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Impact of early adverse experience on complexity of adult-generated neurons

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New neurons continue to be generated in the dentate gyrus (DG) region of the hippocampus throughout adulthood, and abnormal regulation of this process has emerged as an endophenotype common to several psychiatric disorders. Previous research shows that genetic risk factors associated with schizophrenia alter the maturation of adult-generated neurons. Here, we investigate whether early adversity, a potential environmental risk factor, similarly influences adult neurogenesis. During the first 2 weeks of postnatal life, mice were subject to repeated and unpredictable periods of separation from their mothers. When the mice reached adulthood, pharmacological and retroviral labelling techniques were used to assess the generation and maturation of new neurons. We found that adult mice that were repeatedly separated from their mothers early in life had similar rates of proliferation in the DG, but had fewer numbers of cells that survived and differentiated into neurons. Furthermore, neurons generated in adulthood had less complex dendritic arborization and fewer dendritic spines. These findings indicate that early adverse experience has a long-lasting impact on both the number and the complexity of adult-generated neurons in the hippocampus, suggesting that the abnormal regulation of adult neurogenesis associated with psychiatric disorders could arise from environmental influence alone, or from complex interactions of environmental factors with genetic predisposition.
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Introduction

Accumulating evidence suggests a link between abnormal adult hippocampal neurogenesis and psychiatric disorders, including schizophrenia, depression and drug addiction, leading to the idea that aberrant integration of new neurons in the adult brain may contribute to mental illness.[1,2] Consistent with this idea, mutations of genes associated with an increased risk of schizophrenia (for example, *DISC1*, *RELN* and *NPAS3*) alter the morphological maturation and migration of adult-generated neurons in the dentate gyrus (DG).[3–5] However, although genes exert a strong influence on the likelihood of developing psychiatric disorders, early environmental factors, such as social exclusion from peers and caregiver abuse or neglect, also have a significant role.[6,7]

Here, we investigate whether adverse experiences early in life can lead to changes in the regulation of hippocampal neurogenesis during adulthood. Early adversity was modelled in mice using a chronic unpredictable maternal separation paradigm,[8,9] in which mouse pups were separated from their mothers for 3 h a day during the first 2 postnatal weeks. When these mice reached adulthood, we assessed the proliferation, survival, differentiation and morphological maturation of new neurons in the DG. We found that, similar to some genetic manipulations, adverse early experiences have a long-lasting impact on both the number and the complexity of hippocampal neurons generated during adulthood.

Materials and methods

Mice. Mice were obtained from a cross between a primiparous 129Svev female mouse and a C57Bl/6 male mouse (Taconic). Breeding occurred in our colony at the Hospital for Sick Children. The day of birth was designated as postnatal day (P) 0, and weaning occurred on P21. After weaning, mice were housed in groups of 2–5, and maintained on a 12-h light/dark cycle (lights on at 0700 hours) with free access to food and water. All experimental procedures were approved by the Animal Care Committee at the Hospital for Sick Children.

Unpredictable maternal separation. Mouse pups were subjected to unpredictable maternal separation for 3 h a day from P1 to P14 ($n = 9$ litters). On each day, dams were removed from their home cages and individually placed in separate cages located in a different room. The home cages containing the pups were placed on heating pads to maintain the nest temperature at 30–31 °C. The separation occurred randomly at one of the three possible times during the day (0900–1200, 1200–1500 or 1500–1800 hours). Control pups were left undisturbed in their home cages with their dam, except for weekly cage changes ($n = 10$ litters). There were no differences between separated and control group in litter size (separated: 7.6 ± 0.5 pups; control: 7.8 ± 0.6 pups; group effect: $P > 0.05$) or sex ratio within litters (separated: 4.4 ± 0.6 males, 3.1 ± 0.4 females; control: 3.6 ± 0.5 males, 4.2 ± 0.4

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females; group × sex interaction: $P > 0.05$). To reduce the possibility of litter effects on dependent measures,[10] the separated and control mice used for each experiment were sampled from several different litters (proliferation $n = 15$ litters; survival $n = 14$ litters; morphology $n = 8$ litters).

5-Bromo-2′-deoxyuridine (BrdU) injection. Cells undergoing division were labelled with BrdU, a thymidine analog that incorporates into the DNA of cells during the S-phase of the cell cycle. BrdU was dissolved in 0.1 M phosphate-buffered saline (PBS) at a concentration of 10 mg ml^{-1}. At P60, mice received two injections of BrdU per day (100 mg kg^{-1}; 5 h apart) for 3 consecutive days. To assess cell proliferation, mice were perfused 24 h after the last BrdU injection (separated: $n = 15$, 7 male, 8 female; control: $n = 15$, 6 male, 9 female). To assess cell survival and differentiation, mice were perfused 4 weeks after the last BrdU injection (separated: $n = 16$, 9 male, 7 female; control: $n = 18$, 9 male, 9 female). After deep anesthesia with chloral hydrate, mice were perfused transcardially with PBS followed by 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde for 48 h at 4 °C and then transferred to PBS. Serial coronal sections (40 μm) were obtained across the entire anterior–posterior extent of the hippocampus using a vibratome. Sections were stored in PBS with 0.01% sodium azide at 4 °C.

Green fluorescent protein (GFP) retrovirus infusion. New neurons were labelled by CAG promoter-driven GFP expression, following infection with a replication-deficient retroviral vector based on the Moloney murine leukemia virus.[11] The retrovirus was prepared in our laboratory by transfecting Plat-gp cells with plasmids containing an amphotropic envelope (vsvg) and the transgene (pCAG-GFP). Ultra-high-speed centrifugation was used to concentrate the virus into a high-titer solution ($5 \times 10^{8-9}$ infection units ml^{-1}). At P60, mice (separated: $n = 5$, 3 male, 2 female; control: $n = 5$, 2 male, 3 female) were treated with atropine (0.1 mg kg^{-1}) and anesthetized with chloral hydrate (400 mg kg^{-1}). Using stereotaxic procedures, 1.0 μl of the retrovirus was infused into the DG bilaterally (−2.2 mm AP, ±1.6 mm ML and 2.0 mm DV relative to bregma). The retrovirus was delivered using a glass micropipette, and a pump maintained the infusion rate at 0.15 μl min^{-1}. The pipette was left in place for 5 min after each infusion. Mice were postoperatively treated with ketoprofen (5 mg kg^{-1}). Mice were perfused 4 weeks after retrovirus infusion. Serial coronal sections (50 μm) were obtained across the entire anterior–posterior extent of the hippocampus using a vibratome.

Immunohistochemistry. For BrdU labelling, free-floating sections were treated with 1 N HCl at 45 °C for 30 min, 1% H_2O_2 at room temperature (RT) for 15 min and 0.2 M glycine in PBS at RT for 10 min. Sections were incubated with the primary antibody (rat anti-BrdU, 1:1000, monoclonal antibody, Accurate Chemicals, Westbury, NY, USA) at RT overnight and the secondary antibody (Alexa-488 goat anti-rat, 1:1000, Molecular Probes, Carlsbad, CA, USA) at RT for 2 h. For the 4-week survival group, sections were also labelled with neuronal-specific nuclear protein (NeuN) using an additional primary antibody (mouse anti-NeuN, 1:1000, Chemicon, Billerica, MA, USA) and a secondary antibody (Alexa-568 goat anti-mouse, 1:1000, Molecular Probes). Antibodies were diluted in blocking solution (2.5% bovine serum albumin, 5% normal goat serum and 0.3% Triton X-100 in PBS). Sections were counterstained with 4′,6-diamidino-2-phenylindole (1:10 000) and mounted on slides with Permafluor anti-fade medium (ThermoScientific, Waltham, MA, USA).

For GFP labelling, free-floating sections were incubated with the primary antibody (rabbit anti-GFP, 1:500, Invitrogen, Carlsbad, CA, USA) at RT overnight and the biotinylated secondary antibody (goat anti-rabbit, 1:1000, Jackson Immuno Research, West Grove, PA, USA) at RT for 2 h. Cells were visualized using the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA), followed by 3,3′-diaminobenzidine. Sections were mounted on gel-coated slides, dehydrated and coverslipped with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI, USA).

BrdU quantification. BrdU-labelled cells were quantified on a Nikon (Melville, NY, USA) fluorescent microscope with a ×40 objective. Using a modified stereological procedure, BrdU+ cells in the subgranular zone and granule cell layer of the DG were counted manually from every sixth section, and an estimate of the total number of BrdU+ cells in the DG was obtained by multiplying the average number of BrdU+ cells per section by the number of 40-μm sections comprising the entire DG.[12] For the 4-week survival group, BrdU+/NeuN+ cells were also counted manually and were expressed as a proportion of the total number of BrdU+ cells.

Morphological analysis. Morphological analysis of adult-generated granule neurons was performed on an Olympus (Tokyo, Japan) light microscope using a ×100 objective. GFP+ neurons in the granule cell layer of the DG were selected for analysis if they had at least one third-order dendrite. Using NeuroLeucida software, the entire dendritic arbor, including dendritic spines, was traced. A series of concentric spheres spaced 20 μm apart was centered on the soma, and the number of times that each sphere was intersected by a dendrite and the total dendritic length within each sphere were quantified.[13] Spine density was calculated by dividing the spine number by dendritic length. Between one to seven neurons were traced per mouse, and dependent measures were averaged within each mouse.

Statistical analysis. Data were subjected to either two-way analysis of variance with group and sex as between-subject factors or three-way analysis of variance with group and sex as between-subject factors and distance from soma as a within-subject factor We found no significant effects involving sex; therefore this factor was dropped from the analysis.

Results

To determine whether early life adversity reduces the number of adult-generated cells in the DG, we injected adult mice with BrdU to label proliferating cells. At 1 day after injection, maternally separated and control mice had similar numbers of

proliferating cells (Figure 1a; group effect: $P > 0.05$). However, at 4 weeks after injection, maternally separated mice had fewer surviving cells (Figure 1b; group effect: $F_{1,32} = 7.88$, $P = 0.008$) and a smaller proportion of surviving cells that expressed NeuN, a marker of mature neurons (Figure 1c; group effect: $F_{1,32} = 6.90$, $P = 0.013$), indicating that maternal separation persistently reduces the survival of adult-generated granule neurons in the DG.

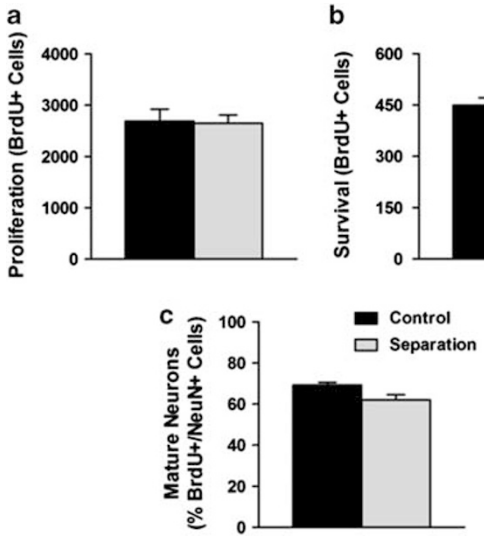

Figure 1 Adult-generated cells in the DG were labelled by injections of BrdU. Maternal separation did not affect the number of proliferating cells 1 day after BrdU injection (**a**), but reduced the number of cells that survived for 4 weeks (**b**) and differentiated into neurons (**c**).

Next, to examine whether early life adversity also affects the maturation of adult-generated neurons, we labelled proliferating cells in adult mice by microinfusion of a GFP-expressing retrovirus into the DG. The robust and persistent expression of GFP in the soma and processes allowed for the reconstruction and detailed morphological analysis of infected neurons at a later time point. The complexity of dendritic arborization was assessed by quantifying the number of dendritic intersections and dendritic length as a function of distance from the soma using Sholl analysis (Figures 2a and b).

We found that repeated and unpredictable periods of separation from the mother led to profound changes in the complexity of adult-generated granule neurons. Four weeks post infection, new neurons in maternally separated mice had fewer dendritic intersections (Figure 2c; group effect: $F_{1,8} = 9.09$, $P = 0.017$; group $\times$ distance interaction: $F_{14,112} = 3.73$, $P < 0.001$) and reduced dendritic length (Figure 2d; group effect: $F_{1,8} = 7.50$, $P = 0.026$; group $\times$ distance interaction: $F_{14,112} = 2.71$, $P = 0.002$) compared with control mice. Although spine density was unaffected (spines per 10 μm; separation: 5.54 ± 0.60; control: 5.93 ± 0.24; group effect: $P > 0.05$), the reduction in dendritic complexity resulted in fewer total numbers of spines per neuron (Figure 2e; group effect: $F_{1,8} = 5.59$, $P = 0.046$), suggesting that adult-generated granule neurons in maternally separated mice form fewer synapses with incoming fibers.

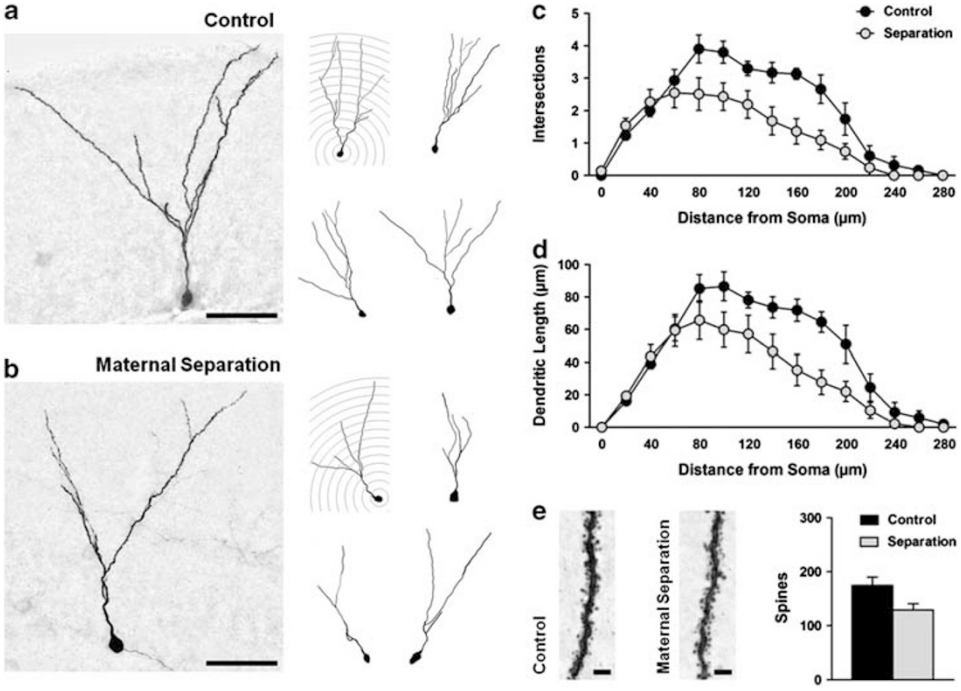

Figure 2 Adult-generated neurons in the DG were labelled by a GFP-expressing retrovirus and visualized using 3,3′-diaminobenzidine staining 4 weeks after infection. Representative images of adult-generated granule neurons from (**a**) control mice and (**b**) mice that were repeatedly and unpredictably separated from their mothers during the first 2 weeks of life (left: $\times$ 20 magnification, scale bar = 50 μm; right: manual tracings). Sholl analysis of adult-generated neurons revealed that maternal separation reduced the complexity of dendritic arborization, as evidenced by (**c**) fewer intersections and (**d**) less dendritic length. (**e**) Representative images of dendritic spines (scale bar = 3 μm) from control and maternally separated mice. Maternal separation reduced the total number of spines per neuron.

Discussion

Previous studies in rodents report that early adversity, in the form of separation from the mother during the initial postnatal weeks, alters the number and morphology of developmentally generated DG neurons.[14–17] Here, we show that the effects of early adversity on the generation and maturation of new DG neurons persist long after the adverse events initially take place. Specifically, rodents that experience maternal separation early in life not only possess fewer new DG neurons during adulthood,[16,18–21] but also possess adult-generated DG neurons with less complex dendritic arborization and fewer dendritic spines. Early adverse experiences, therefore, can exert a long-lasting influence on the regulation of neurogenesis during adulthood, potentially interfering with hippocampal function and leading to behavioral abnormalities later in life. Indeed, rodents that experience maternal separation early in life show several behavioral disturbances during adulthood, including cognitive deficits,[16,19,22] decreased sociability,[23,24] increased anxiety,[22,25,26] and the presence of depression- and schizophrenia-like behaviors,[22,23,27,28] which is consistent with the possibility that the neurobiological consequences of early adverse experiences may contribute to the onset of psychiatric symptoms later in life.

Our finding that the influence of early maternal separation on adult neurogenesis is observable long after the adverse experiences have taken place suggests that the effects of early adversity may be mediated through epigenetic modifications of DNA.[29,30] Such modifications could result in persistent changes in levels of stress hormones or neurotrophic factors that are known to influence the generation and growth of new neurons in the adult brain.[31–33] For instance, adult rodents that experienced maternal separation during the initial postnatal weeks have higher circulating levels of corticosterone[34–36] and reduced hippocampal levels of brain-derived neurotrophic factor,[21,35,37] nerve growth factor[34] and neurotophin-3,[34] thereby possibly resulting in reductions in the number and complexity of newly generated DG neurons. Furthermore, maternal separation and poor maternal care early in life have been found to produce changes in DNA methylation[38–40] that are passed onto the next generation of offspring,[8] suggesting that the impact of early adversity on the brain can persist throughout the lifespan and even into future generations.

Whereas previous studies demonstrate that mutations of genes associated with increased risk of schizophrenia alter the number and complexity of adult-generated neurons in the DG,[3–5] we show here that early adversity, an environmental risk factor, can produce similar changes. The abnormal regulation of adult neurogenesis following early adverse experiences could contribute to psychopathology by disrupting the balance between pattern separation and pattern completion along the entorhinal cortex–DG–CA3 pathway, leading to spurious associations, and hence the formation of memories with psychotic content.[41] Our finding that neurogenesis during adulthood can be shaped by experiences early in life suggests that the abnormalities in adult neurogenesis observed in individuals with schizophrenia, including fewer new hippocampal cells *in vivo*[42] and reduced connectivity of new neurons reprogrammed from fibroblasts *in vitro*,[43] could arise from environmental influence alone or from complex interactions of environmental factors with genetic predisposition.

Conflict of interest

The authors declare no conflict of interest.

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