LETTER

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Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation

Amar Sahay[1,2], Kimberly N. Scobie[1,2], Alexis S. Hill[1,2], Colin M. O'Carroll[1,2], Mazen A. Kheirbek[1,2], Nesha S. Burghardt[1,2], André A. Fenton[3], Alex Dranovsky[1,2] & René Hen[1,2,4]

Adult hippocampal neurogenesis is a unique form of neural circuit plasticity that results in the generation of new neurons in the dentate gyrus throughout life[1,2]. Neurons that arise in adults (adult-born neurons) show heightened synaptic plasticity during their maturation[3] and can account for up to ten per cent of the entire granule cell population[4]. Moreover, levels of adult hippocampal neurogenesis are increased by interventions that are associated with beneficial effects on cognition and mood, such as learning[5], environmental enrichment[6], exercise[6] and chronic treatment with antidepressants[7–10]. Together, these properties of adult neurogenesis indicate that this process could be harnessed to improve hippocampal functions. However, despite a substantial number of studies demonstrating that adult-born neurons are necessary for mediating specific cognitive functions[11], as well as some of the behavioural effects of antidepressants[8–10,12,13], it is unknown whether an increase in adult hippocampal neurogenesis is sufficient to improve cognition and mood. Here we show that inducible genetic expansion of the population of adult-born neurons through enhancing their survival improves performance in a specific cognitive task in which two similar contexts need to be distinguished. Mice with increased adult hippocampal neurogenesis show normal object recognition, spatial learning, contextual fear conditioning and extinction learning but are more efficient in differentiating between overlapping contextual representations, which is indicative of enhanced pattern separation. Furthermore, stimulation of adult hippocampal neurogenesis, when combined with an intervention such as voluntary exercise, produces a robust increase in exploratory behaviour. However, increasing adult hippocampal neurogenesis alone does not produce a behavioural response like that induced by anxiolytic agents or antidepressants. Together, our findings suggest that strategies that are designed to increase adult hippocampal neurogenesis specifically, by targeting the cell death of adult-born neurons or by other mechanisms, may have therapeutic potential for reversing impairments in pattern separation and dentate gyrus dysfunction such as those seen during normal ageing[14,15].

The dentate gyrus subregion of the hippocampus is a substrate for both cognition and mood regulation. Convergent lines of evidence from neuroanatomical, computational, electrophysiological, behavioural and human brain imaging studies suggest a crucial role for the dentate gyrus in the formation of new episodic memories. The dentate gyrus is thought to transform similar experiences or events into discrete, non-overlapping representations, a process known as pattern separation[16]. In addition, overexpression of neurotrophins or transcription factors in the dentate gyrus elicits antidepressant-like behavioural effects[17,18]. Consistent with these functions of the dentate gyrus, ablation of adult hippocampal neurogenesis impairs pattern separation[11,19] and blocks some of the behavioural effects of antidepressants[8–10,12,13]. It is unclear, however, how the converse process, selectively increasing adult hippocampal neurogenesis, affects cognition and mood. Addressing this question has proven difficult owing to a lack of available strategies that selectively increase adult neurogenesis.

Here we developed a genetic gain-of-function strategy to inducibly augment the survival of adult-born neurons in a cell-autonomous manner (Fig. 1a). Because 60–80% of young adult-born neurons undergo programmed cell death, for which the pro-apoptotic gene *Bax* is required[20], we used a transgenic mouse line in which the tamoxifen (TAM)-regulatable recombinase CreER^{T2} is expressed under the control of a 5.26-kilobase fragment of the rat nestin (*Nes*) gene promoter (A. Dranovsky *et al.*, manuscript in preparation) together with a *Bax* conditional knockout mouse line[21] to ablate *Bax* selectively in neural stem cells in the adult brain (Supplementary Fig. 1). Using an inducible reporter, enhanced yellow fluorescent protein (EYFP), as a surrogate marker for *Bax* recombination, we found that 57.5 ± 3.3% of doublecortin (DCX)-expressing neurons expressed EYFP (Supplementary Figs 1 and 2).

Adult hippocampal neurogenesis was analysed in mice carrying two *loxP*-flanked *Bax* alleles (*Bax*$^{f/f}$) and one *Nes-CreER*^{T2} allele (denoted NCff mice) that had been injected with TAM (referred to as *iBax*^{Nes} mice) or injected with vehicle as a control (Fig. 1b). These mice showed comparable levels of stem cell proliferation in the dentate gyrus (Supplementary Fig. 3). By contrast, we found a marked increase in the survival of adult-born neurons at 8 weeks after TAM injection. Analysis of the population of 1–3-week-old adult-born neurons by DCX immunohistochemistry uncovered a significant (1.8-fold and 2-fold) increase in the total number of DCX$^+$ neurons and DCX$^+$ neurons that have at least tertiary dendrites, respectively (Fig. 1c).

Quantification of long-term survival by 5-bromodeoxyuridine (BrdU) pulse–chase experiments revealed a 3.6-fold increase in BrdU-labelled cells in the granule cell layer of the dentate gyrus of *iBax*^{Nes} mice, with no change in the proportion of adult-born neurons (BrdU-labelled neuron-specific nuclear protein (NeuN)$^+$ cells; Supplementary Fig. 5). In accordance with these findings, there was a 3-fold increase in the population of EYFP-labelled adult-born neurons at 6 weeks after TAM injection (Fig. 1d). The larger increase in the number of DCX$^+$ cells in *iBax*^{Nes} mice that was observed at 8 weeks after TAM injection compared with 4 weeks (Supplementary Fig. 4) is consistent with the fact that CreER^{T2}-mediated recombination occurs in both type I neural stem cells (which divide slowly and self-renew) and type II neural progenitors (which are transit amplifying cells) (Supplementary Fig. 1). The expansion of the reservoir of adult-born neurons along the septotemporal axis of the hippocampus in *iBax*^{Nes} mice is comparable to, if not greater than, that observed following chronic antidepressant treatment[7–10], environmental enrichment[6] or exercise[6]. In addition, *iBax*^{Nes} mice show an increase in adult-born cell survival in the olfactory bulb (Supplementary Fig. 5). Control mice and *iBax*^{Nes} mice have a similar body weight, brain architecture and expression of dentate gyrus markers such as calbindin (Supplementary Fig. 6). Surprisingly, the volume of the granule cell layer in the dentate gyrus of both groups is comparable, suggesting that neuronal packing density may be increased in *iBax*^{Nes} mice (Supplementary Fig. 6). In the absence of TAM, no recombination was observed at the

[1]Departments of Neuroscience and Psychiatry, Columbia University, New York, New York 10032, USA. [2]Division of Integrative Neuroscience, The New York State Psychiatric Institute, New York, New York 10032, USA. [3]The Robert F. Furchgott Center for Neural and Behavioural Science, Departments of Physiology and Pharmacology and Neurology, State University of New York, Downstate Medical Center, New York, New York 11203, USA. [4]Department of Pharmacology, Columbia University, New York, New York 10032, USA.

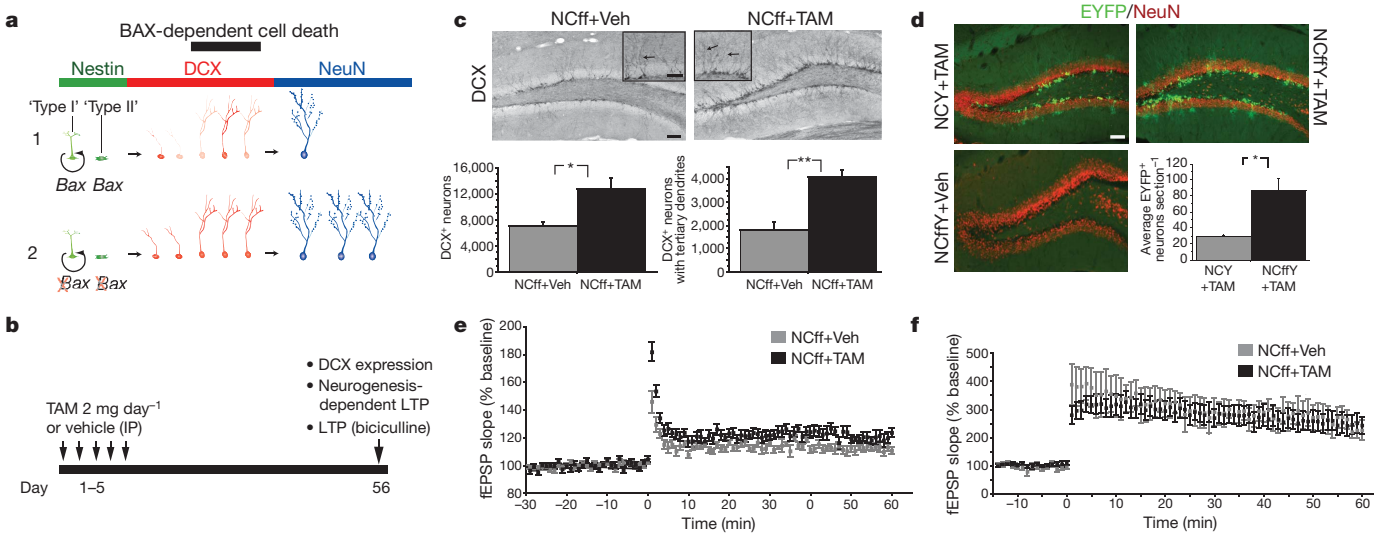

Figure 1 | *Bax* ablation in neural stem cells in the adult brain increases hippocampal neurogenesis and neurogenesis-dependent LTP. **a**, Schematic illustrating genetic gain-of-function strategy to increase adult hippocampal neurogenesis. (1) In the adult dentate gyrus, a substantial fraction of adult-born neurons undergo BAX-dependent programmed cell death (pale red). (2) *Nes-CreER^{T2}*-mediated ablation of *Bax* in type I and type II cells results in the generation of adult-born neurons that lack BAX, thereby preventing their death. **b**, Experimental design. IP, intraperitoneal. **c**, Representative coronal hippocampal sections immunostained for DCX from vehicle (Veh)- and TAM-treated NCff mice (top). Insets are at higher magnification; arrows in insets indicate DCX$^+$ neurons with at least tertiary dendrites. Quantification of DCX$^+$ population (bottom): total number of DCX$^+$ neurons, 6,974 ± 600 (NCff+Veh mice) and 12,636 ± 1764 (NCff+TAM) (*, $P = 0.038$, unpaired two-tailed Student's *t*-test; $n = 3$ mice per group); and total number of DCX$^+$ neurons with at least tertiary dendrites, 1,800 ± 340 (NCff+Veh) and 4,090 ± 285 (NCff+TAM) (**, $P = 0.006$; $n = 3$ mice per group). **d**, Representative coronal hippocampal sections immunostained for EYFP and NeuN from TAM-treated NCY and NCffY mice and Veh-treated NCffY mice. Quantification of EYFP$^+$ neuronal population: mean number of EYFP$^+$ neurons per section, whole hippocampus 29.3 ± 2.1 (NCY+TAM) and 87.6 ± 14.3 (NCffY+TAM) (*, $P = 0.015$, unpaired two-tailed Student's *t*-test); septal 28.76 ± 2.3 (NCY+TAM) and 76.3 ± 15.4 (NCffY+TAM) (*, $P = 0.03$) (data not shown); and temporal 30.66 ± 5.7 (NCY+TAM) and 103.6 ± 20 (NCffY+TAM) (*, $P = 0.02$) (data not shown). Mice per group, $n = 3$. **e**, NCff+TAM mice show enhanced medial-perforant-path–dentate-gyrus LTP compared with NCff+Veh mice. Treatment with TAM has a significant effect: $F_{(1,17)} = 5$, $P = 0.039$ (ANOVA with repeated measures, 50 min). Post-tetanic potentiation differed significantly between the two groups: $P = 0.003$. (NCff+Veh, $n = 8$ slices, six mice; NCff+TAM, $n = 11$ slices, seven mice.) fEPSP, field excitatory postsynaptic potential. **f**, Medial-perforant-path–dentate-gyrus LTP induced in the presence of bicuculline was similar among the groups: $F_{(1,8)} < 1$ (ANOVA with repeated measures, last 30 min). NCff+Veh, $n = 4$ slices, three mice; NCff+TAM, $n = 6$ slices, three mice. **c**–**f**, Results are presented as mean ± s.e.m. **c**, **d**, Scale bar, 100 μm.

conditional locus *ROSA26^{fstopEYFP}* in NCffY mice, which are transgenic for *Nes-CreER^{T2}; Bax$^{f/f}$; ROSA26$^{fstopEYFP/+}$* (Fig. 1d).

We next examined the morphological maturation of adult-born neurons after *Bax* ablation in neural stem cells in the adult brain. Sholl analysis of genetically labelled 6-week-old adult-born neurons in NCffY mice and NCY mice (which are transgenic for *Nes-CreER^{T2}; ROSA26$^{fstopEYFP/+}$*) indicated that apical dendrite maturation and retraction of basal dendrites were normal (Supplementary Fig. 7). Analysis of the mossy fibres of young adult-born neurons that had been genetically labelled with tau–enhanced green fluorescent protein (EGFP) expressed under the control of the pro-opiomelanocortin-α gene promoter (*Pomc-τ-EGFP*) in *iBax^{Nes}* mice and control mice indicated that there was normal axonal extension and targeting in the CA3 subregion of the hippocampus (Supplementary Fig. 8). To assess the functional integration of adult-born neurons in *iBax^{Nes}* mice, we examined a form of long-term potentiation (LTP) at the synapses of medial perforant path axons onto dentate granule neurons; this form of LTP depends on young adult-born neurons[22,23] and is enhanced by chronic treatment with fluoxetine[24]. A significant enhancement in neurogenesis-dependent LTP occurred in *iBax^{Nes}* mice at 4–6 weeks (Supplementary Fig. 9) and at 8 weeks (Fig. 1e) after injection with vehicle or TAM. By contrast, LTP of mature granule neurons in the dentate gyrus, recorded in the presence of bicuculline (Fig. 1f), and basal synaptic transmission (Supplementary Fig. 9) were similar in both groups. These findings suggest that increasing the number of adult-born neurons is sufficient to enhance neurogenesis-dependent LTP and that additional adult-born neurons in *iBax^{Nes}* mice have functionally integrated into the hippocampal network.

To investigate whether there is a causal relationship between increased hippocampal neurogenesis and hippocampus-dependent learning and memory, we tested *iBax^{Nes}* mice and control mice in object recognition and spatial learning and memory tests. Both *iBax^{Nes}* mice and control mice showed comparable levels of exploration of a novel object, as well as comparable levels for a similar object (Supplementary Fig. 10). Increasing adult hippocampal neurogenesis did not affect spatial learning and memory in the reference version of the Morris water maze, during reversal learning or in the active place avoidance task (Supplementary Figs 11 and 12).

To test whether increased adult hippocampal neurogenesis influences rapid contextual encoding, we subjected *iBax^{Nes}* mice and control mice to a single trial contextual fear-conditioning test (Fig. 2a). Control mice and *iBax^{Nes}* mice showed indistinguishably increased levels of freezing in training context 'A' at 24 h after being trained in A, suggesting that both groups acquired and retained contextual fear conditioning equally well (Fig. 2b and Supplementary Fig. 13) (analysis of variance (ANOVA), $F_{(1,26)} < 1$; mean freezing, 34.74 ± 7.18% (NCff+Veh) and 35.26 ± 6.6% (NCff+TAM)). Like control mice, *iBax^{Nes}* mice showed negligible levels of freezing behaviour in a distinct context, 'C', that had few features in common with training context A (Fig. 2b) (ANOVA context C, $F_{(1,26)} < 1$; mean freezing, 4.89 ± 0.78% (NCff+Veh) and 4.03 ± 0.82% (NCff+TAM); comparison of freezing in contexts A and C, two-way ANOVA (context), $F_{(1,52)} = 38.3$, $P < 0.0001$). These results indicate that fear conditioning in both groups was specific to the training context and that increasing adult hippocampal neurogenesis does not affect the ability of an animal to distinguish between two markedly different contexts. Control experiments using mice that were homozygous for the *loxP*-flanked *Bax* allele (*Bax$^{f/f}$*; denoted ff mice) showed that TAM treatment alone does not affect contextual fear conditioning (Fig. 2b and Supplementary Fig. 13) (ANOVA, $F_{(1,29)} < 1$; mean freezing (context A), 33.8 ± 4.9% (ff+Veh) and 28.9 ± 4.7% (ff+TAM);

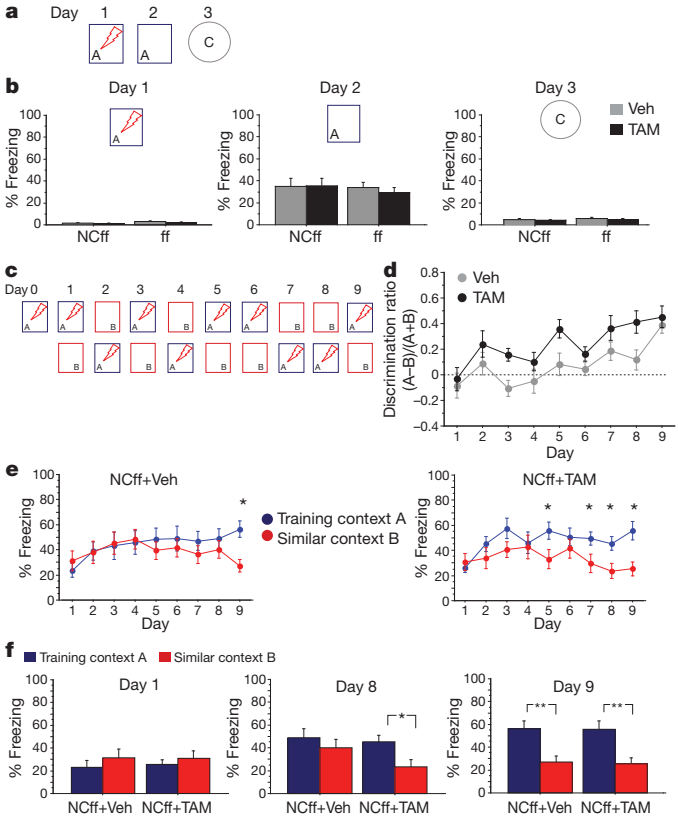

Figure 2 | Increasing adult hippocampal neurogenesis is sufficient to improve discrimination between similar contexts. **a**, Experimental design to test rapid one-trial contextual encoding. **b**, On day 1, both groups showed negligible levels of freezing in context A before a single 2-s, 0.75-mA foot shock (denoted by the red lightning bolt) was delivered. Controls (NCff+Veh) ($n = 14$) and mice with more adult-born neurons (NCff+TAM) ($n = 14$) showed comparable levels of conditioning to training context A and negligible levels of freezing in a distinct context, C. TAM treatment alone did not affect contextual encoding, as reflected in the similar levels of freezing of ff+Veh ($n = 15$) and ff+TAM ($n = 16$) mice in contexts A and C. **c**, Experimental design to test discrimination between two similar contexts, A and B. **d**, Analysis of discrimination ratios. NCff+TAM mice show significantly higher levels of discrimination between the two contexts than do NCff+Veh mice. **e**, Freezing behaviour of mice with increased adult hippocampal neurogenesis (NCff+TAM) and controls (NCff+Veh) over the duration of the experiment. Although both groups show comparable and extensive generalization between the two contexts at the beginning of the experiment, NCff+TAM mice ($n = 11$) distinguished between contexts A and B more rapidly than did NCff+Veh mice ($n = 9$). **f**, NCff+Veh mice were able to discriminate between the two contexts by day 9 of testing: *, $P < 0.05$; **, $P < 0.01$. **b, d–f**, Results are presented as mean ± s.e.m.

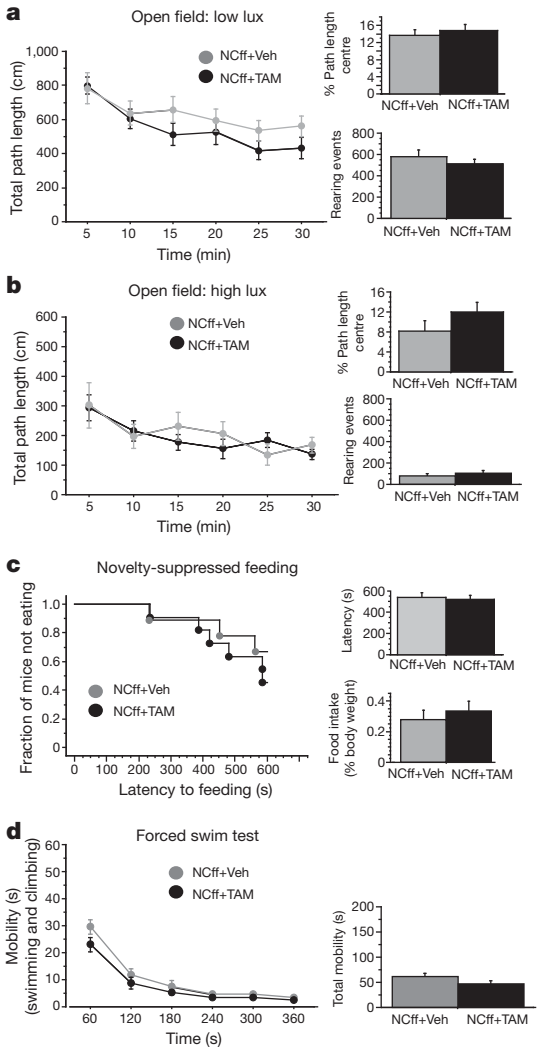

Figure 3 | Increasing adult hippocampal neurogenesis does not produce anxiolytic or antidepressant-like behavioural effects. **a**, **b**, NCff+Veh and NCff+TAM mice showed comparable locomotor activity, rearing events and anxiety-like behaviour in the open field test under two different lighting conditions (with per cent path length centre being the ratio of the distance travelled in the centre of the open field and the total distance travelled in the open field). **c**, NCff+Veh and NCff+TAM mice show similar anxiety-like behaviour in the novelty-suppressed feeding test. **d**, The total mobility of NCff+Veh and NCff+TAM mice did not differ significantly in the forced swim test. Left, swimming and climbing behaviour are shown as a function of time. Right, mobility for the entire session is shown. **a–d**, $n = 9–14$ mice per group; results are presented as mean ± s.e.m.

comparison of freezing in contexts A and C, two-way ANOVA (context), $F_{(1,58)} = 55.23$, $P < 0.0001$).

We next investigated whether increasing the number of adult-born neurons affects a form of learning that requires an animal to distinguish between similar contexts. We chose a contextual fear-discrimination learning task, because it has been proposed to require pattern separation in the dentate gyrus–CA3 circuit[25]. We first established that this learning task depends on adult hippocampal neurogenesis by testing ff mice in which adult hippocampal neurogenesis, but not subventricular zone neurogenesis, was abolished by X-ray irradiation of the hippocampus. Mice lacking adult-born dentate granule neurons were impaired in their ability to distinguish between two similar contexts (Supplementary Fig. 14).

We then examined whether increasing adult hippocampal neurogenesis is sufficient to improve contextual fear-discrimination learning (Fig. 2c). On day 1, both *iBax*^{Nes} mice and control mice showed comparable levels of freezing in context A and context B, suggesting that context B shared enough features with context A to elicit generalization of contextual fear in both groups (Fig. 2e, f) (two-way ANOVA of context and treatment, (context) $F_{(1,18)} = 1$, $P = 0.3$; (treatment) $F_{(1,18)} < 1$, $P = 0.85$; (context × treatment) $F_{(1,18)} < 1$, $P = 0.82$).

Analysis of discrimination ratios for each animal in both groups over the nine days of testing (see Methods), however, revealed that *iBax*^{Nes} mice had significantly higher levels of discrimination between the two contexts than control mice (Fig. 2d) (two-way repeated measures ANOVA of treatment over days, (treatment) $F_{(1,18)} = 6.15$, $P = 0.023$; (day) $F_{(1,18)} = 9.89$, $P < 0.0001$). Analysis of freezing behaviour over days of each group in both contexts showed that NCff+TAM mice had significantly lower levels of freezing in context B than in context A four days earlier than the NCff+Veh group (Fig. 2e, f) (two-way repeated measures ANOVA of context and day followed by Fisher's predicted least-square difference post hoc tests, NCff+Veh (context)

$F_{(1,16)} < 1, P = 0.46$; NCff+Veh (day) $F_{(7,112)} < 1, P = 0.53$; NCff+Veh (context × day) $F_{(7,112)} = 3.1$, $P = 0.004$; NCff+TAM (context) $F_{(1,20)} = 3.4$, $P = 0.07$; NCff+TAM (day) $F_{(7,140)} = 4.3$, $P = 0.0002$; NCff+TAM (context × day) $F_{(7,140)} = 3.8$, $P = 0.0008$). Furthermore, *iBax*^{Nes} mice showed better discrimination than control mice in two other versions of the contextual fear-discrimination learning task (Supplementary Fig. 15).

The enhanced contextual fear-discrimination learning of *iBax*^{Nes} mice was not accompanied by changes in extinction learning (Supplementary Fig. 16), and increasing adult hippocampal neurogenesis did not facilitate the erasure of previously encoded memories[16] (Supplementary Fig. 17). Together, these gain-of-function studies demonstrate that increasing the number of adult-born neurons is sufficient to enhance contextual fear-discrimination learning, which is indicative of improved pattern separation.

Next, we tested a separate cohort of *iBax*^{Nes} mice and control mice in a range of tests that assess anxiety-like and depression-like behaviours and that are used in behavioural screens for antidepressants. Increasing adult hippocampal neurogenesis did not affect exploratory behaviour (as assessed by locomotor activity and rearing events) or anxiety-like behaviour (in the open field, light–dark, elevated plus maze and novelty-suppressed feeding tests) (Fig. 3a–c and Supplementary Fig. 18). No difference in depression-like behaviour was found between *iBax*^{Nes} mice and control mice in the forced swim test (Fig. 3d and Supplementary Fig. 18). As a control, treatment of ff mice with TAM had no effect on anxiety-like and depression-like behaviours (Supplementary Fig. 19). Together with previous studies[8–10,12,13], these results suggest that stimulation of adult hippocampal neurogenesis may be necessary, but is not sufficient, to produce the behavioural effects of antidepressants.

To determine whether changes in mood are observed after combining the genetic expansion of adult hippocampal neurogenesis with an environmental intervention that is known to stimulate network activity and hippocampal neurogenesis, we exposed both *iBax*^{Nes} mice and control mice to a voluntary exercise regimen (Fig. 4a). As expected, voluntary exercise increased adult hippocampal neurogenesis in both groups (compare Fig. 4b and Fig. 1c). After voluntary exercise, compared with control mice, *iBax*^{Nes} mice showed a modest increase in the DCX$^+$ population and a 4.4-fold increase in the number of surviving adult-born neurons but similar neuronal and glial ratios (Fig. 4b and Supplementary Fig. 20).

Surprisingly, *iBax*^{Nes} mice showed a marked increase in exploratory behaviours and decreased anxiety-like behaviour in the open field test compared with control mice (Fig. 4c) (for total path length, two-way repeated measures ANOVA, (treatment) $F_{(1,19)} = 11.23$, $P = 0.003$; (treatment × minute) $F_{(11,209)} = 1.9$, $P = 0.03$; for rearing events, ANOVA, $F_{(1,19)} = 7.54$, $P = 0.01$; for per cent path length centre, ANOVA, $F_{(1,19)} = 4.5$, $P = 0.04$; for time in centre, ANOVA, $F_{(1,19)} = 5.01$, $P = 0.037$). However, in the home cage, *iBax*^{Nes} mice showed similar levels of locomotor activity to control mice (Supplementary Fig. 21). The reduction in anxiety-like behaviour by *iBax*^{Nes} mice in the open field test may result from changes in exploratory behaviour rather than from anxiety itself because *iBax*^{Nes} mice showed normal anxiety-like behaviour in the light–dark and novelty-suppressed feeding tests (Supplementary Fig. 21). Control mice and *iBax*^{Nes} mice showed similar antidepressant-like behaviour in the forced swim test after voluntary exercise (Supplementary Fig. 21). A change in the number of adult-born neurons is unlikely to be solely responsible for the increased exploratory behaviour of *iBax*^{Nes} mice because the increase in survival of adult-born neurons in *iBax*^{Nes} mice after exercise relative to control mice is not much greater than that observed without exercise (Supplementary Fig. 5). Instead, it may be that exercise modifies the properties of the already expanded reservoir of young, excitable adult-born neurons in *iBax*^{Nes} mice.

Determining the impact of increasing adult hippocampal neurogenesis on cognition and mood is pivotal to defining the therapeutic potential of strategies aimed at stimulating the production of new dentate granule neurons in the adult brain. Here we show that selectively increasing the survival of adult-born neurons improves cognitive performance when an animal must distinguish between two similar contexts. By contrast, cognitive gains are not produced when the contexts are markedly different or in other forms of learning, such as object recognition or spatial and reversal learning. Our findings are consistent with the proposed role of the dentate gyrus in pattern separation[16].

Our studies on mood regulation uncover a previously unexpected dissociation between the effect of increased adult hippocampal neurogenesis on learning and on mood. Although *iBax*^{Nes} mice show enhanced pattern

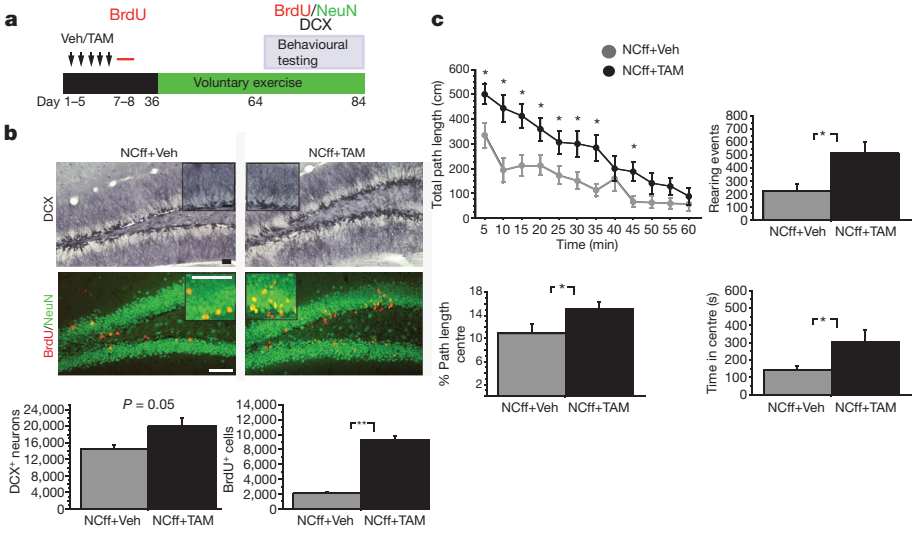

Figure 4 | Mice with more adult-born neurons display increased exploratory behaviour and decreased anxiety-like behaviour in the open field test following a voluntary exercise regimen. **a**, Experimental design. Mice were transferred to cages with running wheels 5 weeks after treatment with Veh or TAM. **b**, Representative images of DCX-immunostained and BrdU- and NeuN-immunostained coronal hippocampal sections from Veh-treated or TAM-treated NCff mice (top). Insets show the granule cell layer at a higher magnification. Scale bars, 100 μm. Quantification of DCX$^+$ neuron population (bottom, $n = 4$–5 mice per group): total DCX$^+$ neurons, 14,527 ± 987 (NCff+Veh) and 19,893 ± 2,022 (NCff+TAM) ($P = 0.05$, unpaired two-tailed Student's *t*-test). Total number of BrdU$^+$ cells in the granule cell layer: 2,119 ± 204 (NCff+Veh) and 9,324 ± 463 (NCff+TAM) (**, $P < 0.0001$). **c**, NCff+TAM mice showed significantly greater locomotor activity, reduced anxiety-like behaviour and a significant increase in rearing events in the open field test compared with NCff+Veh mice: *, $P < 0.05$ (NCff+Veh, $n = 10$ mice; NCff+TAM, $n = 11$). Results are presented as mean ± s.e.m.

separation, they do not show anxiolytic or antidepressant-like behaviours. Because *iBax*^{Nes} mice show only a subset of the modifications that are induced by chronic antidepressant treatment (such as enhanced survival of adult-born neurons and increased neurogenesis-dependent LTP)[7,8,24], it is probable that other antidepressant-dependent modifications of neural circuitry[24,26,27] act in concert with increased adult hippocampal neurogenesis to produce the behavioural effects of antidepressants. Consistent with this idea, increasing adult hippocampal neurogenesis enhances exploratory behaviour when combined with voluntary exercise but not under baseline conditions. This result may reflect a role for the dentate gyrus in the modulation of exploratory behaviour[28].

Recent studies have found dentate gyrus dysfunction and pattern separation impairments during normal ageing in non-human primates[14] and humans[15], respectively. Deficits in pattern separation may affect not only learning, but also anxiety-related behaviours. In fact, impaired contextual fear discrimination (pattern separation) may result in a bias towards encoding ambiguous cues as threatening and may underlie the excessive generalization that is observed in post-traumatic stress disorder and panic disorder[29,30]. Stimulating adult hippocampal neurogenesis may therefore be a novel therapeutic strategy for treating such anxiety disorders, as well as age-related memory impairments.

METHODS SUMMARY

Transgenic and conditional knockout mouse lines were used to recombine *Bax* in stem cells in the adult brain (ref. 21 and A. Dranovsky *et al.*, manuscript in preparation). The impact of *Bax* ablation in stem cells on adult hippocampal neurogenesis and on the morphological maturation of adult-born neurons was characterized using various genetic reporter lines in combination with BrdU pulse–chase labelling and standard immunohistochemistry techniques (see Methods). Assessment of LTP at synapses of medial perforant path axons onto dentate granule neurons was performed as described previously[23]. Focal X-ray irradiation of the hippocampus was performed[23] using sodium pentobarbital as an anaesthetic agent. Behavioural testing was carried out using hippocampus-dependent learning tests (contextual fear conditioning, contextual fear-discrimination learning[25], object recognition, and spatial learning and reversal learning) and tests for anxiety-like and depression-like behaviour. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Columbia University and the New York State Psychiatric Institute. The details of all of the experimental techniques used in this study are available in the Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Generation of mouse lines. The generation and characterization of the *Nes-CreER^{T2}* transgenic mouse line is described in detail elsewhere (A. Dranovsky *et al.*, manuscript in preparation). The colony of NCff mice (that is, *Nes-CreER^{T2}; Bax$^{f/f}$* mice) was maintained by interbreeding *Nes-CreER^{T2}; Bax$^{f/f}$* mice and *Bax$^{f/f}$* mice. The *Bax$^{f/f}$* mice generated from these crosses were used to assess the CreER^{T2}-independent effects of TAM on mouse behaviour. An EYFP reporter line (*ROSA26^{fstopEYFP}*)[31] was used in all experiments in which adult-born neurons were inducibly genetically labelled. Specifically, NCffY and NCY mice were generated as littermates by interbreeding *Nes-CreER^{T2}; Bax$^{f/+}$; ROSA26$^{fstopEYFP/+}$* mice and *Bax$^{f/+}$; ROSA26$^{fstopEYFP/+}$* mice. NCff mice were maintained on a mixed (C57BL/6 and 129/SvEv) genetic background. The *Pomc-τ-EGFP* transgenic mouse line was obtained from GENSAT (http://www.gensat.org) and used to generate NCff; *Pomc-τ-EGFP* mice. To induce CreER^{T2}-mediated recombination of *Bax* and/or *EYFP* in neural stem cells in the adult brain, mice (*Nes-CreER^{T2}; Bax$^{f/f}$* mice, *Nes-CreER^{T2}; Bax$^{f/f}$; ROSA26$^{fstopEYFP/+}$* mice, *Nes-CreER^{T2}; ROSA26$^{fstopEYFP/+}$* mice or *Nes-CreER^{T2}; Bax$^{f/f}$; Pomc-τ-EGFP* mice) of at least 8 weeks of age were given 2 mg TAM intraperitoneally, once a day for 5 consecutive days. TAM (10 mg ml^{-1}, Sigma, T-5648) solution was prepared in corn oil containing 10% ethanol. For vehicle, an identical volume of corn oil with 10% ethanol was injected intraperitoneally, once a day for 5 consecutive days. Mice were housed four to five per cage in a 12 h (6 a.m. to 6 p.m.) light–dark colony room at 22 °C and had free access to food and water. For the voluntary exercise regimen, four to five mice were housed per cage (29.2 cm × 19 cm × 12.7 cm), and each cage was equipped with two running wheels. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Columbia University and the New York State Psychiatric Institute.

Electrophysiological recordings. Electrophysiological recordings in the dentate gyrus were performed as previously described[23,24]. Brains were collected from animals after inducing deep anaesthesia with halothane followed by decapitation, and transverse hippocampal slices (400 μm) were prepared using a vibratome. The slices were incubated in an interface chamber at 32 °C and perfused with oxygenated artificial cerebrospinal fluid (ACSF) (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4 and 11 mM glucose). Slices were allowed to equilibrate for 2 h before positioning the electrodes and beginning stimulation. To record from the dentate gyrus, the medial perforant path (MPP) was stimulated using a stimulation isolation unit and a bipolar tungsten electrode (World Precision Instruments). Evoked potentials were recorded in the molecular layer above the upper blade of the dentate gyrus using a glass capillary microelectrode filled with ACSF (and with a tip resistance of 1–3 MΩ). Isolation of the MPP was confirmed by assessing paired-pulse depression of the MPP–dentate gyrus synaptic connection at 50 ms, which generated the highest level of depression. Input–output curves were obtained after recordings had been stable for 10 min. The stimulation intensity that produced one-third of the maximal response was used for the test pulses and tetanus. After a stable baseline response to test stimulation (once every 20 s) had been observed for 15 min, the ability to elicit LTP was assessed. LTP was induced with a weak stimulation protocol consisting of four trains of 1 s each, at 100 Hz within the train, repeated every 15 s[23]. Responses were recorded every 20 s for 60 min after LTP induction. A similar protocol was used to elicit and record LTP of mature dentate granule neurons except that 10 μM bicuculline (bicuculline methobromide, Sigma, B7561) was added to the ACSF to block $GABA_A$-receptor-mediated inhibition.

Immunohistochemistry and confocal microscopy. To assess the survival of adult-born neurons in the dentate gyrus, BrdU was administered intraperitoneally, once a day in 0.9% NaCl for 2 or 10 days at 150 mg kg^{-1} body weight. Mice were anaesthetized with ketamine or xylazine (100 and 7 mg kg^{-1} body weight, respectively) and transcardially perfused (with cold saline, followed by 4% cold paraformaldehyde in PBS). Brains were postfixed overnight in 4% paraformaldehyde at 4 °C, then cryoprotected in 30% sucrose and stored at 4 °C. Coronal serial sections (40 μm) of the entire hippocampus and sagittal sections (40 μm) of the olfactory bulb were obtained using a cryostat and stored in PBS. For BrdU or NeuN immunohistochemistry, sections were mounted onto SuperFrost Plus charged glass slides. Following pretreatment with 10 mM citrate buffer, sections were subjected to antigen retrieval in 10 mM citrate buffer, using a boiling protocol. After cooling to room temperature, sections were rinsed three times in PBS and blocked in PBS with 0.3% Triton X-100 and 10% normal donkey serum (NDS) for 2 h at room temperature. Incubation with primary antibodies was carried out at 4 °C overnight (for BrdU, rat anti-BrdU antibody at 1/100 dilution, Serotec; for NeuN, mouse, 1/500, Chemicon). Fluorescent-label-coupled secondary antibodies (Jackson ImmunoResearch) were used at a final concentration of 1/400 in PBS. For GFAP, NeuN and EYFP triple immunohistochemistry, floating sections were used. Briefly, sections were washed three times in PBS, blocked in PBS buffer containing 0.3% Triton X-100 and 10% NDS, and incubated in primary antibodies overnight, with shaking at 4 °C (GFAP, rabbit, 1/2000, DAKO; NeuN, mouse, 1/500, Chemicon; GFP, chicken, 1/500, Abcam). The next day, sections were washed three times in PBS and incubated with fluorescent-label-coupled secondary antibodies (Jackson ImmunoResearch) for 2 h at room temperature. For GFP immunohistochemistry alone (rabbit, 1/500, Invitrogen) was used. For calbindin immunohistochemistry, a similar protocol was used (mouse, 1/5,000, Swant). For DCX and Ki67 immunohistochemistry, floating sections were first quenched to remove endogenous peroxidase activity (with 1% H_2O_2 in 1:1 PBS:methanol). Sections were then washed in PBS, blocked (in PBS containing 0.3% Triton X-100 and 10% NDS) and incubated with primary antibody overnight at 4 °C (DCX, goat, 1/500, SantaCruz Biotechnology; Ki67, rabbit, 1/100, Vector Labs). Following washes in PBS, sections were incubated with horse-radish-peroxidase-coupled, biotinylated secondary antibodies. Following incubation with ABC solution (Vector Labs), the colour reaction was carried out using a DAB kit (Vector Labs). An unbiased and blinded quantification protocol was used to quantify DCX$^+$ and BrdU$^+$ cells in the granule cell layer of the dentate gyrus along the septotemporal axis[24]. For quantification of survival of adult-born cells in the main olfactory bulb, two high magnification (×20) images of randomly selected regions in the granule cell layer were obtained from six matched sagittal sections for each mouse. BrdU$^+$ cells were quantified using a cell counter plug-in for the software ImageJ (NIH), and surface density was computed. Bright-field images were obtained using an Axioplan-2 upright microscope (Zeiss). For quantification of EYFP$^+$ neurons in NCY and NCffY mice, five to six (dorsal) and three (ventral) matched sections were selected, and the mean number of EYFP$^+$ neurons per section was computed. Type I neural stem cells expressing EYFP were not included in the analysis. All analyses of mice with the inducible genetic reporter *ROSA26^{fstopEYFP}* were performed at 6 weeks post injection of vehicle or TAM. Phenotyping of BrdU-expressing cells in the granule cell layer of the dentate gyrus entailed the scanning of at least 80 cells from the dorsal and ventral hippocampus of each mouse using an LSM 510 META scanning confocal microscope (Zeiss). To determine the number of BrdU$^+$ cells expressing GFAP or NeuN, *z*-stack analysis was performed using the LSM 510 image browser. To compute the percentage of DCX-expressing cells that also expressed EYFP in NCffY mice, approximately 120 DCX$^+$ neurons per mouse dentate gyrus were scanned using a FluoView 1000 confocal microscope (Olympus) (×40 magnification and numerical aperture (NA) of 1.3). To determine the number of DCX$^+$ neurons expressing EYFP, *z*-stack analysis was performed using FluoView 1000 v1.5 software. Three mice were used for the analysis. A one-in-six series of adjacent sections stained with nuclear fast red (Vector Labs) was used to measure the volume of the granule cell layer in the dentate gyrus.

Quantification of the granule cell layer volume and mossy fibre length in the dentate gyrus. The surface area of the granule cell layer was traced in ImageJ from ×10 images of hippocampal sections spanning the septotemporal axis, and the volume was determined by multiplying the surface area of the granule cell layer by the distance between sections sampled (240 μm). Four mice per group were used for this analysis. To measure the length of axons of young adult-born neurons, we used NCff; *Pomc-τ-EGFP* mice, in which axons are genetically labelled with EGFP. Mossy fibre length was determined by tracing the stratum lucidum along the inner edge of the stratum pyramidale. For measurements, the starting point was the intersection of the trace and a line between the tip of the inner and outer blades of the dentate gyrus[32]. Four dorsal sections from each mouse were used for these measurements.

Sholl analysis. Five to six EYFP$^+$ neurons with complex dendritic trees were chosen from each mouse (from both the dorsal and ventral dentate gyrus) and scanned using a FluoView 1000 (Olympus) (×40, 1.3 NA or ×60, 1.42 NA). Images of collapsed *z*-stacks were imported into Adobe Illustrator CS3, and dendritic trees were reconstructed using the tracing tool. Dendritic complexity was analysed from 8-bit images by using the ImageJ Sholl Analysis plug-in (http://www-biology.ucsd.edu/labs/ghosh/software/). The centre of all concentric circles was defined as the centre of the cell's soma. The parameters used were starting radius (10 μm), ending radius (300 μm from the centre) and interval between consecutive radii (10 μm). Three to four mice per group were used.

Focal X-ray irradiation of the hippocampus. Ten-week-old *Bax$^{f/f}$* mice were anaesthetized with sodium pentobarbital (administered intraperitoneally at 42 mg kg^{-1} body weight, once per day on each of three days that were spaced apart by 3–4 days), placed in a stereotaxic frame and exposed to cranial irradiation using a Stabilopan X-ray system (Siemens) operated at 300 kVp and 20 mA. Animals were protected with a lead shield that covered the entire body, but a 3.22 × 11 mm^2 treatment field above the hippocampus (interaural 3.00 to 0.00) was left unshielded and exposed to X-rays. Dosimetry was done using an electrometer ionization chamber (model PR-06G, Capintec) and Ready Pack Radiographic XV films (Kodak). The corrected dose rate was approximately 1.8 Gy min^{-1} at a source-to-skin distance of 30 cm. The procedure lasted 2 min 47 s, delivering a total of 5 Gy. Three 5-Gy doses were delivered, on days 1, 4 and 8. Behavioural testing was carried out 4 months after hippocampal X-ray irradiation.

Behavioural testing. Behavioural testing was performed using male and female mice that were 14–18 weeks of age at the time of testing, unless otherwise specified. All experiments and analyses were performed blind to genotype or treatment.

Tests for anxiety-like and depression-like behaviours. Testing in the open field test, light–dark test, elevated plus maze, novelty-suppressed feeding and forced swim tests was carried out at 8 or 10 weeks after vehicle or TAM treatment.

The open field test is a standard test of both anxiety and locomotor behaviour. It consists of a simple square enclosure that is equipped with infrared detectors to track animal movement in the horizontal and vertical planes. Measures of total distance travelled and rearing events are used as an index of exploratory activity[33], whereas the proportion of time or distance spent in the centre is construed as a measure of anxiety-like behaviour. Mice were placed in the corner of the open field, and activity was recorded for 30 or 60 min. Testing took place either under low light (200 lx) or bright light (1,000–1,200 lx) conditions.

The novelty-suppressed feeding test has been validated as a model that is sensitive to chronic, but not acute, antidepressant treatment[8]. Mice were food deprived in their home cages for 24–26 h before testing. The testing apparatus consisted of a plastic arena (45 cm long, 15 cm high and 30 cm wide) whose floor was covered with an approximately 2-cm depth of wood-chip bedding. A single food pellet (familiar laboratory mouse chow) was placed on a circular piece of white filter paper (12 cm in diameter) positioned in the centre of the arena. The test began with a mouse being placed in a corner of the arena, and the latency to approach the pellet and begin feeding was recorded (for a maximum time of 10 min). Testing was carried out under bright light conditions. Each mouse was weighed before food deprivation and just before testing to assess changes in body weight. Immediately after the test, each mouse was transferred to its home cage, and the amount of food consumed within 5 min was measured. When appropriate, survival analysis was performed, and statistical differences between the latencies were determined using the Kaplan–Meier product-limit method.

The elevated plus maze[23] and the light–dark test[34] were done as described previously.

For the forced swim test, mice were placed for 6 min in transparent plastic buckets (19 cm in diameter and 23 cm deep) that had been filled with water at 23–25 °C, and their behaviour was recorded using an automated video-tracking system. Testing was carried out over two consecutive days, with the first day serving the purpose of pre-exposure. Mobility (swimming and climbing behaviour) on the second day was analysed using ViewPoint Life Sciences Software.

Object recognition test. At 8 weeks after *Bax* ablation in neural stem cells, separate cohorts of mice were tested for similar and novel object recognition behaviour. NCff mice were tested 8 weeks after TAM or vehicle treatment. Testing entailed placing mice in an arena (45 cm long, 15 cm high and 30 cm wide) with two distinct objects, for seven sessions (each of 7 min) spaced apart by a 3-min intertrial interval. Mice became habituated to the objects during sessions one to six, and one of the objects was then replaced with a novel or a similar object in session seven. Objects and object positions were counterbalanced during testing. The objects that were selected for testing elicited comparable levels of exploration and were categorized as novel or similar based on the exploration levels evoked in NCff mice in pilot experiments. Sessions were videorecorded, and videos were manually scored for locomotion (grid crossings) and object exploration (when an animal's snout was 2 cm or less from the object).

Spatial and reversal learning. A cohort of mice was tested at 8 weeks after *Bax* ablation in neural stem cells. Testing using the reference version of the Morris water maze was performed as described elsewhere[35]. The task was performed with three training phases executed in succession: visible platform (2 days); acquisition phase (4 days), with a hidden platform in the training quadrant (Q3); and transfer/reversal phase (reversal learning, 4 days), with a hidden platform in the opposite quadrant (Q1). Each phase comprised four trials (120 s maximum and 15-min intertrial interval) per day. The start location was in a different quadrant in each trial so that no single start location was used in consecutive trials. Shaping was carried out before the first trial of the visible platform and the acquisition phases. A probe trial (60 s and no platform) was performed 24 h after the last trial of the acquisition and transfer phase. The animals' trajectories were recorded with a videotracking system (HVS Image Analysing VP-118).

Active place avoidance. Spatial learning was also tested using an active place avoidance task, which is sensitive to hippocampal dysfunction[36]. The place avoidance training apparatus consists of a slowly rotating (clockwise at 1 r.p.m.) circular platform (40 cm in diameter) within which a non-rotating 60° region of the room is a shock zone (Supplementary Fig. 12, delineated in red). Visual cues are located on the walls of the room. Mice walk freely on the rotating platform and learn to avoid the shock zone based on the visual cues. When the mouse enters the shock zone, it receives a brief foot shock at a constant current (500 ms, 60 Hz, 0.2 mA) that is scrambled across pairs of parallel rods located on the platform floor. Additional shocks of the same intensity and duration are administered every 1.5 s until the mouse leaves the shock zone. The position of the mouse is tracked by PC-based software that analyses images from an overhead camera and delivers shocks appropriately (Tracker, Bio-Signal Group). Track analysis software is used to compute the number of times each animal enters the shock zone and the number of shocks administered. On the first day of the experiment, mice walked freely on the rotating platform for 10 min while the shock device was turned off (pretraining). Then the shock device was turned on, and mice were given three 10-min training trials with an intertrial interval of 50 min, for two days (trials one to six).

One-trial contextual fear conditioning. Mice were tested at 8 weeks following *Bax* ablation in neural stem cells. Conditioning was conducted on one side of a shuttle box (Med-Associates, ENV-010MC; 20.3 cm × 15.9 cm × 21.3 cm) with a clear plexiglass wall, three aluminium walls and a stainless steel grid as a floor. The chamber was lit from above with a house light (CM1820 bulb), ventilated with a house fan and encased by a sound-dampening cubicle. On the days of testing, mice were brought out of the vivarium and allowed to habituate for 1 h outside the testing room before starting the experiment. Mouse behaviour was recorded by digital video cameras mounted above the conditioning chamber. FreezeFrame and FreezeView software (Actimetrics) were used for recording and analysing freezing behaviour, respectively. The one-trial contextual fear conditioning protocol entailed delivery of a single 2-s foot shock of 0.75 mA at 185 s after placement of the mouse in the training context. The mouse was taken out 15 s after termination of the foot shock and returned to its home cage. For the training context, A, the house fan and lights were switched on; stainless steel grids were exposed; and a mild lemon scent was used as an olfactory cue. Ethanol (70%) was used to clean grids between runs. For the distinct context, C, the stainless steel grid floor was covered with a plastic panel and cage bedding. The chamber walls were covered using plastic inserts, and the house fan and lights were turned off. The chamber door was left ajar during testing. A mild anise scent was used as an olfactory cue, and a non-alcoholic antiseptic was used to clean the chamber between runs. Mice were brought into the testing room in cardboard buckets by a different handler, and the testing room was dimly lit before placement of the mice in the testing chambers. The one-trial contextual fear conditioning protocol was used for extinction learning and memory-clearance experiments. Only males were used for these studies.

Contextual fear-discrimination learning. Mice were tested at 8 weeks after *Bax* ablation in neural stem cells. This test captures an animal's ability to distinguish between two similar contexts, conditions that are most likely to recruit the dentate gyrus[25]. The shock-associated training context, A, and the similar (no-shock) context, B, shared many features, including an exposed stainless steel grid floor (a salient feature of the context) and roof. The similar context differed from the training context in that two plastic inserts were used to cover the walls; the house fan and lights were turned off; and the chamber door was left ajar during testing. A mild mint scent was used as an olfactory cue, and a non-alcoholic antiseptic was used to clean the grids between runs. Mice were brought into the testing room in buckets by the same experimenter who had handled the mice for the training context. In pilot experiments, the similar context was found to evoke comparable levels of freezing behaviour as that observed in the training context, indicative of extensive generalization (pattern completion) between the two contexts. For discrimination learning, mice were exposed to the training context in which they received a single 2-s foot shock of 0.75 mA at 185 s after placement in the chamber. Mice were taken out of the chamber 15 s after termination of the foot shock and returned to their home cage. After 1 h, mice were placed in the similar context, in which they were left for 180 s and were never shocked. Measurement of the freezing levels in both the training context (3-min pre-shock) and the similar context (3 min) each day allowed the assessment of discrimination between the two contexts and was computed as a discrimination ratio: $(\text{Freezing}_{\text{Training context}} - \text{Freezing}_{\text{Similar context}})/(\text{Freezing}_{\text{Training context}} + \text{Freezing}_{\text{Similar context}})$. A score of 0 indicates complete lack of discrimination: that is, freezing levels are the same in the similar and training contexts ($\text{Freezing}_{\text{Similar context}} = \text{Freezing}_{\text{Training context}}$). A score of 1 indicates perfect discrimination: that is, freezing level in the similar context is zero ($\text{Freezing}_{\text{Similar context}} = 0$). Only males were used for these experiments.

Home cage activity. Animal behaviour was recorded for 15 min in the home cage, and videos were manually scored for locomotion (grid crossings).

Statistical analysis. Statistical analysis was carried out using StatView software or Microsoft Excel. Statistical significance was assessed by unpaired two-tailed Student's *t*-tests or ANOVA. Significant main effects or interactions were followed up with Fisher's predicted least-square difference post hoc tests where appropriate: *, $P < 0.05$; **, $P < 0.01$.

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