0.03$, $d = 1.43$ - Fig. 4B). By P28, levels of PV cells in control mice continued to rise and began to approximate levels observed in ELS animals (Student's t -test, $t = -1.93$, $p = 0.09$, $d = 1.16$).

As additional biomarkers of neurodevelopment, we included assessment of changes in expression of genes associated with the development of synaptic plasticity (NR2a:NR2b ratio) and myelination (Myelin basic protein). Over the course of early development, there are significant changes in synaptic maturity and composition. Previous work has demonstrated a developmental shift in the expression of the NMDA receptor subunits NR2a and NR2b (Sheng et al., 1994). Early in development, the NR2b receptor subunit is the predominant form. During the transition to peri-adolescence, expression of the NR2a subunit increases and eventually becomes the predominant form (Liu et al., 2004). As a biomarker of developmental status, we tested for effects of ELS on the timing of the developmental shift in gene expression in ELS relative to control-reared animals.

We observed a significant treatment \times age interaction (ANOVA, $F_{(4,36)} = 3.02$, $p = 0.03$, $\eta^2 = 0.079$). ELS mice had a higher ratio of NR2a:NR2b than control mice at 12 ($p < 0.01$, $d = 3.87$) and 16 ($p < 0.01$, $d = 2.14$) days of age compared with controls, indicating an earlier shift in receptor subunit ratio in ELS mice. In control mice, NR2a became the predominant form (ratio significantly > 1) by 21 day of age (single sample t -test, $\#p < 0.01$, $d = 3.54$). However, in mice exposed to ELS, the ratio of NR2a to NR2b became significantly biased toward NR2a by 16 days of age (single sample t -test, $\#p < 0.05$, $d = 1.60$), indicating that ELS led to an earlier developmental shift in receptor subunit composition (Fig. 4C).

During early postnatal development, there is also a significant increase in myelination of the hippocampus, which based upon imaging studies in mice, is estimated to peak around 30 days of age (Baloch et al., 2009). As a third biomarker of development, we tested for effects of ELS on expression of myelin basic protein (MBP), a key component of the myelin sheath. We observed a significant effect of age (ANOVA, $F_{(4,36)} = 107.02$, $p < 0.01$, $\eta^2 = 0.883$), treatment (ANOVA, $F_{(1,36)} =$

9.678 , $p = 0.004$, $\eta^2 = 0.080$) and a treatment \times age interaction (ANOVA, $F_{(4,36)} = 3.45$, $p = 0.02$, $\eta^2 = 0.029$) on MBP expression. In the hippocampus of ELS mice, MBP levels rose sooner as evidenced by elevated levels relative to control samples at 12 ($p < 0.01$, $d = 1.80$), 16 ($p < 0.01$, $d = 1.64$), and 21 ($p < 0.01$, $d = 1.77$) days of age (Fig. 4D).

ELS leads to an earlier decline in expression of markers of cell proliferation and differentiation

During postnatal development there is a significant decline in rates of cell proliferation and neuronal differentiation as hippocampus development nears completion. Previous work has shown that ELS or exposure to stress hormones leads to a suppression of the proliferation of new cells and increases rates of cell death (Gould et al., 1991c; Gould et al., 1991b). To test the effects of ELS on hippocampus development, we measured changes in the expression of markers associated with cell proliferation (Ki-67 (Kee et al., 2002)) and differentiation (doublecortin (Brown et al., 2003)).

For doublecortin (DCX), a marker expressed by immature neuronal precursor cells, we found a significant effect of age (ANOVA, $F_{(4,36)} = 73.31$, $p = 0.01$, $\eta^2 = 0.817$), treatment (ANOVA, $F_{(1,36)} = 5.40$, $p = 0.03$, $\eta^2 = 0.014$) and treatment \times age interaction (ANOVA, $F_{(4,36)} = 6.27$, $p < 0.01$, $\eta^2 = 0.069$), with ELS leading to lower levels of DCX expression compared with controls at 12 ($p < 0.01$, $d = 3.55$), 16 ($p < 0.01$, $d = 2.06$), and 21 ($p < 0.01$, $d = 1.74$) days of age (Fig. 5A). To confirm that changes in DCX gene expression are indicative of changes in cell density, we used immunohistochemical approaches in a separate cohort of control and ELS 16 day old mice. During this developmental period, DCX-labeling appears diffuse throughout most of the hippocampus, including the dentate gyrus, with more punctate staining emerging later in development (Takacs et al., 2007). Thus, we focused on counting cells in the subiculum, as this region shows clear labeling and allowed for accurate quantification of immuno-positive cells at this developmental time point. Consistent with the gene expression data, we found decreased density of DCX in ELS relative to control-reared mice at 16 days of age (Student's t -test, $t = 3.26$, $p < 0.02$, $d = 2.2$ - Fig. 5B).

For Ki-67, consistent with DCX levels, we found a significant effect of treatment on age (ANOVA; $F_{(4,36)} = 73.89$, $p < 0.01$, $\eta^2 = 0.752$), treatment (ANOVA; $F_{(1,36)} = 11.12$, $p = 0.01$, $\eta^2 = 0.028$) and treatment \times age interaction (ANOVA; $F_{(4,36)} = 12.76$, $p < 0.01$, $\eta^2 = 0.128$). Ki-67 levels were reduced in ELS compared with controls at 12 ($p < 0.01$,

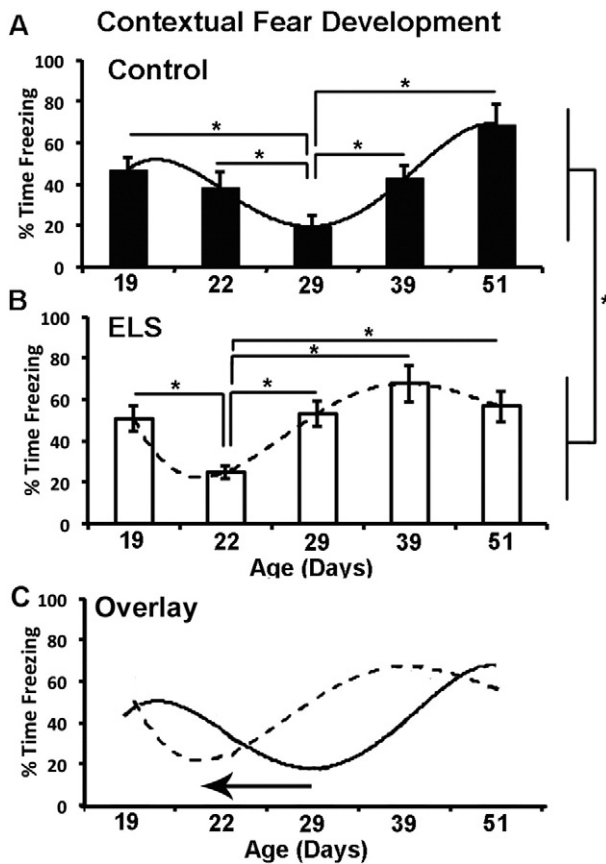


Fig. 3. ELS accelerates the developmental suppression of contextual fear responding. Mean percent duration freezing (\pm SEM) for the 5-minute context test (24 hours post conditioning). Each time point represents a different cohort of mice, sampled from multiple litters. A) Control mice (solid) showed a significant reduction in freezing at 29 days of age, compared with other developmental time points. B) In ELS mice (unfilled), a significant reduction in freezing was observed at 22 days of age compared with mice tested at all other developmental time points. C) Visual comparison of lines of best fit tracking developmental change in freezing behavior for control (solid) and ELS (dashed) mice, show a shift in the trough for freezing behavioral over development, with ELS mice decreasing earlier than control mice. Two-way ANOVA was used to assess overall effects of trial and between groups effects. Within group comparisons ($n = 7$ to 18 mice per group) were computed using one-way ANOVA with post hoc (Tukey's LSD) comparisons to test for effects of age. * $p < 0.05$.

$d = 4.7$) and 16 ($p < 0.01$, $d = 2.5$) days of age followed by elevated levels of Ki-67 in ELS compared with controls at 28 days of age ($p < 0.01$, $d = 2.56$; Fig. 5C).

ELS suppresses expression of glucocorticoid (GR) and mineralocorticoid (MR) receptors in the hippocampus

In rodent models, significant disruptions in the quality or quantity of maternal care, has been associated with suppressed expression of MR and GR (van Oers et al., 1997; van Oers et al., 1998; Avishai-Eliner et al., 1999; Francis et al., 1999; Schmidt et al., 2002; Enthoven et al., 2010; Meaney et al., 2013; Daskalakis et al., 2014), an effect that has also been observed in abused children (McGowan et al., 2009). Effects on MR and GR are thought to impact basal corticosterone levels, feedback inhibition to reduce the stress response, and expression of peptide hormones, effects which may contribute to blunting of neurodevelopment (Champagne and Meaney, 2001; Weaver et al., 2004; Cottrell and Seckl, 2009). Here, we found a significant effect of treatment (ANOVA; $F_{(1,36)} = 5.01$, $p < 0.03$, $\eta^2 = 0.022$) and treatment by age interaction on GR expression (ANOVA; $F_{(4,36)} = 2.99$, $p < 0.03$, $\eta^2 = 0.053$ -Fig. 5A). ELS mice had decreased expression of GR at 12 days of age compared with control-reared animals (Student's t -test, $t = 6.53$, $p < 0.01$,

$d = 4.13$ - Fig. 6A). Similar effects were observed for MR, with decreased levels in ELS hippocampus compared with controls at 12 days of age (Student's t -test, $t = -3.39$, $p < 0.01$, $d = 2.52$ - Fig. 6B).

Discussion

Here, we demonstrate that ELS, in the form of fragmented maternal care, leads to an accelerated profile of hippocampal behavioral development using a fear conditioning paradigm, as well as precocious expression of measures of maturation in the hippocampus. These findings are paired with the observation that ELS results in a more rapid developmental decline in the expression of markers of neuronal proliferation and differentiation in this same structure. Taken together, these novel findings provide the first direct evidence to support the hypothesis that ELS may be driving an earlier transition from processes of growth to processes of maturation at both the behavioral and neural level.

Stress and brain development (growth versus maturation)

ELS has been associated with reduced cell proliferation and increased cell death in the hippocampus (Gould et al., 1991c; Gould et al., 1991b; Tanapat et al., 1998), enhanced dendritic turnover (Liston and Gan, 2011), simplification of dendritic arbors (Brunson et al., 2001; Chen et al., 2008; Liston and Gan, 2011), decreased synaptic density (Andersen and Teicher, 2004), as well as reduced regional volume when measured in adolescence and adulthood (Vythilingam et al., 2002; Frodl et al., 2010; Teicher et al., 2012). Based on these findings, it has been argued that ELS or stress hormone exposure serves to truncate the process of neurodevelopment through suppressive effects on cell birth and survival. Some have posited that this is associated with an earlier onset of excitatory synaptic signaling, which may support development of subpopulations of cells in the hippocampus, however the direct effects of these manipulations on neural maturation were not carried out. In more recent work, ELS or early life exposure to stress hormones have been associated with earlier emergence of defensive behaviors (Takahashi, 1995, 1996), more mature forms of fear-associated learning (Moriceau and Sullivan, 2004) and adult-like fear extinction in rats (Callaghan and Richardson, 2014). In humans, early life adversity has been linked with earlier onset of menarche (Allsworth et al., 2005; Foster et al., 2008) as well as more mature patterns of functional brain activation in children in response to emotional stimuli (Gee et al., 2013). Such observations suggest that ELS may in fact have pro-maturational effects on hormonal, behavioral, and emotional development. However, while many studies have investigated the immediate or the long-term effects of stress on neurodevelopmental outcomes, including cell birth and survival, the effects of ELS on the timing of maturational events has largely been overlooked. This has left open the question of whether more mature patterns of behavior and functional brain activation are in fact due to accelerated neural maturation, or failures in the completion of some secondary developmental process. The data presented here confirms previous reports demonstrating that ELS suppresses measures of growth of the hippocampus and directly tested for effects of ELS on measures of circuit maturation. The current findings support the hypothesis that ELS serves to drive a switch from programs of growth to earlier behavioral and neural maturation in the hippocampus.

Potential mechanisms underlying ELS effects on brain development

A number of pathways have been implicated in mediating the effects of ELS on brain development, including stress-associated changes in corticotropin releasing hormone (CRH), corticosterone, as well as others. In the fragmented maternal care paradigm, ELS is associated with diminished CRH expression within the hypothalamus (Avishai-Eliner et al., 2001; Rice et al., 2008) and increased density of CRH-positive cells in the hippocampus (Ivy et al., 2010). CRH activity in hippocampus has

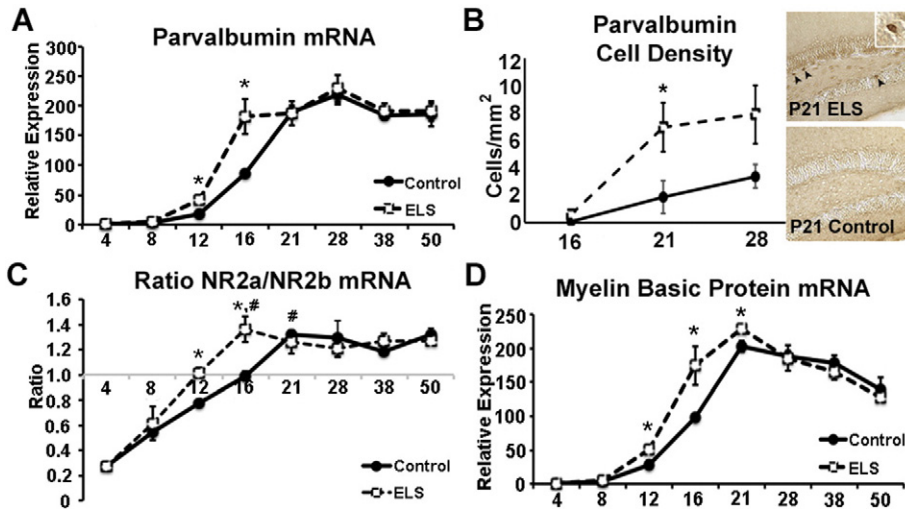


Fig. 4. ELS accelerates the developmental expression of markers of neural and circuit maturation. A) Realtime qPCR (RT-qPCR) tracking developmental change in the mean expression (\pm SEM) of parvalbumin (PV) normalized to beta-actin for control (solid) and ELS (dashed) samples ($n = 5$ per group). B) Quantification of parvalbumin-positive cell density in the dentate gyrus of the hippocampus across development, along with representative photomicrographs of PV-labeling in 21 day old control and ELS brains. C) Plot of the mean ratio of NR2a/NR2b mRNA expression across development for control and ELS hippocampus. D) Plot of relative mRNA expression (\pm SEM) of myelin basic protein (MBP) over development. Two-way ANOVA was used to assess overall effects of age and treatment \times age interactions. Developmental shift in the ratio of NR2a:NR2b was assessed using a single sample *t*-test (relative to 1). # indicates first point in which the ratio of NR2a:NR2b is significantly above 1 (equal ratio). Follow-up comparisons of treatment effects within age were carried out with planned un-paired Student's *t*-tests. * $p < 0.05$. # $p < 0.05$.

been implicated in dendritic remodeling (Chen et al., 2004; Chen et al., 2008; Lin and Koleske, 2010), while diminished hypothalamic CRH may contribute to altered regulation of the hormonal stress response, ultimately contributing to elevations in basal corticosterone levels (Avishai-Eliner et al., 2001; Rice et al., 2008). Early life administration of corticosterone has been linked with diminished cell proliferation and increased cell death (Gould et al., 1991c; Gould et al., 1991b; Tanapat et al., 1998), increased turnover and ultimately decreased number of dendritic spines (Brunson et al., 2001; Chen et al., 2008; Liston and Gan, 2011), and decreased synaptic density (Teicher et al., 2006), effects similar to what is observed following chronic stress in adulthood. While most work has focused on the detrimental effects of ELS and corticosterone exposure on cell birth, death, and morphology, repeated administration of corticosterone, or disruptions in maternal care, have also been shown to be associated with more mature patterns of behavior and functional brain activation in juvenile animals (Moriceau and Sullivan, 2004; Callaghan and Richardson, 2014). Thus, in the developing brain, corticosterone may be playing a functional role in both the regulation of the timing of cell birth and development as well as stimulating the onset of processes of circuit maturation. Such effects could be the consequence of direct activation of adrenal hormone receptors on

cellular signaling, or through downstream effects on gene expression, which may impact activity of other pathways that mediate growth and maturation such as BDNF.

Our observation of a transient elevation in basal corticosterone may have emerged as a function of failures in suppression of HPA axis activity. In mice exposed to ELS, we observed elevations in MR and GR expression, receptors whose function include regulating feedback inhibition to suppress corticosterone release. In previous work, using a variety of models to induced ELS, including maternal separation, maternal deprivation, reduced licking and grooming, as well as maternal bedding restriction, MR and GR expression have been reported to be suppressed, associated with elevated basal corticosterone levels, and lead to poor feedback inhibition when exposed to subsequent stressors (van Oers et al., 1997; van Oers et al., 1998; Avishai-Eliner et al., 1999; Francis et al., 1999; Schmidt et al., 2002; Enthoven et al., 2010; Meaney et al., 2013; Daskalakis et al., 2014). Our observation of altered receptor expression could support the transient elevations in corticosterone observed here, as well as have played a role in driving changes in gene expression that both suppress growth as well as stimulate maturation. However, more work will be required to test the unique role of altered receptor expression on our observed measures.

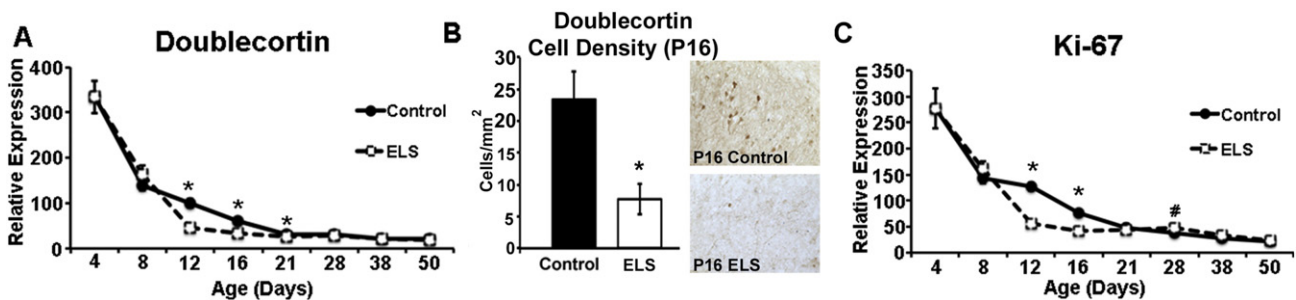


Fig. 5. ELS accelerates the developmental decline in expression of markers of growth. A) RT-qPCR to assess doublecortin (DCX) mRNA levels across development in hippocampus of control and ELS mice. B) Quantification of DCX-positive cell density in subiculum of 16 day old control and ELS mice, along with representative photomicrographs of DCX-labeling. C) Plots of the developmental expression of Ki-67, a marker expressed by proliferating cells. All data are presented as mean (\pm SEM). RT-qPCR were normalized to beta-actin and derived from $n = 5$ samples per group. Two-way ANOVA was used to assess overall effects of age and treatment \times age interactions. Follow up comparisons of treatment effects within age were carried out with planned un-paired Student's *t*-tests. * $p < 0.05$.

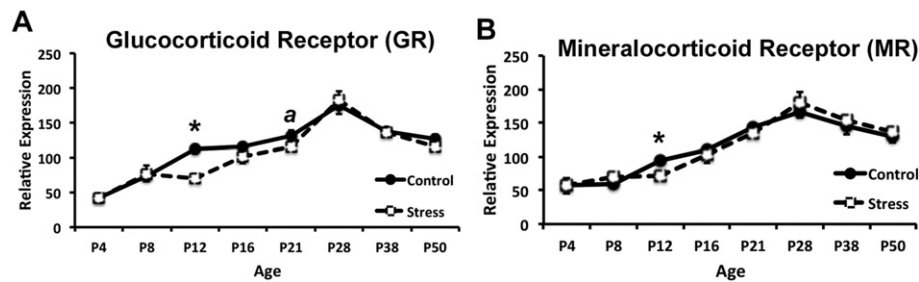


Fig. 6. ELS leads to suppressed expression of GR and MR in the hippocampus. RT-qPCR to assess gene expression in hippocampus across development. Mean expression (\pm SEM) normalized to beta-actin is presented for control (solid) and ELS (dashed) samples ($n = 5$ per group). A) developmental expression of glucocorticoid receptor (GR) and B) developmental expression of mineralocorticoid receptor (MR). Two-way ANOVA was used to assess overall effects of age and treatment \times age interactions. Follow up comparisons of treatment effects within age were carried out with planned un-paired Student's *t*-tests. * $p < 0.05$.

Possible adaptive benefits of accelerated neurobehavioral maturation

Chronic engagement of the stress response in adults can result in significant effects on brain and neuronal morphology, with particularly detrimental effects on the hippocampus. These effects have been described as toxic effects of stress that compromise the health of cells and increases their sensitivity to toxic challenge (Lupien et al., 2009). A possible alternative hypothesis is that morphological changes and suppressed growth could be an adaptation by which the body reallocates available resources to sustain sympathetic arousal at the expense of maintaining metabolically demanding neural tissue. Regardless, the effects of ELS may compromise neural and neuroendocrine development, contributing to enhanced vulnerability to disorder later in life (Lupien et al., 2009). However, such interpretations are difficult to rectify with what appears to be an accelerated rate of neural maturation, observed here. An earlier switch to maturation may represent an adaptation, in which ELS drives an earlier transition from growth to maturation, promoting earlier functional development, in order to support egress from a stressful early environment. This early transition from growth to maturation may provide short-term advantages, but carry with it consequences for long-term functioning and contribute to increased risk for later pathology. However, more work will be required to draw a direct link between the effects of stress on the timing of circuit development and pathology.

Here we focused on measures of hippocampus development and the development of hippocampus-associated behaviors. The hippocampus was chosen based upon its relevance for memory, regulation of emotion, and previous work demonstrating significant sensitivity of this structure to both stress and stress hormones. However, more expansive studies will be required that investigate potential generalizability of these effects to other brain regions, such as amygdala and frontal cortex. We provide behavioral, genetic, and immunohistochemical measures to test for effects of ELS on the timing of developmental events. However, additional work will be required to understand the functional consequences of these changes, such as if the earlier shift in NR2a:NR2b ratio has consequences for electrophysiological plasticity.

In the current investigation, we only report data from male mice. It is well known that males and females respond differently to stress, and that differences in outcome depend upon the type and timing of the stressor. It has also been demonstrated in clinical populations that females are at increased risk of developing stress-associated pathology (Gater et al., 1998; Weissman et al., 2005). If accelerated maturation contributes to later risk for the development of affective or cognitive disturbance, more work will be required to test if ELS-associated acceleration in neural maturation applies equally to both males and females. If accelerated maturation represents a compensation of the system to cope with early adversity, and significant sex differences are observed in response to ELS, this could represent a potential neural basis for sex disparity in risk for disorder and a possible mechanism to target for treatment of stress-associated pathology.

Conclusions

In summary, the studies presented here represent the first direct demonstration that ELS is associated with an acceleration of both behavioral and neural maturation of the hippocampus. Such findings reveal novel effects of ELS on processes of growth and maturation and identify potential mechanisms regulating these effects. Such insights could be important for informing the way we think about stress effects on developmental trajectories and possible mechanism underlying later development of pathology. The current findings demonstrate how stress might be driving changes in developmental process and possibly stimulating maturation, as opposed to simply truncating the process of growth.

Conflict of interest

The authors declare no competing interest.

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