Chronic Early Life Stress Alters Developmental and Adult Neurogenesis and Impairs Cognitive Function in Mice

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ABSTRACT: **Early life stress (ES) increases vulnerability to psychopathology and impairs cognition in adulthood. These ES-induced deficits are associated with lasting changes in hippocampal plasticity. Detailed information on the neurobiological basis, the onset, and progression of such changes and their sex-specificity is currently lacking but is required to tailor specific intervention strategies. Here, we use a chronic ES mouse model based on limited nesting and bedding material from postnatal day (P) 2–9 to investigate; (1) if ES leads to impairments in hippocampus-dependent cognitive function in adulthood and (2) if these alterations are paralleled by changes in developmental and/or adult hippocampal neurogenesis. ES increased developmental neurogenesis (proliferation and differentiation) in the dentate gyrus (DG) at P9, and the number of immature (NeurD1$^+$) cells migrating postnatally from the secondary dentate matrix, indicating prompt changes in DG structure in both sexes. ES lastingly reduced DG volume and the long-term survival of developmentally born neurons in both sexes at P150. In adult male mice only, ES reduced survival of adult-born neurons (BrdU/NeuN$^+$ cells), while proliferation (Ki67$^+$) and differentiation (DCX$^+$) were unaffected. These changes correlated with impaired performance in all learning and memory tasks used here. In contrast, in female mice, despite early alterations in developmental neurogenesis, no lasting changes were present in adult neurogenesis after ES and the cognitive impairments were less prominent and only apparent in some cognitive tasks. We further show that, although neurogenesis and cognition correlate positively, only the hippocampus-dependent functions depend on changes in neurogenesis, whereas cognitive functions that are not exclusively hippocampus-dependent do not. This study indicates that chronic ES has lasting consequences on hippocampal structure and function in mice and suggests that male mice are more susceptible to ES than females. Unraveling the mechanisms that underlie the persistent ES-induced effects may have clinical implications for treatments to counteract ES-induced deficits.** © 2014 Wiley Periodicals, Inc.

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INTRODUCTION

Early life stress (ES) programs the brain for life. Both clinical (Chugani et al., 2001; Nelson et al., 2007; Yasik et al., 2007; Gatt et al., 2009; Mueller et al., 2010; Alastalo et al., 2013; Pesonen et al., 2013) and pre-clinical studies (Brunson et al., 2005; Aisa et al., 2007; Oomen et al., 2010), reveal a very strong association between stressful early life experiences and impaired cognitive function and emotional health later in life. Sadly, stressful early life experiences (e.g., experiencing parental loss or family disruption, physical/sexual abuse, neglect, war, poverty, illness, or institutionalization) concern children anywhere in the contemporary world. For example, in the United States, every year more than 1 million children are exposed to sexual or physical abuse or severe neglect (Sedlak et al., 2013). Hence, from both a societal as well as an economic point of view, it is critical to better understand the underlying mechanisms to find effective interventions to prevent or reverse the lasting consequences of ES on mental health.

There is increasing evidence that the ES-induced cognitive deficits might result from permanent alterations in structure and function of the hippocampus. This brain region is key for cognitive functions (Squire, 1992) and continues its development into the postnatal period (Pleasure et al., 2000), making it highly sensitive to the early life environment. Indeed, both human as well as animal studies have shown that ES exposure is associated with persistently diminished hippocampal volume (Bremner et al., 1997; Vythilingam et al., 2002; Andersen et al., 2008; Gatt et al., 2009; Frodl et al., 2010; Herpfer et al., 2012; Teicher et al., 2012) and with altered hippocampal dendritic complexity and synaptic integrity (Huot et al., 2002; Brunson et al., 2005; Leventopoulos et al., 2007; Champagne et al., 2008; Ivy et al., 2010; Oomen et al., 2011), indicative of an inappropriate development and altered structure of this brain region.

A subregion of the hippocampus, the dentate gyrus (DG), is critically involved in learning and memory (Zhao et al., 2008) and in regulating the stress response (Snyder et al., 2011; Fitzsimons et al., 2013). It is also one of the very few brain regions that

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maintains the ability to generate new neurons throughout adult life. This process, named adult neurogenesis, originates from the proliferation of neuronal progenitor cells located in the subgranular zone (SGZ), which is followed by the differentiation and maturation of a subset of these newly generated cells into fully functional neurons that subsequently integrate into the existing hippocampal circuitry. It is well established that the newly formed neurons make critical contributions to hippocampal functioning and are involved in specific aspects of hippocampus-dependent learning and memory (Deng et al., 2010; Oomen et al., 2014), such as long-term retention of spatial memory (Snyder et al., 2005; Imayoshi et al., 2008; Jessberger et al., 2009), object recognition memory (Bruel-Jungerman et al., 2005; Jessberger et al., 2009), and spatial pattern separation (Clelland et al., 2009; Sahay et al., 2011). This unique form of neuronal plasticity is lastingly affected by the early life environment (Korosi et al., 2012), pointing to an important role for adult neurogenesis in mediating ES-induced cognitive impairments. The negative effects of ES on hippocampal neurogenesis in adulthood (Mirescu et al., 2004; Oomen et al., 2010) and/or in (early) puberty (Nair et al., 2007; Oomen et al., 2009; Suri et al., 2013) have been established in rat models of acute or recurrent early life stress (e.g., maternal separation or maternal deprivation). These studies indicated that the various phases of the adult neurogenic process can be persistently altered by exposure to stress early in life. However it is not known if ES also disturbs levels of developmental neurogenesis (between P0 and P15; Gould et al., 1991; Tanapat et al., 1998). This could be important because altered developmental neurogenesis might affect DG structure and adult neurogenic capacity. In fact, while both developmentally and adult-generated granule cells follow similar maturation steps and reach high degree of functional similarity eight weeks after they are born (Laplagne et al., 2006), important differences between developmental and adult neurogenesis exist. Indeed, during the early postnatal period there is a higher rate of neurogenesis in a proliferative zone, next to the ventricle, which is not yet restricted to the SGZ (Mathews et al., 2010). These developmentally born cells will migrate to form the granular cell layer (GCL) and the future neurogenic niche. To understand whether alterations in postnatal and/or adult neurogenesis after ES are indeed involved in the later cognitive decline, we wondered: (i) if the ES-induced alterations in neurogenesis have an early onset, (ii) if these early changes then result in altered levels of adult neurogenesis, and importantly (iii) if the ES-induced cognitive impairments are neurogenesis dependent or independent (Lazic, 2012). In other words, are the changes in cognition and neurogenesis just merely correlative or are the ES-induced changes in neurogenesis determinant in the ES-induced cognitive impairments?

A final issue that needs clarification is the emerging evidence for a sex-dependent effect of ES on hippocampal structure and function (Llorente et al., 2009; Oomen et al., 2009, 2010, 2011; Frodl et al., 2010; Mak et al., 2013) with males generally being more vulnerable to ES. This phenomenon might underlie existing sex differences in stress-related psychopathologies such as schizophrenia and depression (Bale et al., 2010). However, very little is known about the nature of these differences as very few studies compare the ES effects of female and males simultaneously (Oomen et al., 2009). Here, we study both male and female offspring to reveal if ES has sex-specific effects on hippocampal structure and function.

In this study, we will address effects of early life stress in mice. As mentioned above, rat models have been used most frequently but given the current availability of (recombinant) inbred strains of mice, expanding our knowledge of ES effects in mouse models becomes increasingly relevant. To this end, we will use a chronic ES mouse model, which recapitulates important elements of human neglect/abuse situations, where the mother is present but provides unpredictable and fragmented care (Rice et al., 2008; Baram et al., 2012). We will here present how chronic ES affects (i) DG volume, (ii) levels of developmental as well as adult neurogenesis, (iii) the long-term survival and cell fate of developmentally generated granule cells, and (iv) adult cognitive function. We will further study sex-specific effects and thereby also address for the first time effects of chronic ES in female mice offspring. Finally, for the first time we will apply a novel and validated statistical causal model (Lazic, 2012) to test whether the ES-induced cognitive impairments are neurogenesis-dependent.

Our findings provide evidence for early onset alterations in neurogenesis after ES exposure, increased vulnerability of male mice to the lasting consequences of chronic ES, and a functional relevance of altered levels of neurogenesis in the observed cognitive impairments in adulthood.

MATERIALS AND METHODS

Mice and Breeding

A total of 26 litters (13 Ctl and 13 ES litters), containing five-six pups each, was used. All mice were kept under standard housing conditions (temperature 20–22°C, 40–60% humidity, with standard chow, and water ad libitum). Animals were kept on a standard 12/12h light/dark schedule (lights on at 8 AM). For the long-term studies, animals were allowed to mature; these animals were weaned at P21 and housed in groups of the same sex and age (2–3 animals/cage). All experimental procedures were conducted under national law and European Union directives on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam.

To standardize the perinatal environment, mice were bred in house; 8-weeks old male and 10-week old female C57Bl/6J mice were purchased from Harlan Laboratories B.V. (Venray, The Netherlands) and allowed to habituate for one week before breeding. After acclimatization, two females and one male were housed together for one week to allow mating. After another week of paired-housing, pregnant primiparous females were housed individually in a standard cage covered with a filtertop and monitored every 12 h for the birth of pups. When a litter

was born before 9.00 AM, the previous day was designated the day of birth (postnatal day 0; P0).

Early Life Stress Paradigm

The early life stress paradigm consisted of the limited nesting/bedding-material procedure described previously (Rice et al., 2008) ES model was initiated on the morning of P2. After the litters were culled to six pups (including both sexes), the dams and pups were weighted and housed in the ES or control condition. Throughout all procedures, manipulation was kept to a minimum to avoid handling effects. Control cages were equipped with standard amounts of sawdust bedding and nesting material (one square piece of cotton nesting material (5 × 5 cm; Technilab-BMI, Someren, The Netherlands). In the ES cages, the bottom was covered with a little amount of sawdust bedding and a fine-gauge stainless steel mesh was placed 1 cm above the cage floor. On top of the mesh, half a square piece of cotton nesting material (2.5 × 5 cm) was placed (see Fig. 2a). Cages were covered with a filtertop. Both groups were left completely undisturbed until P9.

Maternal behavior was observed twice daily from P2 till P8: in the light phase (9.00 AM) and in the dark phase (8.30 PM) during 48-min observation sessions. Levels of activity of the dam were scored every third minute, resulting in 16 one-minute epochs per observations session. The behaviors that were scored, included: exits of the dam from the nest, nursing behavior (arched back nursing, low nursing, and side nursing), licking and grooming of the pups, carrying the pups, time spent reconstructing the nest (nesting behavior), and dam-related behaviors off the nest (eating, drinking, self-licking, or grooming).

On the morning of P9, dams and pups were moved to standard cages. For short-term studies, animals were sacrificed on the morning of P9 (between 8.00 and 9.30 AM) by rapid decapitation or by transcardial perfusion (see below). The pups used to measure levels of stress hormones, were rapidly removed from their cage, weighted, and decapitated within 2 min of their disturbance. Trunk blood was collected in ice-cold EDTA-coated tubes (Sarstedt, Etten-leur, The Netherlands), placed on ice and centrifuged at 13,000 rpm for 15 min after which plasma was stored at −20°C. Basal plasma corticosterone (CORT) levels were measured using a commercially available radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands). Intra-assay coefficiency of variation was 0.2–10.6%. The adrenal glands and thymus were dissected and weighted.

Cell Birth Dating

The cell birth date marker 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) was used to study cell fate and survival of both developmentally and adult-born neurons. In all experiments, BrdU stock solutions (10 mg/mL dissolved in sterile saline + 0.007M NaOH) were freshly made on the morning of the day of injection. To examine cell fate and long-term survival of developmentally born cells, pups were IP injected once per day with 50 mg/kg BrdU for five consecutive days (P9–P13) and killed by transcardial perfusion at P150. To examine cell fate and survival of adult born cells, a different group of adult mice was IP injected three weeks after the final behavioral test with 100 mg/kg BrdU three times per day for two consecutive days (at P220–P221) and killed by transcardial perfusion four weeks after the last injection at P250.

Tissue Preparation

Mice of 9, 150, and 250 days old were anaesthetized by an IP injection of pentobarbital (Euthasol® 120 mg/kg) and transcardial perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB = 0.1M, pH = 7.4). Thymus and adrenals were dissected and weighted. Brains were removed and postfixed overnight in PFA/0.1M PB at 4°C and stored in PB with 0.01% sodium azide at 4°C until further processing. After overnight cryoprotection in 30% sucrose/0.1M PB, frozen brains were cut in 40 μm thick coronal sections using a sliding microtome and stored in antifreeze solution (30% Ethylene glycol, 20% Glycerol, 50% 0.05M PBS) at −20°C until processing. Pup brain sections were divided over 4 parallel series, adult brains were divided over 6 parallel series.

DAB Immunohistochemistry

To determine the direct and lasting effects of chronic ES on neurogenesis, immunohistochemistry was used to identify cells in different stages of neurogenesis (proliferation, differentiation, and survival) in P9 and adult mouse brain tissue. All stainings were performed on parallel series from the same brains within an age group.

Immunocytochemistry for the protein Ki67, which is expressed during all phases of the cell cycle, was used to identify proliferating cells. Previous to Ki67 staining, sections were mounted on glass (Superfrost Plus slides, Menzel, Braunschweig, Germany) and first antigen retrieval was performed by heating the sections in 0.1M citrate buffer (pH = 6.0) in a standard microwave (Samsung M6235) to a temperature of ∼95°C for 15 min (5 min at 800 Watt, 5 min at 400 Watt, and 5 min at 200 Watt).

NeuroD1, a basic helix-loop-helix protein that acts as a transcription factor during neuronal commitment, was used as a marker of differentiating neuronal cells in P9 tissue. Calretinin (CR) was used as a marker of differentiating neuronal cells in P9 tissues because CR expression is restricted to a shorter time-window as NeuroD1, which allows detecting differences in the number of newborn neuronal cells during a late stage of neurogenesis. In adult mouse brains, the microtubule-associated protein doublecortin (DCX), which is expressed in newborn, migratory cells from the progenitor stage to the CR-positive stage, was used as a marker for young, differentiating neurons. For NeuroD1, CR, and DCX stainings were conducted using free-floating sections.

For all stainings, both premounted and free-floating sections were incubated with 0.3% H_2O_2 for 15 min to block

endogenous peroxidase activity. After 3 × 5 min rinsing in wash buffer (0.3% Triton X-100 in 0.05M TBS for P9 tissue; 0.05M TBS for adult tissue), sections were incubated for 30 min in blocking buffer and subsequently incubated with primary antibody for one hour at room temperature followed by overnight incubation at 4°C. Primary antibodies were used as follows: polyclonal rabbit anti-Ki67 (Novocastra NCL-L-Ki67_MM1, 1:20,000 in Supermix (0.5% Triton X-100, 0.25% gelatine in 0.05M TBS); blocking buffer: 2% milk powder in TBS), polyclonal goat anti-NeuroD1 [SantaCruz Biotechnology sc-1084; 1:1,000 in blocking buffer (1% bovine serum albumin, 0.3% Triton X-100 in 0.05M TBS)], polyclonal rabbit anti-CR [Swant 7697; 1:1,000 in blocking buffer (2% normal goat serum, 0.3% Triton X-100 in 0.05M TBS)], polyclonal goat anti-DCX (SantaCruz Biotechnology sc-8066; 1:800 in Supermix; blockingbuffer: 2% milkpowder in TBS). Then, sections were rinsed 5 × 5 min with wash buffer and incubated with biotinylated secondary antibodies in Supermix (1:200 goat anti-rabbit, Vector Laboratories or 1:200 donkey anti-goat, Jackson Laboratories) for 2 h at room temperature followed by a 90 min incubation with avidin-biotin complex (ABC kit, Elite Vectastain Brunschwig Chemie, Amsterdam, 1:800). For Ki67 and DCX, additional amplification with tyramide (1:500, 0.01% H_2O_2, once, for 30 min) was performed. Subsequent chromogen development was performed with diaminobenzidine (20 mg per 100 mL 0.05M Tris, 0.01% H_2O_2).

Fluorescent Immunohistochemistry

For survival and cell fate determination, BrdU-labeled cells were detected and double-labeled with the neuronal nuclear marker NeuN and the proliferation marker Ki67. For all stainings, antigen retrieval was performed on premounted sections as described above. After 3 × 5 min rinsing with 0.05M TBS, sections were incubated for 30 min with blocking buffer (1% bovine serum albumin in TBS) and subsequently incubated with primary antibody in incubation mix (1% bovine serum albumin, 0.3% Triton X-100 in 0.05M TBS) for one hour at room temperature followed by overnight incubation at 4°C. Primary antibodies were used as follows: monoclonal rat anti-BrdU (Accurate Chemical and Scientific Corporation OBT0030, 1:200), polyclonal rabbit anti-Ki67 (Abcam 15580; 1:1,000), and monoclonal mouse anti-NeuN (Millipore MAB 377; 1:1,000). After rinsing 5 × 5 min with wash buffer, sections were further stained with Alexa fluor-conjugated secondary antibodies (Invitrogen; 1:1,000) for 2 h at room temperature and covered with DAPI-containing Vectashield.

Imaging and Quantification

For DAB immunohistochemistry, quantification procedures were performed blindly on a Zeiss Axiophot light microscope with Microfire camera (Coptronics) using StereoInvestigator software (MicroBrightField, Germany). Coronal sections of matched anatomical levels along the rostro-caudal axis were used for analysis. Quantification was performed in both hemispheres on six sections/animal for pups with an intersection distance of ~160 μm and on eight sections/animal for adults with an intersection distance of ~240 μm. $Ki67^+$, CR^+, and DCX^+ immunoreactive cell counts were performed manually by means of a modified stereological procedure, using a 20× objective (200× magnification), the total cell-counts were multiplied by 4 (for pups) or 6 (for adults) to obtain an estimation of the total number of immunoreactive cells per DG. For quantification of $NeuroD1^+$ expression in the pup DG, stereological counting was performed using optical fractionator software (StereoInvestigator, MicroBrightField) with a 40× objective (400× magnification). The following optimized settings were applied: distance between sampled sections: 320 μm, counting frame: 10 × 10 μm, stepping size: 80 × 80 μm, dissector: 8 μm. The mean calculated coefficient of error in this experiment was 0.07, thereby indicating a high precision in the estimated total number of $NeuroD1^+$ cells.

Immunoreactive cells were counted in the various anatomical (and functional) subregions of the DG: granular, subgranular, and hilar region, supra versus infrapyramidal and rostral versus caudal and location. All sections rostral to bregma −2.92 (Franklin and Paxinos, 2007) were considered rostral, and all other sections were considered caudal. Cell counts were represented as: (i) total number of immunoreactive cells per unilateral DG (hilus + SGZ + GCL) and (ii) unilateral granular zone (SGZ + GCL) and as numerical density (number of immunoreactive cells/mm^3 of the granular zone).

To obtain volume estimations of the DG and the granular zone in mm^3, the Cavalieri principle was applied: a surface estimation (in mm^2) of the DG at each level was obtained by boundary contour tracings using Stereoinvestigator software, these were first multiplied by 4 (for pups) or 6 (for adults), as sections were split into 4/6 series, and then multiplied by the thickness of the sections (0.04 mm). To estimate numerical densities of $NeuroD1^+$ cells in the hippocampal migratory stream (HMS), the length of the HMS was measured by means of a straight-line measurement from the secondary dentate matrix in the hippocampal subventricular zone to the lateral corner of the infrapyramidal blade of the DG. The number of $NeuroD1^+$ cells in the HMS is normalized to this length measurement.

For counting BrdU, overview images of the fluorescent BrdU double-labeled DG were taken on a Leica CTR5500 microscope (20× objective) using the Leica MM AF program (MetaMorph, version 1.6.0). Stitched Z-stack images were acquired using the multidimensional acquisition function with the following settings: FluoA4 (DAPI), gain of 1, exposure time of 15 ms; FluOL5 (BrdU), gain of 1, and exposure time of 150 ms.

NeuN/BrdU coexpression was then evaluated in 200–250 BrdU-nuclei per animal. From each animal ($N = 6$/condition), a total of 15 Z-stack images were taken; from five hippocampal sections, the suprapyramidal blade, the infrapyriamidal blade and the crest were imaged using a Zeiss LSM 510 confocal laser-scanning microscope with a 63× oil objective. The ratio of NeuN/BrdU double-positive cells over the number of BrdU

single-positive cells was expressed as a percentage. The same images were also used to analyze the intranuclear BrdU-expression pattern to categorize BrdU+ cells into: (i) first generation cells, that we classified based on the presence of incorporated BrdU and a homogenous BrdU-expression over the entire nucleus, indicating the cell had not further divided afterwards and (ii) a BrdU positive cell that divided once or more after the incorporation of BrdU and displayed a clustered pattern of nuclear BrdU-expression.

Western Blot

For quantification of Ki67 expression levels in P9 DG ($N = 5$/condition, only male pups) via Western blot, samples were prepared as follows. Upon rapid decapitation on the morning of P9, hippocampi were quickly dissected in ice-cold saline and then cut in 4–5 slices using equally spaced razor blades to allow for DG dissection under a binocular. Tissue was immediately frozen on dry-ice and stored on −80°C until further use. Samples were homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, and pH = 6.8) using small pellet mixers, then incubated for 10 min at room temperature and subsequently sonicated for 2× 30 s at maximum intensity, again incubated for 10 min and than centrifuged for 1 min with 10,000*g*. The supernatant was collected and the lysate protein concentration for each sample was determined using a BCA Protein Assays (Pierce); 20 μg of whole cell lysate were separated by electrophoresis on 15% polyacrylamide-SDS gels (Biorad MiniPROTEAN system) with 5% stacking gels and transferred to nitrocellulose for 2 h at 75 V in Towbin Buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH = 8.3). The membranes were cut in strips according to later probing and blocked in TBST (TBS + 0.1% Tween-20) containing 10% milk powder (ELK) at 4°C overnight. After blocking, blots were rinsed with TBST and incubated with the primary antibodies ON at 4°C. Blots were then washed with TBST and incubated for 2 h with primary antibody species matching Licor IRDyes (goat anti-rabbit 680LT, Licor 926–68021; goat anti-mouse 800CW, Licor 926–32210), 1:10,000 in TBST. After thorough washes with TBST and a final wash with TBS, signal was developed using a Licor Odyssey FC machine and Licor Image Studio software. Signal intensities were measured using ImageJ software (NIH), the expression levels per animal were calculated from the means of four technical replications within one experiment.

Behavioral Analyses

Five month old male (Ctl $n = 6$, ES $n = 6$) and female (Ctl $n = 9$, ES $n = 8$) mice were tested in a behavioral test battery including the following tasks (in order of testing): elevated plus maze (EPM), object recognition task (ORT), object location task (OLT), Morris water maze (MWM), and the forced swim task (FST). During testing, behaviors were recorded by a video camera connected to a computer with Ethovision software (Noldus, The Netherlands) and in addition manually scored by an experimenter that was blind to the condition of the animals. Males and females were separately tested. For female mice, vaginal smears were taken after the last trial of each task to determine estrous cycle phase.

Because mice are nocturnal animals, all behavioral testing was conducted during the dark (active) phase between 1 and 4 PM in a clean testing room lit by red two red spots (EGB, 25 Watt). The mice that underwent behavioral testing were group-housed in a room with a reversed light/dark schedule (lights on at 8 PM) starting one month before the testing. Before the first test, mice were handled five minutes per day for five consecutive days. On the last two days of this handling period, mice were also transferred, in their home cage, from their housing room to an adjacent testing room, where they were kept for 60 min to familiarize with the testing room.

Basal exploration and anxiety-like behavior were tested in the EPM. Every mouse was placed in the center (5 × 5 cm) of the plus maze (100 cm above the floor), facing one of the open arms (35 × 5 cm) and allowed to freely explore the maze for 5 min. Velocity, exploration time per arm, and open/closed arm entries were scored to assess exploration patterns.

The object recognition test (ORT) is a nonspatial, emotionally neutral memory test in which mice had to discriminate between a novel object and an object that they had explored previously (familiar). After a five-day interval during which the mice were handled daily for 2 min, mice were tested in the ORT. During the last three days of the interval, mice were habituated for 5 min/day to the testing arena that consisted of a rectangular plastic box (dimensions: 23.5 × 31 cm, height wall: 27 cm). The floor of the arena was covered with one cm clean sawdust (which was refreshed after every animal). On the training day, mice had 5 min to explore two identical objects (9.5 cm high glass bottles) that were placed 12 cm from each other and 11 cm from the wall. The time spent exploring each object was measured using stopwatches. On the testing day 24 h post-training, one object was exchanged by a novel object (constructed of yellow Lego Duplo bricks) placed in exactly the same position, and mice were reintroduced into the arena for 5 min to explore the novel and the familiar object. The ratio of novel/familiar object exploration time (on day two) was used as an index of memory; a ratio>1 indicates a preference for the novel object, indicating remembrance of the object observed on day one. Mice that spend <10 s exploring the objects during either day one or day two were excluded from the analysis.

Object location tests

For the OLT, the arena and habituation protocol were identical to those used in the ORT; two completely novel identical objects (white coffee cups) were placed 12 cm from each other and 11 cm from the wall. On the training day, mice had 5 min to explore the objects and their location. On the testing day, 24 h post-training one object was moved to a novel position in the arena and mice were reintroduced into the arena for five min to explore. Time spent exploring was recorded as

before. In this task, two completely novel objects (compared with ORT) were used.

Morris water maze

After an interval of five days during which the mice were handled daily for 2 min, the mice were tested in the MWM (Rice et al., 2008). A circular water maze (110 cm in diameter) was filled with opaque water (23 ± 1°C, with non-toxic paint). On day one, mice were subjected to two cued trials (of maximally 60 s or till the mouse reached the platform) in which the platform was placed one cm above the water surface, visible for the animal in the center of the pool, but without visual cues on the walls surrounding the pool. On day two, the acquisition phase started with two training trials per day with an inter-trial-interval of 10 minutes during six consecutive days. During the acquisition phase, the platform was hidden in the NW-quadrant of the pool one cm below the water surface. Geometric visual cues (22 × 22 cm) were placed on the walls surrounding the pool. Between trials starting points were varied between one of the three quadrants without the platform (i.e., NE, SW, and SE) to prevent the animals from using an egocentric search strategy (Morris, 1984). At the start of each trial, the mouse was placed in the water facing the wall of the pool. The animal was allowed to search for the platform for maximally 60 s. On day eight, the platform was removed from the pool for a single probe trial, in which the swim path and the time spent in the target quadrant was recorded. On day nine, the platform was placed back in the pool but this time in the SW-quadrant to test reversal learning in two trials with an interval of 10 min.

FST: after an interval of ten days, mice were tested in the FST for their ability to cope with a physical challenge (depressive-like behavior). Mice were individually placed in a glass cylinders (40 × 14.5 cm) filled with 30 cm deep water (23 ± 1°C) for the duration of 5 min (pretest) and tested 24 h later (test) for 5 min. Behavior was videotaped and afterwards scored by an independent researcher for immobility duration. Immobility was defined as floating passively in the water with no additional movements other than those necessary to keep the head above the water surface, for at least 2 s. Latency to float was defined as the time until a phase of more than 20 s immobility was reached. After testing, mice were placed back in their housing room and left undisturbed for three weeks before BrdU-labeling.

Statistical Analysis

Data were analyzed using SPSS 20.0 (IBM software), Graphpad Prism 5 (Graphpad software), and R (version 3.1.0). All data are expressed as mean ± standard error of the mean (SEM). Data were considered statistically significant when $P < 0.05$.

Maternal behavior was analyzed using repeated measure One-way ANOVA. Physiological, behavioral and immunohistochemical data were compared using two-way ANOVA with two fixed factors: condition (Ctl vs. ES) and sex (male vs. female). If a significant difference was detected, post-hoc analyses were performed using Bonferroni multiple comparison tests. For measurements of thymus and adrenal gland weights, data was expressed as percentage of total bodyweight.

Animals from multiple litters were included in each experiment and nested under the condition factor (three litters in each of the Ctl and ES groups). Models with and without litter included as a random factor were compared to assess the degree to which litter effects influenced the outcome variables (Lazic and Essioux, 2013). Litter effects were negligible for most histological and behavioral outcomes, with the exception of the EPM (likelihood ratio = 14.4, $P < 0.001$). Litter was therefore included as a random factor when analyzing the EPM data.

For behavioral data from the female mice, first the effect of estrous cycle stage was evaluated by a two-way ANOVA with estrous cycle stage (estrous vs. nonestrous) and condition (Ctl vs. ES) as fixed factors. Since no main effects of estrous cycle stage were detected, all female mice were included in the analysis as one group.

For the ORT and OLT, one-sample T-tests were used to compare the exploration ratio of novel/familiar to one (no discrimination). Learning and memory in the MWM was analyzed using a three-way repeated measures ANOVA, with two 'between subjects' factors (Ctl vs. ES and male vs. female) and one "within-subjects" factor (training day). To analyze cognitive performance during the probe trial, the percentage of the total time swum in the target quadrant was calculated and one-sample T-tests were used to compare mean quadrant percentages to chance (25%).

An ANCOVA model was used to estimate the association between the number of BrdU+ cells and behavior, which is the appropriate method for testing an association in the presence of heterogeneous groups (Lazic, 2012) In these models "number of $BrdU^+$ cells" was used as a continuous predictor and included as a covariate, "behavioral outcome" was the continuous response variable and "condition" and "sex" were used as fixed categorical factors. A significant effect of neurogenesis indicates that there is a neurogenesis-behavior association within the different experimental conditions (Lazic, 2012). Bayesian graphical models were used to separate the effect of ES on behavior into the component that can be attributed to neurogenesis and to neurogenesis-independent mechanisms, stratified by sex. These models were fit in R (version 3.0.3) using the R2jags package (version 0.04-01) and JAGS (Just Another Gibbs Sampler; version 3.4.0). Measures of neurogenesis and behavior were standardized to have a mean of zero and standard deviation of one. The Markov chain Monte Carlo sampling used three chains of 1,000,000 iterations each, a burn-in period of 5000 iterations, and every tenth value was saved. The three chains were well mixed (Gelman-Rubin statistic ≤ 1.01 for all parameters). Noninformative priors were used and the results were not sensitive to the form of the prior. Further details of this modeling approach applied to neurogenesis data can be found in Lazic (2012).

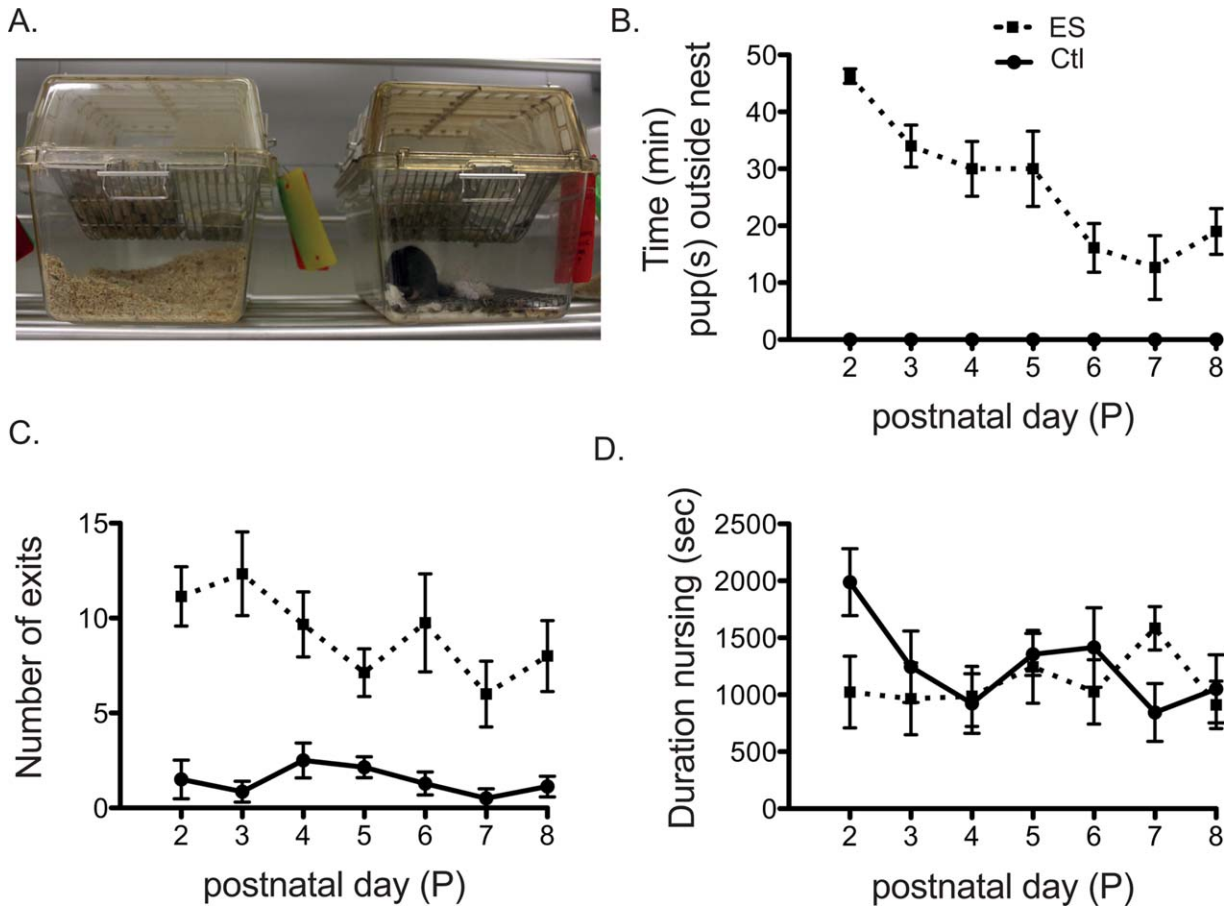

FIGURE 1. Limited nesting material induces erratic maternal care. A: Photograph demonstrating a standard Ctl cage (left) and a limited nesting-bedding cage (right) in which the ES litters (dam with six pups) are housed during P2-P9. B: The total time per 48 min-observation period for each postnatal day during which at least 1 pup was outside the nest. While in Ctl litters this barely happens (0.0 ± 2.0 min/observation period), ES pups are frequently found outside the nest (26.2 ± 1.8 min/observation period, repeated measures ANOVA $F_{1,16} = 95.871$, $P < 0.001$). C: During P2-P9, dams housed in the ES condition exit the nest more frequently than control dams (9.1 ± 0.5 exits/observation period in ES vs. 1.2 ± 0.5 exits/observation period in Ctl, repeated measures ANOVA $F_{1,16} = 119.925$, $P < 0.001$). D: There is no difference in the total time spent nursing between Ctl dams (1260 ± 104 s/observation period) and ES dams (1093 ± 93 s/observation period, repeated measures ANOVA $F_{1,16} = 1.457$, $P = 0.245$). Data expressed as mean $\pm$ SEM, Ctl $n = 8$, ES $n = 10$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS

Limiting Nesting and Bedding Material Leads to Fragmentation of Maternal Care

Housing litters in a cage with limiting nesting and bedding material (Fig. 1a) from P2-P9 induced erratic maternal care. During this period, ES dams: (i) were not able to keep all the pups in the nest area (Fig. 1b); (ii) showed an increased frequency in exiting the nest (Fig. 1c) and (iii) showed 2.5 times more "nesting behavior" trying to construct a rudimentary nest (266 ± 20 s/observation period in ES vs. 105 ± 22 s/observation period in Ctl, repeated measures ANOVA $F_{1,16} = 29.899$, $P < 0.001$). Aside from this erratic maternal behavior, the total duration of nursing behavior was not different between conditions (Fig. 1d), indicating a reduction in the quality, but not the total quantity of maternal care expressed by the dam. These findings confirm and extend on the original findings by Rice et al. (2008).

Fragmentation of Maternal Care Results in Chronic Early Life Stress in the Pups

Exposure to the limited nesting and bedding material cage resulted in physiological signs of chronic early life stress in the pups at P9 (Table 1). ES pups exhibited on average a 36% reduction in bodyweight gain from P2 till P9 (two-way ANOVA, main effect of condition: $F_{1,126} = 172.81$, $P < 0.001$, no effect of sex: $F_{1,126} = 0.27$, $P = 0.607$). In addition, ES pups had elevated basal plasma CORT levels on the morning of P9 compared with controls [ES ($n = 13$): 10.6 ± 3.0 ng/mL vs. Ctl ($n = 12$): 3.3 ± 0.3 ng/mL], two-way ANOVA revealed a main effect of condition ($F_{1,21} = 4.44$, $P = 0.047$), but no main effect of sex ($F_{1,21} = 0.06$, $P = 0.802$).

TABLE 1.

Physiological Effects of Chronic ES Exposure in Pup (P9) and Adult Mice (P150)

Pups	Ctl P9 (*n*)		ES P9 (*n*)	
Body weight gain P2–P9 (g)	3.6 ± 0.4 (74)		2.3 ± 0.6[a] (56)	
Thymus weight (% of BW)	0.554 ± 0.001 (23)		0.463 ± 0.002[a] (34)	
Adrenal gland weight (% of BW)	0.026 ± 0.003 (24)		0.026 ± 0.002 (34)	
Basal plasma CORT (ng/mL)	3.3 ± 0.3 (12)		10.6 ± 3.0[a] (13)	
Adult mice	Ctl males P150 (*n*)	Ctl females P150 (*n*)	ES males P150 (*n*)	ES females P150 (*n*)
Bodyweight (g)	33.78 ± 0.55 (14)	22.68 ± 0.51[b] (15)	31.66 ± 1.42 (14)	21.69 ± 0.45[b] (15)
Thymus weight (% of BW)	0.148 ± 0.004 (11)	0.230 ± 0.011[b] (12)	0.149 ± 0.006 (12)	0.236 ± 0.010[b] (18)
Adrenal gland weight (% of BW)	0.012 ± 0.001 (11)	0.030 ± 0.001[b] (12)	0.015 ± 0.001 (14)	0.029 ± 0.001[b] (18)
Basal plasma CORT (ng/mL)	24.56 ± 6.99 (9)	47.33 ± 8.48[b](23)	21.20 ± 5.24 (10)	46.39 ± 11.47[b] (14)

[a]$P < 0.05$, Two-way ANOVA, main effect for condition.
[b]$P < 0.05$, Two-way ANOVA, main effect for sex. Data expressed as mean ± SEM.

Relative thymus weight was significantly reduced in ES pups (two-way ANOVA, main for condition $F_{1,53} = 15.365$, $P < 0{,}001$, no effect for sex $F_{1,53} = 0.449$ $P = 0.506$), a typical sign of experiencing chronic stress. The relative size of the adrenal glands at P9 was not different between Ctl and ES pups (two-way ANOVA, no main effect of condition: $F_{1,54} = 0.002$, $P = 0.965$ or sex: $F_{1,54} = 0.022$; $P = 0.884$).

The physiological signs of chronic stress present at P9 had disappeared by five months of age. Adult bodyweight (at P150) was similar between Ctl and ES mice; two-way ANOVA revealed no main effect for condition ($F_{1,54} = 3.598$; $P = 0.063$), but a main effect of sex was found ($F_{1,54} = 167.396$; $P < 0.001$) as adult males were heavier than females. In addition, relative thymus weight was no longer different between Ctl and ES mice (two-way ANOVA, no main effect of condition: $F_{1,49} = 0.104$; $P = 0.748$) and only differed between males and females (main effect of sex: $F_{1,49} = 87.419$, $P < 0.001$). Similarly, relative adrenal gland weight was affected by sex ($F_{1,55} = 169.628$, $P < 0.001$) but not by condition ($F_{1,55} = 0.625$, $P = 0.433$). Finally, basal plasma CORT levels at P150 were no longer different between Ctl and ES mice (two-way ANOVA, no main effect of condition: $F_{1,52} = 0.045$, $P = 0.833$), however a main effect of sex on basal plasma CORT levels was detected as well ($F_{1,52} = 5.532$ $P = 0.022$).

Volume of the Hippocampal DG is Lastingly Reduced by Chronic ES

Chronic ES had a direct effect on total DG volume, which was significantly smaller in ES pups compared with Ctl (two-way ANOVA, main effect of condition $F_{1,22} = 10.468$ $P = 0.004$, no main effect of sex $F_{1,22} = 0.591$ $P = 0.450$). On average, estimated DG volume (including granular zone, hilus, and molecular layer) was reduced by 18.2% in ES mice compared with Ctl mice. Analysis of the volume of only the granular zone (GZ: granular cell layer + SGZ) also showed a reduction of GZ volume after ES exposure at P9 (Fig. 2a, two-way ANOVA, main effect of condition $F_{1,22} = 9.470$; $P = 0.006$, no main effect of sex $F_{1,22} = 0.470$; $P = 0.500$).

An ES-induced reduction of 11% in GZ volume was also present at P150, in both male and female mice (Fig. 2b). A two-way ANOVA revealed a main effect of condition ($F_{1,29} = 13.990$; $P < 0.001$) and a main effect of sex ($F_{1,29} = 22.115$; $P = 0.001$), as male mice have a slightly larger GZ volume than female mice. No significant interaction of condition and sex was found ($F_{1,29} = 0.037$; $P = 0.849$).

Levels of Developmental Neurogenesis in the DG is Increased after Chronic ES Exposure

Postnatal proliferation

The absolute number of proliferating ($Ki67^+$) cells in the whole DG was significantly increased at P9 in pups that were exposed to chronic ES (two-way ANOVA, main effect of condition $F_{1,22} = 4.681$; $P = 0.042$, main effect of sex $F_{1,22} = 8.232$; $P = 0.009$, no interaction effect). Taking into account the ES-induced reduction in DG volume, and thus expressing levels of neurogenesis as numeric densities, the amount of proliferating cells in the whole DG (GZ + hilus) was significantly increased in mice that were exposed to chronic ES (two-way ANOVA, main effect of condition $F_{1,22} = 19.229$; $P < 0.001$, main effect of sex $F_{1,22} = 6.339$; $P = 0.020$, no interaction effect, Fig. 2c). These results were confirmed by Western Blot of male DG tissue (Student's T-test, $N = 5$/condition, average intensity 1.12 ± 0.07 in Ctl vs. 1.58 ± 0.08, $P = 0.002$, data not shown). The increase in proliferating cells in ES pups was present in both the suprapyramidal and infrapyramidal blade of the GCL (Fig. 2d) and no differences along the rostral-caudal axis were detected (data not shown).

Absolute numbers of $Ki67^+$ in the paraventricular nucleus of the hypothalamus (PVN) at P9 were not affected by ES in neither of the sexes (two-way ANOVA, no effect of condition $F_{1,22} = 2.233$; $P = 0.152$, no effect of sex $F_{1,22} = 1.127$;

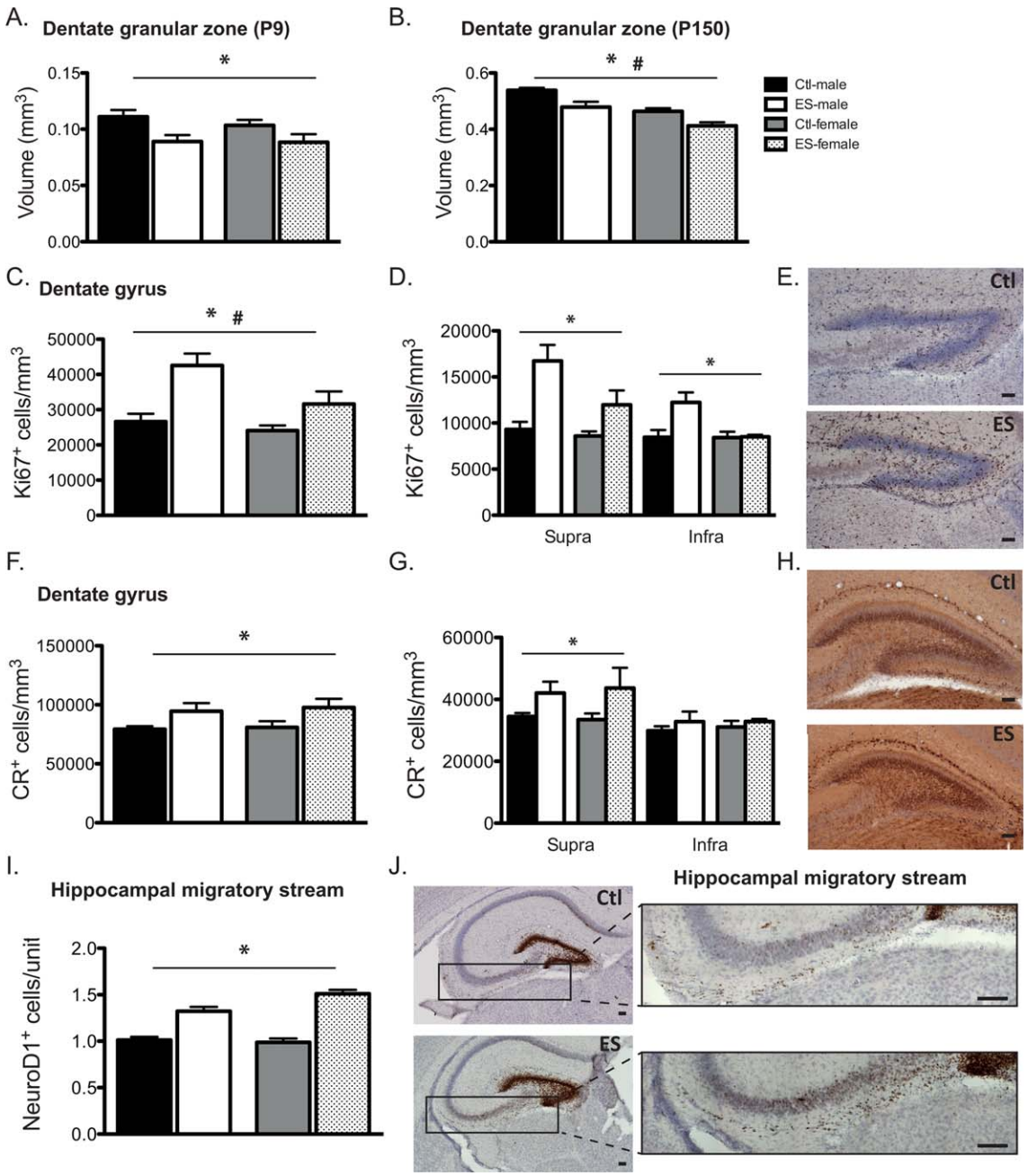

FIGURE 2. Volume of the granular zone is reduced and developmental neurogenesis is increased by chronic ES exposure. A: At P9, ES had caused a significant reduction in dentate granular zone volume (*: main effect of condition, $F_{1,22} = 9.470$; $P = 0.006$). B: At P150, dentate granular zone volume was significantly reduced by exposure to ES (*: main effect of condition, $F_{1,29} = 13.990$ $P < 0.001$) and significantly different between males and females: (#: main effect of sex, $F_{1,29} = 22.115$; $P = 0.001$). C: ES increased the number of proliferating cells/mm^3 in the DG at P9 (*: main effect of condition, $F_{1,22} = 19.229$; $P < 0.001$, #: main effect of sex, $F_{1,22} = 6.339$; $P = 0.020$, no interaction effect). D: Proliferating cells/mm^3 are increased the suprapyramidal blade of the GZ (*: main effect of condition $F_{1,22} = 19.683$ $P < 0.001$) and the infrapyramidal blade of the GZ (*: main effect of condition $F_{1,22} = 5.050$; $P < 0.035$). E: Representative images of Ki67 immunostaining of proliferating cells in the DG of 9 day old Ctl and ES male mice. F: ES increased the number of differentiating cells/mm^3 in the DG at P9 (*: main effect of condition, $F_{1,18} = 8.492$; $P = 0.009$, no main effect of sex: $F_{1,18} = 0.180$; $P = 0.676$, no interaction effect). G: Differentiating cells/mm^3 are increased the suprapyramidal blade of the GZ (*: main effect of condition $F_{1,18} = 8.646$; $P = 0.009$, no main effect of sex $F_{1,18} = 0.013$; $P = 0.912$). No effects of condition and/or sex were found in the infrapyramidal blade (no main effect of condition $F_{1,18} = 8.646$; $P = 0.338$, no main effect of sex $F_{1,18} = 0.013$; $P = 0.791$, no interaction effect). H: Representative images of CR immunostaining of proliferating cells in the DG of 9 day old Ctl and ES male mice. I: ES increased the number of NeuroD1$^+$ cells migrating from the hippocampal subventricular zone towards the DG, cell numbers were normalized for the length of the migratory stream (*: main effect of condition $F_{1,21} = 78.666$; $P < 0.001$, no main effect of sex $F_{1,21} = 3.010$; $P = 0.097$). J: Representative images of NeuroD1 immunostaining of differentiating/migrating cells in the DG and HMS of 9 day old Ctl and ES male mice. Data are expressed as mean ± SEM, scale bars represent 100 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

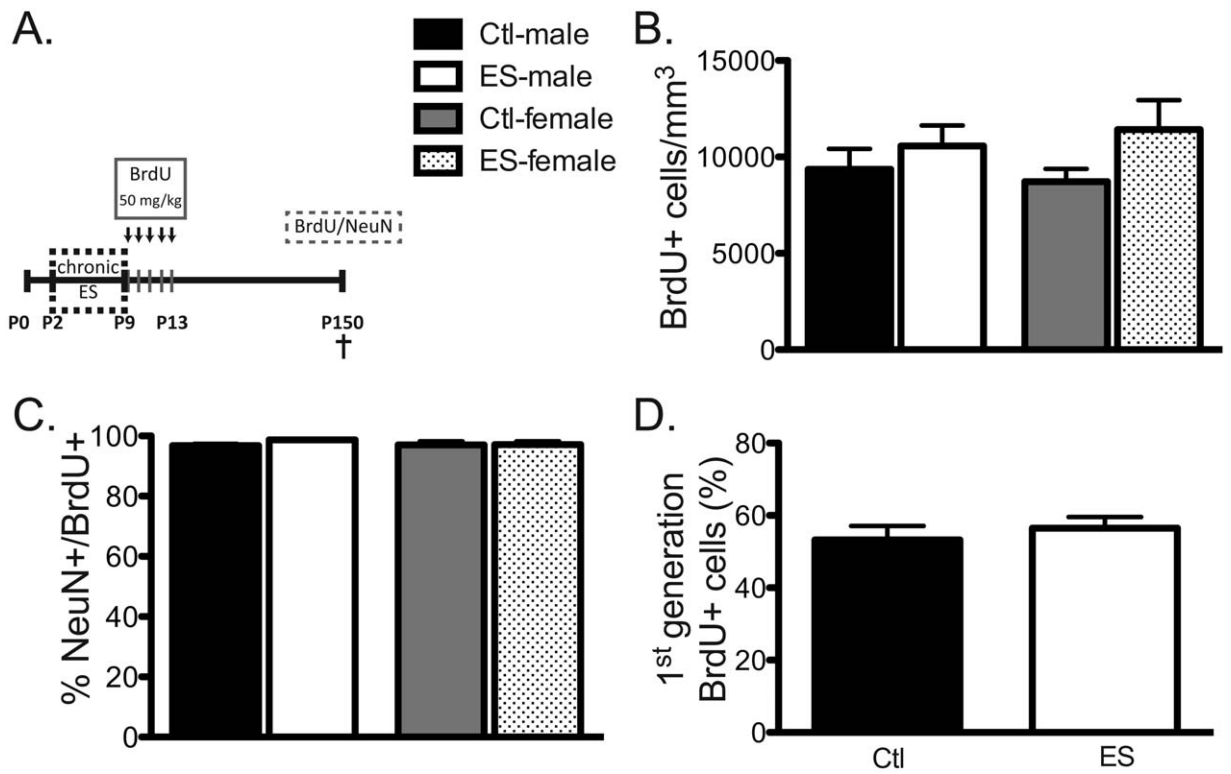

FIGURE 3. Chronic ES and long-term survival of developmentally born neurons. A: To study the cell fate and survival rate of developmentally born cells, mice were injected directly after the Ctl/ES period with BrdU (50 mg/kg once per day from P9 till P13). The animals were left undisturbed until perfusion at P150. Double-labeling with BrdU-NeuN was used to determine survival rate and neuronal cell fate of the cells born during P9–13. B: Numerical densities of BrdU$^+$ cells in the GZ are not different between conditions (two-way ANOVA, no effect of condition $F_{1,29} = 2.680$; $P = 0.112$, no effect of sex $F_{1,29} = 0.009$; $P = 0.926$, NS, no interaction effect). C: The percentage of BrdU/NeuN doublepositive cells was not different between conditions (two-way ANOVA, no effect of condition $F_{1,14} = 1.357$; $P = 0.264$, no effect of sex $F_{1,14} = 1.088$; $P = 0.315$, no interaction effect $F_{1,14} = 1.098$ $P = 0.312$). D: The percentage of first generation BrdU$^+$ cells (= cells exhibiting a homogenous nuclear BrdU staining, indicating no subsequent cell division after BrdU-incorporation) was unaltered by ES (independent T-test, $n = 9$/condition $P = 0.518$). Data are expressed as mean ± SEM.

$P = 0.302$, no interaction effect, data not shown), suggesting that effects of ES on proliferation are specific for the DG.

Postnatal Differentiation

Exposure to chronic ES also increased numeric densities of differentiating immature granule cells indicated by an increase in CR$^+$ cells in the whole DG (two-way ANOVA, main effect of condition $F_{1,18} = 8.492$; $P = 0.009$, no main effect of sex $F_{1,18} = 0.180$; $P = 0.676$, no interaction effect, Fig. 2f). Separate analysis of the numeric density of CR+ cells in the two blades of the granular zone revealed subregion-specific effects. There was a significant increase in the numeric density of differentiating cells in the granular zone in the suprapyramidal blade of the DG (two-way ANOVA, main effect of condition $F_{1,18} = 8.646$; $P = 0.009$, no main effect of sex $F_{1,18} = 0.013$; $P = 0.912$), but no effect of ES in the infrapyramidal blade (two-way ANOVA, no main effect of condition $F_{1,18} = 8.646$; $P = 0.338$, no main effect of sex $F_{1,18} = 0.013$; $P = 0.791$, Fig. 2g). Furthermore, the effect of ES was significant in the rostral part of the granular zone (two-way ANOVA, main effect of condition $F_{1,18} = 5.833$; $P = 0.027$, no main effect of sex $F_{1,18} = 0.004$; $P = 0.948$) but not in the caudal part (no main effect of condition $F_{1,18} = 1.471$; $P = 0.241$, no main effect of sex $F_{1,18} = 0.110$; $P = 0.744$). In contrast, the numeric density of NeuroD1$^+$ cells (which has a broader time window of expression than CR) in the DG was not affected by ES (two-way ANOVA, no main effect of condition $F_{1,21} = 0.128$; $P = 0.725$, no main effect of sex $F_{1,21} = 3.190$; $P = 0.089$, no interaction effect), suggesting that ES might affect a specific stage of differentiation.

Interestingly, the number of differentiating, NeuroD1$^+$ neurons that migrate from the hippocampal subventricular zone via the HMS to form granule neurons and progenitor cells in the ipsilateral DG, was also increased after ES (two-way ANOVA, main effect of condition $F_{1,21} = 78.666$; $P < 0.001$, no main effect of sex $F_{1,21} = 3.010$; $P = 0.097$; Figs. 2i,j). A significant interaction between sex and condition was detected ($F_{1,21} = 5.217$; $P = 0.033$), as the number of NeuroD1$^+$ cells was increased by 30.6% in males and by 53% in females.

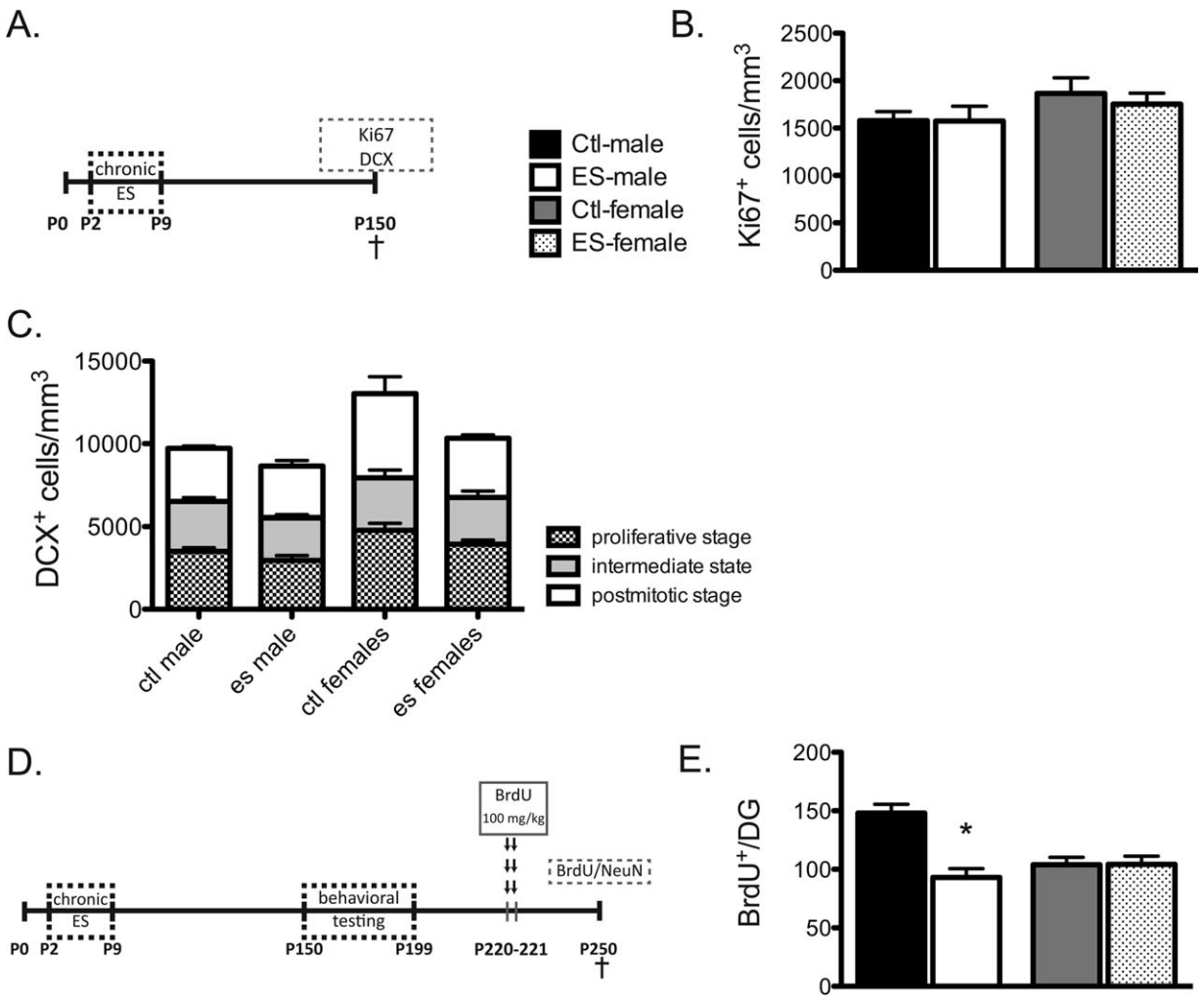

FIGURE 4. Survival of adult born neurons is reduced by chronic ES exclusively in male mice. A: Experimental timeline: levels of proliferating (Ki67+) and differentiating (DCX+) cells were measured in adult animals at P150. B: Numeric densities of Ki67$^+$ cells in the adult DG were not different between conditions ($F_{1,20} = 0.180$; $P = 0.676$). C: Numeric densities of DCX$^+$ cells in the adult DG were not different between conditions ($F_{1,20} = 3.014$ $P = 0.098$). D: Experimental timeline: in order to study survival of cells born in adulthood, a different group of adult mice was injected three weeks after the final behavioral test with 100 mg/kg BrdU three times per day for two consecutive days and killed by transcardial perfusion four weeks after the last injection. E: The number of BrdU$^+$ cells, four weeks after BrdU injection was significantly reduced in ES male offspring when compared with controls (Figs. 6d,e; *: $F_{1,24} = 14.593$; $P = 0.001$).

Chronic ES Reduces Long-Term Survival of Developmentally Born Neurons

What happens with the ES-induced increase in number of postnatally proliferating and differentiating neurons later in life? The long-term survival of the cells generated at the end of the ES period (P9) was reduced. In fact, the number of cells that were detected five months after BrdU-labeling (Fig. 3a) did not differ between conditions. Neither absolute numbers of BrdU$^+$ cells (two-way ANOVA, no effect of condition $F_{1,29} = 0.298$ $P = 0.589$, no effect of sex $F_{1,29} = 1.740$, $P = 0.198$, and no interaction effect), nor the numerical densities of BrdU$^+$ cells in the GZ (Fig. 3b, two-way ANOVA, no effect of condition $F_{1,29} = 2.680$; $P = 0.112$, no effect of sex $F_{1,29} = 0.009$; $P = 0.926$, no interaction effect) were different between Ctl and ES adult offspring. The strong ES-induced increase in the number of proliferating/dividing cells at P9 (see above) suggests that BrdU incorporation during P9–13 is drastically increased. Thus, the absence of an increased BrdU expression in the adult GZ of mice exposed to ES indicates a reduction in the long-term survival of developmentally generated cells. To determine cell fate of these postnatally generated BrdU$^+$ cells, co-labeling with a marker for mature neurons (NeuN) was performed. NeuN/BrdU coexpression revealed that the large majority (97.6%) of the developmentally born cells that survived had become neurons. No differences between conditions were present (Fig. 3c, two-way ANOVA, no effect of condition $F_{1,14} = 1.357$; $P = 0.264$, no effect of sex $F_{1,14} = 1.088$; $P = 0.315$, no interaction effect).

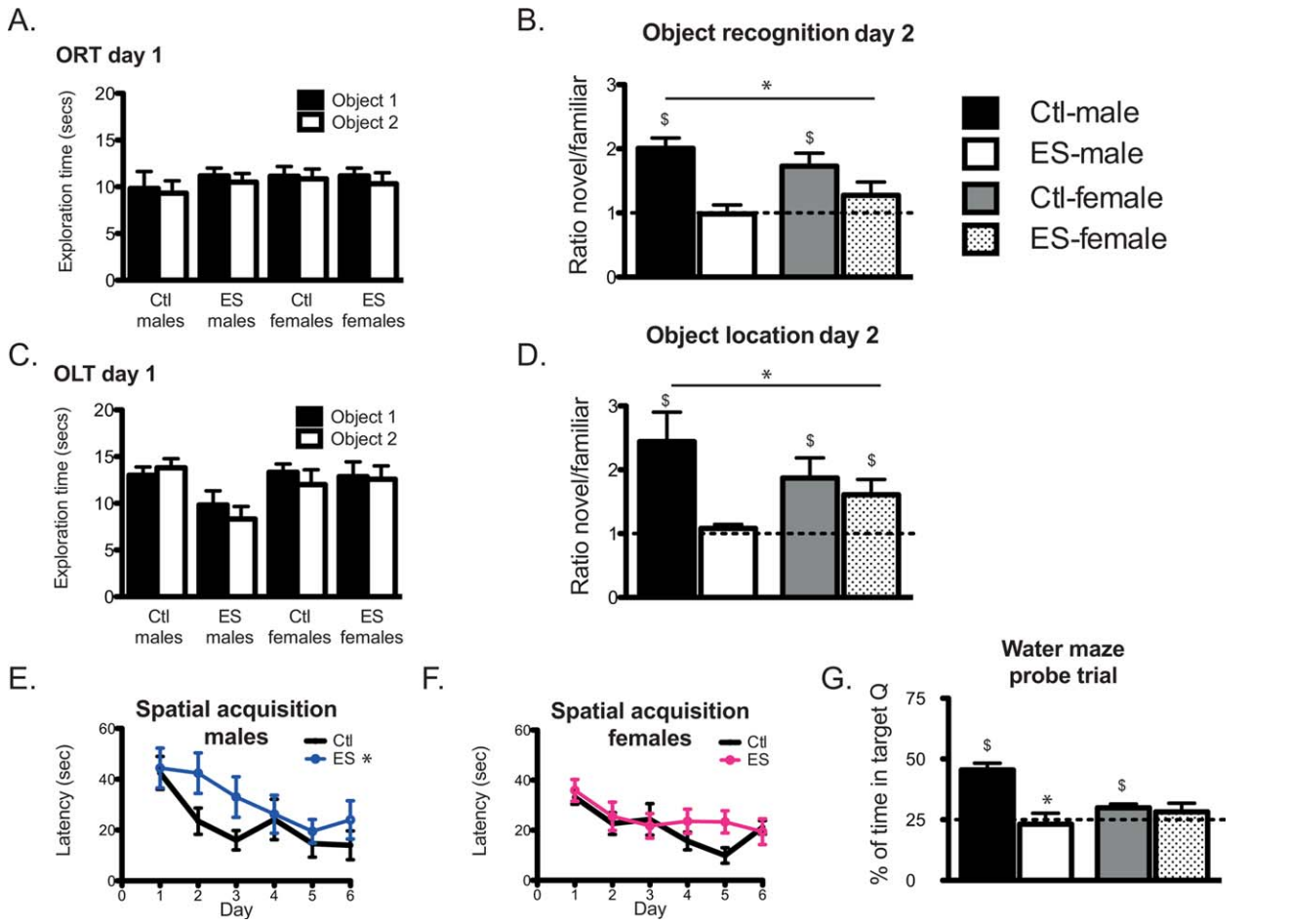

FIGURE 5. Cognitive functions in adulthood are impaired by chronic ES exposure. A: On day 1 of the ORT mice spent equal amounts of time (seconds) exploring the two identical objects. B: On day two of the ORT, the ratio novel/familiar object exploration time was significantly above 1 (indicated with $) for the Ctl males (One-sample T-test, $N = 6$, and $P = 0.002$) and Ctl females (One-sample T-test, $N = 6$, and $P = 0.015$), indicating recognition of the familiar object. There is a significant effect of condition on discrimination ratio (*: main effect of condition, $F_{1,21} = 16.236$, and $P = 0.001$). C: On day 1 of the OLT mice spent equal amounts of time (seconds) exploring the two identical objects. D: On day two of the OLT, the ratio novel/familiar object exploration time was significantly above 1 (indicated with $) for the Ctl males (One-sample T-test, $N = 5$, and $P = 0.036$), Ctl females (One-sample T-test, $N = 6$, and $P = 0.040$), and ES females (One-sample T-test, $N = 9$, and $P = 0.036$). Discrimination ratios differ significantly between conditions (*: main effect of condition ($F_{1,22} = 12.643$, $P = 0.002$). E: Average time per training day needed to find the hidden platform is longer in ES males compared to Ctl ($P = 0.019$*). F: Average time per training day needed to find the hidden platform is not different between Ctl and ES females ($P = 0.244$). G: Percentage of time spent swimming in the target quadrant is above chance level ($) in Ctl males and females. Performance in the probe trial is impaired by ES ($F_{1,24} = 13.942$ and $P = 0.001$) in the male mice (post-hoc comparison: $P < 0.001$*) but not females (post hoc comparison: $P = 0.666$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In addition, careful analysis of the intranuclear BrdU-expression pattern allowed for categorization of BrdU+ cells into first generation cells and cell that divided once or more after incorporating BrdU (Manders et al., 1996). No differences were found between conditions (Fig. 5d, two-way ANOVA, no effect of condition $F_{1,14} = 0.023$; $P = 0.881$, no effect of sex $F_{1,14} = 0.023$; $P = 0.881$, no interaction effect).

Survival of Adult-Born Neurons is Reduced after Chronic ES Exclusively in Male Mice

Adult proliferation and differentiation

Levels of proliferation in adulthood were not affected by ES exposure as no differences were detected between conditions, neither in absolute Ki67$^+$cell counts (two-way ANOVA, no effect of condition $F_{1,20} = 0.820$ $P = 0.376$, no effect of sex $F_{1,20} = 0.190$; $P = 0.668$, no interaction effect) nor in numeric densities (Fig. 4b, two-way ANOVA, no effect of condition $F_{1,20} = 0.180$; $P = 0.676$, no effect of sex $F_{1,20} = 2.917$, $P = 0.103$, no interaction effect). No subregion-specific effects were detected (data not shown). Similarly, levels of differentiating immature granule cells were not altered by ES exposure, neither absolute DCX$^+$ cell counts (two-way ANOVA, no effect of condition $F_{1,20} = 4.028$ $P = 0.058$, no effect of sex $F_{1,20} = 0.274$, $P = 0.607$, no interaction effect) nor the numeric densities (Fig. 4c, two-way ANOVA, no effect of condition $F_{1,20} = 3.014$; $P = 0.098$) were different between conditions. Interestingly, females expressed slightly higher levels of DCX compared with

males (main effect of sex $F_{1,20} = 5.287$; $P = 0.032$). Based on their morphology DCX^+ were classified to be in: (i) a proliferative stage, (ii) an intermediate stage, (iii) a postmitotic stage (based on Plümpe et al., 2006). No effect of condition and/or sex on the maturation of DCX+ cells was detected.

Survival of adult born neurons

ES exposure significantly reduced survival of adult born cells. The number of $BrdU^+$ cells, four weeks after BrdU injection was significantly reduced in ES offspring when compared with controls (Figs. 4d,e, $F_{1,24} = 14.593$ $P = 0.001$). Two-way ANOVA also revealed a significant effect of sex ($F_{1,24} = 5.286$; $P = 0.031$) and a significant interaction effect of sex and condition ($F_{1,24} = 14.997$; $P = 0.001$). In fact there was a 37% reduction in cell survival in male adult offspring (post hoc comparison: $P < 0.001$) while experiencing ES did not alter levels of survival in female adult offspring (post hoc comparison: $P = 0.968$).

Emotional Function in Adulthood is not Affected by ES

Exposure to chronic ES did not affect anxiety-like behavior in adulthood according to the performance in the EPM. The amount of open arm entries relative to total arm entries were not different between the groups (two-way ANOVA, no main effect of condition: $F_{1,23} = 0.872$, $P = 0.360$; no main effect of sex: $F_{1,23} = 0.170$, $P = 0.684$, no interaction effect). In addition, analysis of the time spent in the open arms relative to total exploration time in the EPM, revealed no differences between the groups (two-way ANOVA, no main effect for condition: $F_{1,23} = 3.626$, $P = 0.069$; no main effect for sex: $F_{1,23} = 0.008$, $P = 0.929$, no interaction effect). Exposure to ES did not affect general level of activity as no differences were detected between groups in velocity (two-way ANOVA, no main effect of condition: $F_{1,23} = 1.227$, $P = 0.279$; no main effect for sex: $F_{1,23} = 2.478$, $P = 0.129$) or the total distance moved (two-way ANOVA, no main effect of condition: $F_{1,23} = 1.860$, $P = 0.186$). Female mice were more active than males, as they moved a longer distance in the EPM (two-way ANOVA, main effect of sex: $F_{1,23} = 9.198$ and $P = 0.006$).

Depressive-like behavior, assessed by performance in FST, was unaffected by history of ES. Indeed on day one ES and Ctl male mice expressed equal durations of immobility (two-way ANOVA, no main effect of condition on floating time: $F_{1,25} = 3.228$, $P = 0.084$, a main effect for sex was detected: $F_{1,25} = 9.152$, $P = 0.006$). Previous exposure to the FST significantly increased immobility time on day two in all groups, indicating that all mice acquired a coping strategy regardless of condition (repeated measures ANOVA, effect of day $F_{1,25} = 199.383$, $P < 0.001$).

Cognitive Function in Adulthood is Impaired

Object recognition test

Object memory was impaired after ES exposure. On the training day (day one), mice had no preference for either of the two identical objects and there was no difference between groups in the total time spent exploring the objects (Fig. 5a). On the testing day (day two), the ratio novel/familiar object exploration time (discrimination ratio) was significantly above 1 for the Ctl males (One-sample T-test, $N = 6$, $P = 0.002$) and Ctl females (One-sample T-test, $N = 6$, $P = 0.015$), but not for the ES males (One-sample T-test, $N = 6$, $P = 0.903$) and ES females (One-sample T-test, $N = 7$, $P = 0.231$; Fig. 5b). Comparison of the discrimination ratios between groups indicated that ES significantly impaired object recognition memory. Two-way ANOVA revealed effect of condition ($F_{1,21} = 16.236$ and $P = 0.001$), with no effect of sex ($F_{1,21} = 0.002$ and $P = 0.967$) and no interaction of condition $\times$ sex ($F_{1,21} = 2.431$ and $P = 0.134$).

Object location test

ES exposure impaired hippocampal-dependent object location memory. On day one, mice had no preference for either of the two identical objects and there was no difference between groups in the total time spent exploring the objects (Fig. 5c). On day two, while for the Ctl males (One-sample T-test, $N = 5$, $P = 0.036$) and Ctl females (One-sample T-test, $N = 6$, $P = 0.040$) and ES females (One-sample T-test, $N = 9$, $P = 0.036$) the ratio exploration time of the novel/familiar location was significantly above 1, this was not the case for the ES males (One-sample T-test, $N = 6$, $P = 0.251$; Fig. 5d). Indicating that, all groups except the ES males remembered the familiar object location. The comparison of the discrimination ratios between groups (two-way ANOVA) indicated a main effect condition ($F_{1,22} = 12.643$, $P = 0.002$), without effect of sex ($F_{1,22} = 0.012$, $P = 0.915$), or interaction of condition $\times$ sex ($F_{1,22} = 1.915$, $P = 0.180$), indicating that exposure to ES impairs OLT performance in both sexes and that despite a trend for a larger ES effect in males, this difference did not reach significance.

Morris water maze

ES impaired hippocampal-dependent spatial learning and memory exclusively in male mice. This was evident both during the acquisition as well as the retrieval of the task. The latency to find the platform decreased significantly between the first and the last training day in all groups (paired T-test, $P < 0.05$) except for the ES males (paired T-test, $P = 0.221$, $t = 1.396$, df = 5) indicating that ES males had more problems to learn the task within six days. Repeated measures ANOVA revealed a significant effect for condition ($F_{1,24} = 7.160$; $P = 0.013$) (Figs. 5e,f). Impaired learning ability in ES mice was not due to differences in swimming ability or motivation, as swim speed was not different between conditions (data not shown). During the probe trial (Fig. 5g) ES males showed no preference over chance for the target quadrant (One-sample T-test, $P = 0.463$) and were significantly different from Ctl males who spent significantly more time in the target quadrant (One-sample T-test, $P = 0.028$). Ctl females, but not ES females, performed significant above chance (One-sample T-test, $P = 0.017$), but the time spent in the target quadrant was only

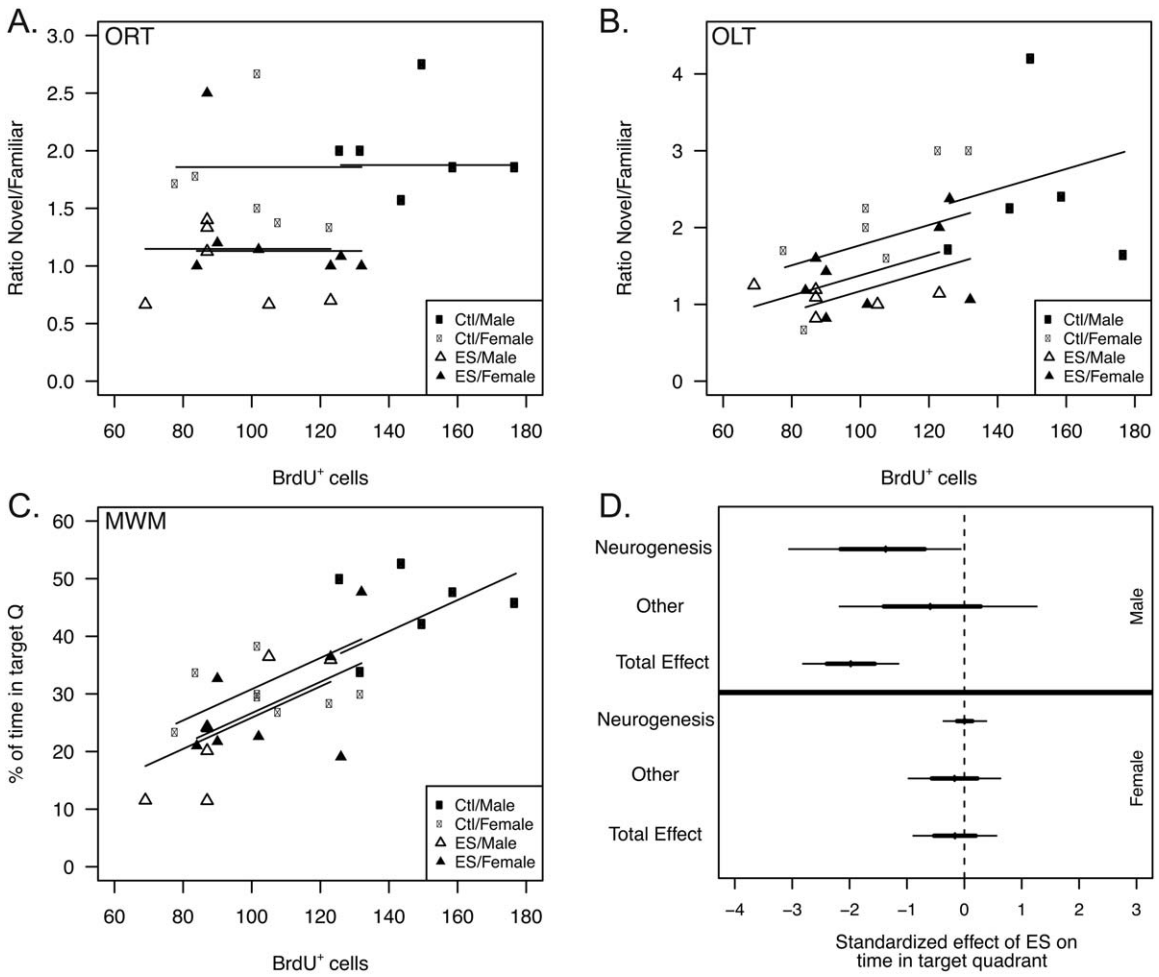

FIGURE 6. ES-induced cognitive impairments in OLT and MWM are neurogenesis dependent. A: There is no significant association between neurogenesis and ORT performance [ANCOVA, $F(1,21) = 0$, and $P = 0.998$]. B: Performance in the OLT was significantly associated with the number of BrdU$^+$ cells at P250 in both males and females [ANCOVA, $F(1,22) = 2.3$, and $P = 0.0272$). C: Performance in the probe trial of the MWM was significantly associated with the number of BrdU$^+$ cells at P250 [ANCOVA, $F(1,24) = 17.5$, and $P = 0.0003$). D: Bayesian analysis revealed that, in the male mice, the majority of the effect of ES on MWM probe test performance was dependent on neurogenesis.

5% above chance level ($30 \pm 0.02\%$). Two-way ANOVA revealed a main effect for condition ($F_{1,24} = 13.942$, $P = 0.001$), which was found to be specific for males (post hoc comparison: $P < 0.001$) but not females (post hoc comparison: $P = 0.666$), no main effect of sex ($F_{1,24} = 2.699$, $P = 0.113$) was found, but there was an interaction of condition and sex ($F_{1,24} = 10.001$, $P = 0.004$), indicating that the effect of ES was larger in males compared with females.

The ES-Induced Impaired Learning is (at Least Partly) Neurogenesis Dependent

An ANCOVA model was used to test whether behavioral performance was associated with the number of BrdU$^+$ cells that survived four weeks after injection. A significant output of such an analysis establishes covariation between neurogenesis and cognitive performance, one of the basic requirements for making causal claims (Lazic, 2012).

For tasks to test emotional functioning, no significant neurogenesis-behavior associations were found using the ANCOVA model [Open-arm time in EPM/BrdU$^+$ cells: $F(1,18) = 4.2$, $P = 0.0563$; FST floating time/BrdU$^+$ cells: $F(1,22) = 1.2$, $P = 0.2816$). For the partially hippocampus-dependent ORT, there was no significant association between neurogenesis and object recognition performance [ANCOVA, $F(1,21) = 0$, $P = 0.998$, Fig. 6a).

Performance in the hippocampus-dependent OLT and in the probe trial of the MWM were significantly associated with the number of BrdU$^+$ cells at P250 in both males and females [ANCOVA, $F(1,22) = 2.3$, $P = 0.0272$; Fig. 6b) and [ANCOVA: $F(1,24) = 17.5$, $P = 0.0003$, Fig. 6c), respectively. To test if these performances were dependent on the number of BrdU$^+$ cells we used a Bayesian analysis. This test revealed a small contribution of neurogenesis to OLT indicating that newborn neurons are (at least partially) involved in recognizing

that an object has been placed to a new location. Interestingly, according to Bayesian analyses the majority of the effect of ES on MWM probe test performance was dependent on neurogenesis (Fig. 6d) revealing a large contribution of neurogenesis in this more complex spatial memory task.

DISCUSSION

The studies described here demonstrate for the first time that exposure to chronic ES in mice leads to: (i) hippocampus-dependent cognitive impairment in adulthood, which is more prominent in males. These deficits are associated, only in males, with (ii) a reduction in survival of adult-born neurons. (iii) Levels of survival not only correlates with the cognitive impairments but also accounts for these cognitive deficits (at least partly), as evident from our statistical causality analyses (Lazic, 2012). Interestingly, despite the difference in severity of the above-mentioned changes in male vs. female mice, ES induced a set of structural changes in the hippocampus in both sexes, including (iv) a lasting reduction in DG volume; (v) an increase in developmental neurogenesis and (vi) a lasting reduction in long-term survival of developmentally born neurons. Taken together, these data suggest a differential vulnerability to the lasting consequences of chronic ES of male and female mice both at structural as well as functional level and point to a functional relevance of the altered levels of survival of adult-born neurons in the observed cognitive impairments. The volumetric and most of the structural hippocampal alterations induced by ES were present in both sexes but the cognitive functions were differentially affected later life in males versus females. This led us to conclude that next to these structural changes, other key elements must be at play to explain the ES-induced cognitive deficits.

Is the Altered Hippocampal Structure and Plasticity the Neurobiological Substrate of the ES-Induced Cognitive Deficits in Mice?

Based on an extensive battery of behavioral tests, our data suggest that while chronic ES does not have lasting effects on adult emotional function (e.g., anxiety-like behavior tested in the EPM and depressive-like behavior tested in the FST), it causes cognitive deficits as evident from impaired performance in OR, OL and MWM, which are overall more robust in male compared to female mice. These findings confirm and extend on the published findings with this same chronic ES mouse model (Rice et al., 2008; Wang et al., 2011). We set out to understand whether the cognitive defects are associated with changes in DG volume and with altered levels of hippocampal neurogenesis and if this form of plasticity had a causal role in the observed cognitive impairments. We also questioned if these structural changes had an early onset and if so, how these progress in time and we studied if alterations in neurogenesis levels could explain the differential vulnerability of males versus females to ES exposure.

First, we found a persistent reduction in volume of the DG in both sexes. This data together with the observed cognitive deficits supports the translational value of the chronic ES mouse model. In fact, clinical studies, using volumetric magnetic resonance imaging techniques, have shown that humans exposed to ES exhibit smaller hippocampal as well as DG volume and that these are associated with lower IQs (Bremner et al., 1997; Andersen et al., 2008; Vythilingam et al., 2002, Teicher et al., 2012). Some have found that the hippocampal volume reduction after ES is more pronounced in males (Frodl et al., 2010). While we indeed found that males are more vulnerable to the lasting consequences of ES concerning survival of adult-born neurons and cognitive performance, we did not detect sex differences in the ES effects on DG volume in our studies. Similarly, a reduction in hippocampal volume has been reported in other early stress models such as maternal separation or deprivation in rats and mice (Fabricius et al., 2008; Aksić et al., 2013, Oomen et al., 2011; Herpfer et al., 2012). The fact that the volume of the DG is so markedly affected by early life experience can be understood when realizing that this hippocampal subregion is developmentally the last hippocampal region to be formed, developing between the last trimester of gestation and 16 years of age in humans (Arnold and Trojanowski, 1996) and between embryonic day 18 and the first two postnatal weeks in mice (Altman and Bayer, 1990) thus rendering its development highly sensitive to stimuli in the early life environment.

A form of plasticity typical for this brain region is adult neurogenesis (Kempermann et al., 2004). This is present in most mammals including humans (Eriksson et al., 1998) and rodents (Abrous, 2005; Lucassen et al., 2010). There is accumulative evidence indicating that increased levels of adult neurogenesis are associated with increased learning and memory capacities (Imayoshi et al., 2008; Jessberger et al., 2009; Deng et al., 2010; Sahay et al., 2011; Oomen et al., 2014). Could then ES exposure affect levels of neurogenesis and could this (at least partly) be the substrate of the cognitive deficits that we observed? ES persistently alters levels of adult neurogenesis, mainly in models of ES in rats (for an overview see: Korosi et al., 2012; Loi et al., 2014). For example, repeated maternal separation or maternal deprivation in rats leads to an age-dependent biphasic effect on neurogenesis with a transient increase in proliferation (Nair et al., 2007) and differentiation (Suri et al., 2013) during adolescence (P21) and a lastingly decrease in levels of proliferation and differentiation in adulthood (Mirescu et al., 2004; Oomen et al., 2010; Suri et al., 2013), without affecting levels of neuronal survival in the DG of the offspring (Mirescu et al., 2004; Greisen et al., 2005) while in another model of early life stress, comparing adult offspring of low-caring with offspring of high-caring rat mothers, the stressed offspring exhibited decreased neuronal survival and increased apoptosis (Weaver et al., 2002; Bredy et al., 2003). We are the first to report such detailed analyses of the effects of ES on neurogenesis in mice.

The above summarized data clearly indicate that exposure to early stress in rats affects levels of various phases of neurogenesis and that these effects seem to be biphasic with an increase during adolescence and a reduction during adulthood. However, a few critical questions remained unanswered. Does ES affect levels of neurogenesis in mice? When is the onset of these alterations; are the early onset alterations in postnatal hippocampal neurogenesis leading to changes in adult neurogenesis? And, most importantly, are these changes causally related to the cognitive impairments caused by ES exposure? In this study, we found increased levels of proliferating ($Ki67^+$) and differentiating (CR^+) cells in the DG and increased number of differentiating ($NeuroD1^+$) cells in the HMS directly after chronic ES at P9. At P9, quantification of DCX-positive cells was not feasible due to the large density and the hectic, intermingled arrangement of DCX positive dendrites at this early postnatal age (personal observation). During normal mouse DG development, proliferation peaks around P5–7 (Navarro-Quiroga et al., 2006). The hippocampal subventricular zone from where precursor cells migrate via HMS to form granule neurons and progenitor cells in the ipsilateral DG and astrocytes in the ipsi- and contralateral DG is depleted by P15 (Navarro-Quiroga et al., 2006). Considering the ongoing development of the DG during P2-P9, it might be more susceptible to the effects of chronic ES during this period than other brain regions that are in a different developmental stage. Indeed, levels of proliferation in the paraventricular nucleus of the hypothalamus were not affected by ES exposure. Hypothalamic developmental neurogenesis indeed mainly occurs between embryonic day 10 and 16 (Shimada and Nakamura, 1973; Saaltink et al., 2012).

The increased levels of developmental neurogenesis that we observed could imply a deviation from the normal developmental sequence. Such an interfering effect of increased postnatal neurogenesis on network formation during development is likely to have lasting consequences on DG structure and function in adulthood. Recently Akers et al. (2014) have shown that reducing neurogenesis after learning improved memory retention in pups at P17. It is an interesting idea to consider that the increased neurogenesis at P9 acts as a compensatory protective mechanism to assist "forgetting" the stressful early life period (Frankland et al., 2013). But how does the increased level of developmental neurogenesis affect the adult neurogenic capacity of the DG, and what happens with this increased number of developmentally generated neurons? The developmentally born cells were labeled with BrdU, immediately after stress exposure and followed for survival and cell fate five months later. Despite the increase in proliferating cells (and thus the increased likelihood to incorporate BrdU) at P9, there were no differences in $BrdU^+$ expression at five months of age in neither of the sexes. This indicates that in the ES condition, a smaller proportion of the developmentally born cells survive after five months. As to the levels of adult neurogenesis in males, but not in females, 4-week survival of adult newborn cells was significantly reduced, while proliferation and differentiation in adulthood were not affected by exposure to ES. This is in line with one of the few mouse studies, which found that repeated maternal separation did not affect the number of differentiating newborn neurons (DCX^+ cells) at P70 (Herpfer et al., 2012). This indicates that while the initial steps of the neurogenic process are not affected by ES, the survival and functional integration of newborn neurons in adulthood is hampered after ES exposure.

What is the functional implication of these changes? It is interesting to consider that the function of a neuron within the dentate granule population is determined by its ontogenetic age, with mature developmentally born cells being specialized in their ability to discriminate between similar contexts, and with mature adult-born cells being involved in spatial problem solving (such as required in the MWM; Tronel et al., in press). Indeed in line with this concept, we find in this study a positive correlation between levels of survival of adult born neurons and learning tasks that depend on the hippocampus as OLT and MWM. However, is this simply a correlation or are the changes in levels of neurogenesis causally involved in the cognitive impairments induced by ES? The idea that newborn neurons qualitatively contribute to hippocampus-dependent learning and memory has been mainly based on correlational evidence (e.g., studies showing that increased neurogenesis is associated with improved cognitive performance (Kempermann et al., 1997) while elimination of neurogenesis is associated with impaired performance in some, but not all hippocampal-dependent tasks (Shors et al., 2002). In fact, over the course of four weeks, newborn granule cells develop similar electrophysiological characteristics to older granule cells and become functionally integrated into the circuitry (van Praag et al., 2002); a reduction in the number of newborn mature neurons could result in impaired cognitive performance. The fact that we investigated both behavior and levels of adult neurogenesis in the same animals enabled linking the two directly. In this study, we showed positive associations between the reduced four-week survival of adult born neurons and impaired performance in the OLT and the MWM probe test. In these tasks, levels of newborn cell survival where predictive for cognitive performance. Indeed, our Bayesian analyses (Lazic, 2012) indicated that the effect of ES was partly mediated by changes in neurogenesis, pointing toward a role of neurogenesis in these exclusively hippocampus-dependent cognitive tasks, but not in partially hippocampus-dependent cognitive tasks such as ORT. Many aspects of spatial learning require an intact and fully functional hippocampus and in particular, neurogenesis seems to contribute to highly specific functional aspects of spatial learning, such as for example probe trial performance in the MWM (Garthe and Kempermann, 2013). Because it has been reported that learning alone can modify the survival and shape of immature neurons that are younger than three weeks old (Tronel et al., 2010), our study design included identical behavioral task exposure for all animals and BrdU-injections >4 weeks after the last learning experience in the MWM to prevent learning from affecting levels of hippocampal neurogenesis. Therefore, the counts of $BrdU^+$ cells are used as a proxy for levels of neurogenesis at the time of behavioral testing.

At this point, it is also important to realize that adult neurogenesis is only one of the forms of structural plasticity occurring in the DG and hippocampus that contributes to the function of this brain region. Indeed ES in rats and mice have been shown to affect synaptic density, dendritic length and long-term potentiation in CA1, CA3 (Huot et al., 2002; Champagne et al., 2008; Bagot et al., 2009; Ivy et al., 2010; Oomen et al., 2010), and DG (Oomen et al., 2011). Thus even though we demonstrate that levels of neurogenesis most probably contribute to the cognitive defects after ES, the end result is probably an integrated effect on all these different forms of plasticity of the hippocampus. How these affect one another and if either of them is dominant over the others remains to be determined. In addition, there has been some evidence from rats that the effects of ES might be adaptive under stressful circumstances (Oomen et al., 2010; Santarelli et al., 2014). As these studies were performed under basal conditions, clearly further research is required to reveal how ES affects the cognitive functions and neurogenic response upon a challenge.

Sex-Differences in Vulnerability to ES

We have converging evidence suggesting sex-differences in the vulnerability to the lasting effects of ES. Strikingly, ES affected levels of survival of adult-born neurons exclusively in males associated with (and determinant for) a more severely impaired cognitive performance in adult ES-male mice compared with females. This is in line with previous clinical and preclinical studies indicating a higher sensitivity of males to ES (Llorente et al., 2009; Oomen et al., 2009; Frodl et al., 2010; Mak et al., 2013; Loi et al., 2014), again reinforcing the translational value of this ES model. It indeed appears that males are more vulnerable to develop mental diseases after exposure to ES. For instance, adverse effects of parental separation during childhood on physical and psychosocial functioning in late adulthood have been found in male but not female subjects (Alastalo et al., 2013), similarly only male (and not female) offspring of mothers exposed to stress of the 1940 invasion in the Netherlands expressed increased risk for psychopathology in adulthood (van Os and Selten, 1998).

Based on the behavioral tasks performed in these study, we have a strong indication that ES alters hippocampus-dependent cognitive function in female mice in a more subtle manner than in male mice, In fact the effect of ES was larger in males for all three learning and memory tests, however, our two-way ANOVA analyses did not detect significant interaction between stress and sex in the OLT and ORT tasks, prompting us to be cautious with our conclusions on the sex-specificity of the ES effects on cognitive functions. Clearly, future studies are required to further address the mechanisms underlying the possible sex-specificity of the effects of chronic ES on cognition.

In both sexes, ES increased developmental neurogenesis (proliferation and differentiation) in the DG and the number of immature cells migrating postnatally from the secondary dentate matrix to the DG, indicating prompt changes in DG structure after ES. However, lasting changes on levels of adult neurogenesis were only found in males. This indicates that while the changes in DG volume and the early changes in postnatal neurogenesis did occur, these were not sufficient to lead to lasting changes in neurogenesis and cognitive deficits in females to the same extent as in males. Probably, some other factors protected the females from the deleterious progression observed in the male mice. This is consistent with evidence from rat early stress models where females as well seem protected against the effects of ES. For example, under basal conditions, levels of neurogenesis at P21 are higher in male than female rats, while in offspring exposed to maternal deprivation (at P3), a reduced proliferation was found in male but not female rats at P21, whereas maturation of newborn cells was increased in males and reduced in females. These early alterations were found to be transient, while there was a persistent reduction in the total amount of granule neurons and cell density in the DG in adulthood (Oomen et al., 2009), in concordance with our current findings. Thus, our results contribute to the accumulating evidence that sex differences are relevant for the effects of postnatal stress on hippocampal plasticity and function and that females are more protected against the deleterious effects of ES.

What could be the reason that the effects ES are different between the sexes? In rats, a maternal attention bias towards males has been described; rat dams preferentially lick and groom male pups more than female pups (Moore and Morelli, 1979; Oomen et al., 2009) indicating that male rodent pups receive higher amounts of maternal sensory stimulation. In this study, we were not able to assess if changes in levels of maternal care directed to male versus female pups had occurred (such investigation would require marking of the individual pups to allow for discrimination between male and female offspring during observations, which would interfere with the ES model). It remains difficult to pinpoint what mechanisms mediate the sex-specific effects of postnatal early life experiences, but ES may have different effects in males and females because the sexual organization of the brain has already taken place in prenatal and early neonatal life. Therefore, the brain is sexually dimorphic and can already respond in a sex-dependent manner to early life experiences, that is, before levels of circulating sex steroids increase during puberty. Clearly, more research is needed to elucidate the underlying mechanisms.

What are the Possible Molecular Mechanisms Responsible for the ES-Induced Changes?

Finally, remaining questions relate to the exact molecular mechanisms mediating these changes in neurogenesis and cognition. So far, the effects of the chronic ES model have been mainly accredited to the altered sensory stimuli from the mother (Fenoglio et al., 2006; Ivy et al., 2008; Rice et al., 2008) and the altered levels of stress hormones (Liu et al., 1997; Weaver et al., 2004; Ivy et al., 2008) and stress related neuropeptides (Rice et al., 2008; Murgatroyd et al., 2009; Korosi et al., 2010; Chen et al., 2012). There is an abundance of literature about the regulating role of CORT on neurogenesis

(Lucassen et al., 2010), even though that is in most cases referring to the effects of CORT in adulthood and mostly pointing to an inhibitory role of CORT on the neurogenic process. Whether the rise in CORT that we observe at P9 (and no longer in adulthood) modulates the neurogenesis process long-term remains to be determined. Similarly, whether the increased levels of CRH are involved in this regulation is an interesting option as well (Ivy et al., 2010; Wang et al., 2011). Next to these key elements, it is interesting to consider alterations in other critical components of the early life environment that are embedded in the dam-pup relationship such as warmth and nutrition. Actually, stress during lactation can alter food-intake and metabolism by the dam, thereby altering the availability of macro- and micronutrients for the pups, which are essential for brain development, neurogenesis, and proper functioning of the epigenetic machinery, which seem to have a crucial role in early life programming of the brain (Lucassen et al., 2013; Parylak et al., 2014). Future studies are required to reveal the interplay of these elements.

In summary, chronic early life stress leads to a lifelong phenotype of impaired cognitive function. This is associated with a transient increase in postnatal neurogenesis in both sexes, which results in a reduced survival of adult born neurons exclusively in males. Understanding the basis of this early life stress induced alterations is profoundly important to mental health and disease and should provide the foundation of future therapeutic interventions.

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