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LETTERS

A role for adult TLX-positive neural stem cells in learning and behaviour

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Neurogenesis persists in the adult brain and can be regulated by a plethora of external stimuli, such as learning, memory, exercise, environment and stress[1]. Although newly generated neurons are able to migrate and preferentially incorporate into the neural network[2–5], how these cells are molecularly regulated and whether they are required for any normal brain function are unresolved questions[6]. The adult neural stem cell pool is composed of orphan nuclear receptor TLX-positive cells[7]. Here, using genetic approaches in mice, we demonstrate that TLX (also called NR2E1) regulates adult neural stem cell proliferation in a cell-autonomous manner by controlling a defined genetic network implicated in cell proliferation and growth. Consequently, specific removal of TLX from the adult mouse brain through inducible recombination results in a significant reduction of stem cell proliferation and a marked decrement in spatial learning. In contrast, the resulting suppression of adult neurogenesis does not affect contextual fear conditioning, locomotion or diurnal rhythmic activities, indicating a more selective contribution of newly generated neurons to specific cognitive functions.

Global deletion of TLX during development leads to microencephaly, retinal dystrophy, blindness and aggression[8,9]. To facilitate an analysis of the function of TLX in adult neural stem cells (NSCs), we created a conditional allele by flanking exon 2 with two *loxP* sites. The resulting mouse strain is hereafter referred to as $Tlx^{f/f}$ (Supplementary Fig. 1).

Using β-gal-based fluorescence-activated cell sorting[7], we isolated *Tlx*-expressing NSCs from adult $Tlx^{f/Z}$ mice, in which one allele of *Tlx* is replaced with the *lacZ* marker and the other allele is flanked by *loxP* sites but is still functional. These purified adult cells have the ability to self-renew and differentiate to all three neural lineages[7]. Infection with a Cre-expressing virus results in specific deletion of the second *Tlx* allele and leads to a >80% reduction in dividing cells after 36 h of infection (Fig. 1a, b), suggesting that TLX is essential for adult NSC proliferation *in vitro*.

We then tested whether TLX is required cell autonomously. Low densities of isolated NSCs were infected with Cre-expressing virus for 10 h, after which uninfected virus was removed and twofold more uninfected wild-type cells were added. After 36 h in growth media, proliferation of infected cells (green fluorescent protein (GFP)-positive) was scored by staining for phosphorylated histone H3 (p-H3), a marker for mitosis. We found that deletion of *Tlx* in infected cells resulted in a more than 80% reduction in proliferation (Fig. 1c, d), indicating that growth factors and the presence of surrounding wild-type cells cannot rescue the loss of TLX function in the infected cells and suggesting that TLX is required cell autonomously for NSC proliferation.

To identify TLX-dependent molecular targets, we isolated two populations of *Tlx*-expressing NSCs from adult male brains. One population, designated $Tlx^{f/z;CreER}$ NSC, harbours a floxed allele of *Tlx* and a constitutively expressed transgene, $CreER^{TM}$, which encodes a fusion of Cre recombinase and a modified, tamoxifen (TM)-responsive ligand-binding domain of oestrogen receptor[10]. Addition of tamoxifen to the culture medium leads to a temporally controlled robust deletion of the floxed allele of *Tlx* (Fig. 2a). The control population ($Tlx^{f/z}$ NSC) does not contain the $CreER^{TM}$ transgene; thus, treatment with tamoxifen has no effect on *Tlx* mRNA (Fig. 2a).

Using total RNA isolated from these two populations of cells after 36 h or 60 h of treatment with tamoxifen or vehicle, we analysed all genes whose expression was altered by at least 1.39-fold. After exclusion of tamoxifen-induced changes in control cells (36 and 99 genes at 36 h and 60 h, respectively), the number of genes with altered expression in response to deletion of *Tlx* was found to be 432 genes at 36 h and 607 genes at 60 h (Fig. 2b). Among these genes, 53.9% and 51.7% are upregulated at 36 h and 60 h, respectively (Supplementary data 1–3). Further analysis revealed that 206 genes had altered expression levels at both 36 h and 60 h after tamoxifen-induced

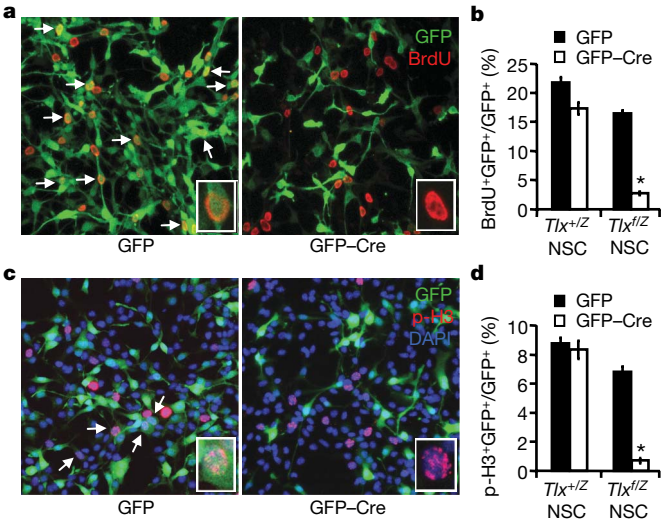

Figure 1 | Cell-autonomous requirement of TLX for adult NSC proliferation. **a**, $Tlx^{f/Z}$ NSCs were stained for GFP and BrdU after infection with adenovirus expressing GFP alone or GFP–Cre. Arrows, GFP^+BrdU^+ cells. Insets are stained cells at a higher magnification. **b**, Percentage of proliferating NSCs among infected cells. $Tlx^{+/Z}$ NSCs with one intact wild-type allele were used as controls. **c**, $Tlx^{f/Z}$ NSCs stained for GFP, mitotic marker phospho-H3 (p-H3) and the nucleus (DAPI) after virus infection. Arrows, GFP and p-H3 co-labelled cells. Insets are stained cells at a higher magnification. **d**, Percentage of infected cells undergoing mitosis. Mean ± s.e.m.; $n = 4$; asterisk, $P < 0.001$.

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deletion of *Tlx*. Of these, 21.8% are involved in regulation of cell cycle, proliferation, or DNA replication (Fig. 2b), some of which were further confirmed by quantitative RT–PCR on independent samples (Fig. 2c).

Because *Tlx* expression is restricted to the forebrain, we could use the inducible *CreER*^{TM} system to test whether it is required for adult neurogenesis *in vivo*[9–12]. Using adult reporter mice treated for 8 days with tamoxifen, we observed high recombination efficiencies in the hippocampus and ventricular regions and in proliferating NSCs (Supplementary Fig. 2a, c). Moreover, there was about an 80% reduction of *Tlx* mRNA in the forebrain and olfactory bulbs but not in the retina (Supplementary Fig. 2b), preserving visual competency (see below).

We treated 8-week-old mice with vehicle or tamoxifen for 8 days and injected 5-bromodeoxyuridine (BrdU) at various time points to label dividing cells. Although careful examination of the body weight, brain weight or brain morphology did not reveal observable changes in any of the treated mice (Supplementary Fig. 3), inducible deletion of *Tlx* resulted in a marked reduction of BrdU-labelled cells in the dentate gyrus when examined at 4 weeks or at 5.5 months (66.2% and 67.6% reduction compared to tamoxifen-treated controls, respectively; Fig. 3a–c). Additional controls were used to exclude the possibility of a Cre-mediated toxic effect[13] (Supplementary Fig. 4).

To test whether the remaining cells still responded to voluntary running with enhanced proliferation[14], we subjected the mice to 3 weeks of running 1 week after treatment and analysed the proliferation of NSCs by BrdU incorporation (Fig. 3d). Despite a marked reduction of BrdU-positive cells, the total number of dividing cells in the exercised *Tlx*-deleted brains was increased (~2-fold) compared to non-runners (compare Fig. 3d to 3b), indicating that voluntary exercise is still able to stimulate the proliferation of the remaining NSCs but cannot fully compensate for those cells lost owing to *Tlx* deletion.

Long-term effects of *Tlx* deletion on NSC survival and adult neurogenesis in the dentate gyrus were analysed 4 weeks after the last injection of BrdU (Fig. 3e–g). We found that the total number of BrdU-positive cells was markedly reduced in *Tlx*-deficient mice (Fig. 3e, 77% decrease compared to tamoxifen-treated controls). In contrast, the survival rate, which was measured as the ratio of BrdU-positive cells 4 weeks after labelling to that at 1 day after BrdU incorporation, was not significantly different from controls, indicating that deletion of *Tlx* has a minimal effect on the residual wild-type NSCs (Fig. 3f). Using NeuN as a mature neuronal marker, we observed a more than 80% reduction of NeuN and BrdU double-labelled cells (Fig. 3g). Together, these data suggest that TLX is essential for maintaining the NSC pool and the associated neurogenesis in adult brains.

Recent data indicate that adult neurogenesis is essential for contextual fear conditioning[15,16]. To test this possibility, we evaluated a cohort of mice 4 weeks after tamoxifen or vehicle treatment in the contextual fear conditioning paradigm. Unexpectedly, the learning ability of neurogenesis-deficient mice (*Tlx*$^{f/f;CreER}$ plus tamoxifen)

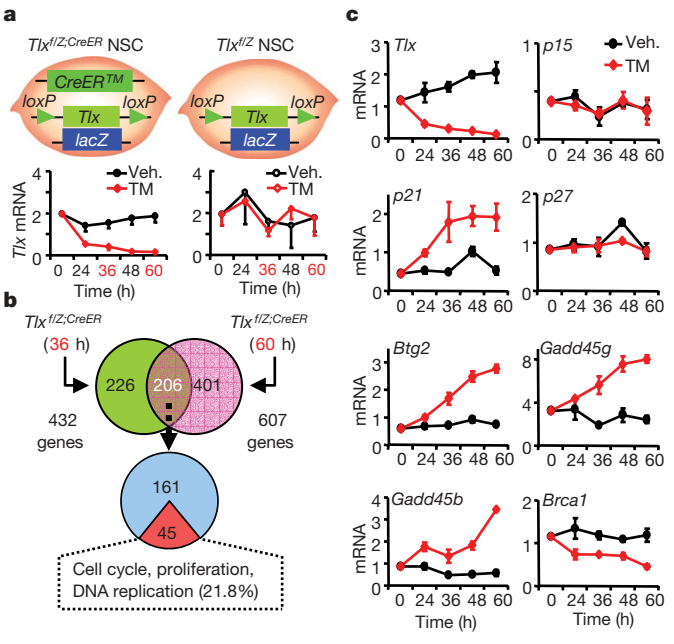

Figure 2 | TLX-regulated genetic programme in adult NSCs. a, Tamoxifen-induced, time-dependent deletion of *Tlx* in adult male *Tlx*$^{f/Z;CreER}$ NSCs but not in cells without the CreER transgene. Total RNAs collected at 36 h and 60 h after treatment were used for global gene expression profiling. TM, tamoxifen; veh., vehicle. *x*-axis, time (h) after treatment; *y*-axis, relative expression of mRNA after normalization to that of *hprt*. **b**, Schematic diagram showing TLX-dependent gene expression. Of the 206 genes that changed expression at both time points, 21.8% are implicated in regulation of cell proliferation based on gene ontology analysis. **c**, Quantitative RT–PCR analysis of selected genes for which expression is dependent on TLX. *p15* and *p27* expression were used as controls. Mean ± s.e.m.; $n = 3$.

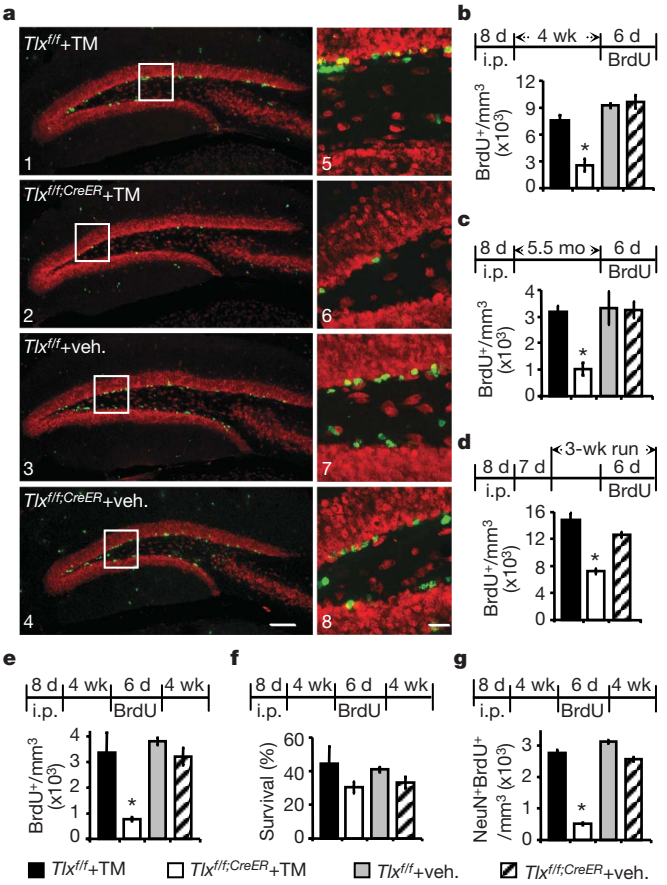

Figure 3 | Deficient NSC proliferation and neurogenesis in the adult hippocampus after inducible deletion of *Tlx*. a, Representative coronal sections of the dentate gyrus stained with neuronal marker NeuN (red) and proliferating marker BrdU (green) 4 weeks after 8-day tamoxifen (TM) or vehicle (veh.) treatment of 8-week-old male mice. Panels 5–8 are images taken from boxed regions in panels 1–4, respectively. Scale bar: 1–4, 100 μm; 5–8, 20 μm. **b–d**, BrdU-labelled cells normalized to the volume of the dentate gyrus after 4 weeks (**b**, asterisk, $P < 0.0001$), 5.5 months (**c**, asterisk, $P < 0.003$) or running for 3 weeks (**d**, asterisk, $P < 0.001$) after treatment. **e**, Surviving BrdU-positive cells at 4 weeks. Asterisk, $P < 0.002$. **f**, Survival rate, represented as the ratio of BrdU-positive cells at 4 weeks to that at 1 day after BrdU injection. **g**, Four-week-old new neurons. Asterisk, $P < 0.002$. Mean ± s.e.m.; $n = 4$ for each group. d, day; wk, week; mo, month; i.p., intraperitoneal injection of tamoxifen or vehicle.

was comparable to their controls after three training sessions (Fig. 4a, $F_{1,16} = 0.34$, $P = 0.57$; Supplementary Fig. 5a; two-way analysis of variance (ANOVA) with repeated measures); they also maintained normal contextual memory after 24 h and 48 h (Fig. 4b, $F_{1,16} = 0.13$, $P = 0.73$ at 24 h and $F_{1,16} = 0.74$, $P = 0.40$ at 48 h; Supplementary Fig. 5b; two-way ANOVA with repeated measures). Tone-cued memory measured 24 h later was also similar between treatment groups (Fig. 4c, $P = 0.37$; see also Supplementary Fig. 5c; single factor ANOVA).

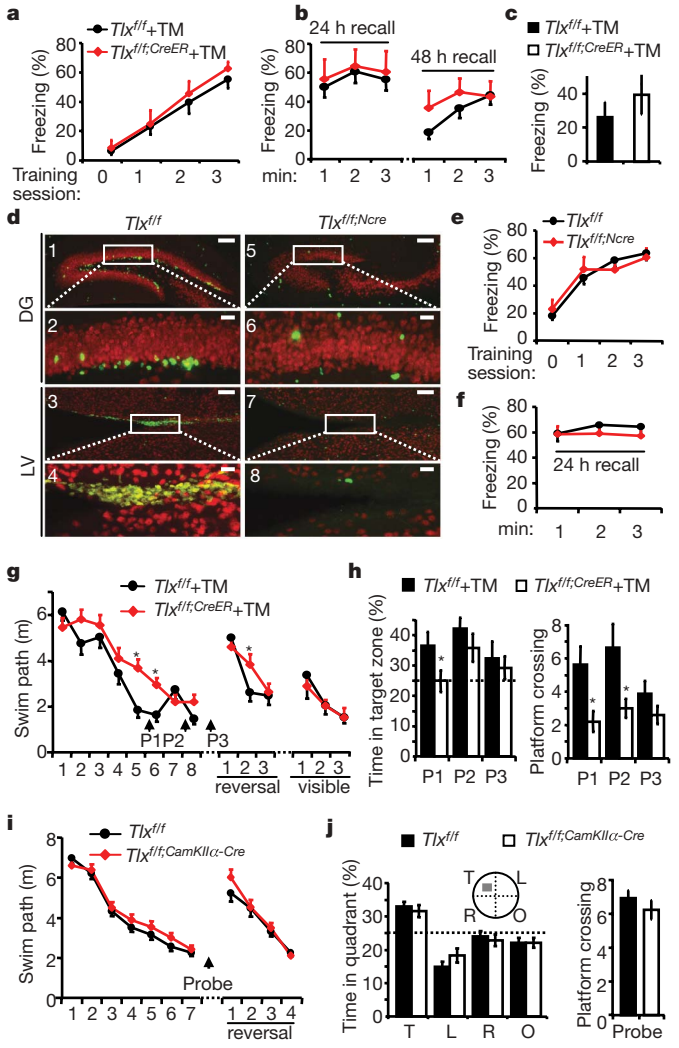

Figure 4 | Normal contextual fear conditioning but impaired spatial learning and memory for mice with defective adult neurogenesis. a–f, New neurons are dispensable for contextual fear conditioning. **a–c**, Freezing behaviour monitored during training (**a**), contextual (**b**) or tone-cued memory tests (**c**). $n \geq 8$. **d**, Confocal images showing proliferating cells (BrdU, green). DG, dentate gyrus; LV, lateral ventricle. Panels 2, 4, 6 and 8 are higher magnification views (scale, 20 μm) of the regions boxed in panels 1, 3, 5 and 7 (scale, 100 μm), respectively. **e, f**, Freezing behaviour monitored during training (**e**) and contextual memory tests (**f**). $n \geq 14$. **g, h**, New neurons in the hippocampus participate in spatial learning. Mice were trained on the water maze (8-day training period) and tested for memory by two probe trials at 12 h (P1, P2) and one probe trial at 3 weeks (P3) after the previous training, followed by 3 days of reversal training and three blocks of training with a visible platform. Dotted line indicates chance level (25%). $n \geq 8$. Asterisk, $P < 0.05$. **i, j**, Neuronal deletion of *Tlx* (*Tlx*$^{f/f;CamKII\alpha\text{-}Cre}$) has no effect on spatial learning and memory. Mice were trained on the water maze, followed by a probe trial at 2 days after the last training and 4-day reversal trainings. $n \geq 28$. T, target zone; L, left zone; R, right zone; O, opposite zone. All data are presented as mean ± s.e.m.

To examine further this unexpected finding, we generated *Tlx*$^{f/f;NCre}$ mice by crossing the conditional allele to a transgenic line carrying the Cre gene under the control of a nestin enhancer that directs expression in the developing central nervous system (CNS)[17]. This early deletion of *Tlx* leads to a hypomorphic dentate gyrus and an absence of BrdU-labelled cells in adult mutant brains (Fig. 4d; see also Supplementary Fig. 6a, b). Unlike the germline deletion of *Tlx* that results in blindness[9], which may contribute to some of the observed behavioural defects[18], visual function was spared in 2- to 4-month-old *Tlx*$^{f/f;NCre}$ mice (Supplementary Fig. 6c–g). Although profoundly impaired in spatial learning and memory (Supplementary Figs 7 and 8), *Tlx*$^{f/f;NCre}$ mice learned and recalled contextual fear conditioning as well as their littermate controls (Fig. 4e, f, $F_{1,34} = 0.00$, $P = 0.97$ for training and $F_{1,34} = 0.30$, $P = 0.59$ for contextual memory; two-way ANOVA with repeated measures). Of note, our protocol can clearly detect the deficit in contextual fear conditioning using a mouse model with normal adult neurogenesis (our own unpublished data). Even when a new cohort of mice was examined using an alternative protocol[15,16], we did not detect a deficit in this behavioural paradigm (Supplementary Fig. 9). Consistent with our data, it has been reported that partial or complete hippocampus-lesioned animals are able to learn and recall as well as controls in contextual fear conditioning[19–21]. Perhaps the observed impairment in contextual fear conditioning in those studies[15,16] is attributable to differences in species or to the side effects of the methods used to knock down neurogenesis[6,22,23].

To test whether adult neurogenesis is involved in spatial learning and memory, we examined a new cohort of mice 4 weeks after an 8-day tamoxifen or vehicle treatment in a more challenging version of the Morris water maze, which uses 40-s learning trials without pre-training. Although tamoxifen-treated *Tlx*$^{f/f;CreER}$ mice performed similarly in the initial phase of learning (day 1 to day 4) and were able to eventually catch up to the controls (overall performance $F_{1,16} = 3.41$, $P = 0.08$; two-way ANOVA with repeated measures), planned comparisons of swim path revealed significant delays in learning on the fifth and sixth day (Fig. 4g). Indeed, probe trials carried out 12 h after the fifth day session demonstrated a major deficiency in short-term memory for these mutant mice, measured by time in the target zone or platform crossings (Fig. 4h, P1). They also made fewer crosses through the platform location during a second probe trial, indicating a less robust search strategy (Fig. 4h, P2). However, long-term memory examined 3 weeks after the last training session was not affected (Fig. 4h, P3). Notably, tamoxifen-treated *Tlx*$^{f/f;CreER}$ mice continued to show delayed learning on the second day of a reversal training, as revealed by planned comparisons, suggesting that they could not efficiently associate previous experience with the new task and/or were deficient in cue discrimination (Fig. 4g, reversal 1–3). Such learning and memory impairments are probably not due to a lack of motivation, visual disability or locomotor dysfunction, as these mice performed as well as their littermate controls on a visible version of the water maze, swim velocity, visual cliff, light/dark transition and circadian activity tests (Fig. 4g, visible 1–3; see also Supplementary Figs 10 and 11).

Because tamoxifen-induced recombination also occurred in mature neurons in the dentate gyrus and parts of the cortex (Supplementary Fig. 2a), where *Tlx* shows sporadic expression (Supplementary Fig. 12b, c), the above observed delay in spatial learning may come from the neuronal function of TLX. To examine this possibility, we first analysed the neuronal cell types in which *Tlx* has expression by staining brain sections and found that *Tlx* is specifically expressed in excitatory neurons but not in inhibitory interneurons (Supplementary Fig. 13). This observation was confirmed by immunostaining of cultured hippocampal neurons from postnatal day 0 pups (Supplementary Fig. 14). To delete *Tlx* in these neurons, we crossed *Tlx*$^{f/f}$ mice to a *CaMKIIα-Cre* transgenic line, which gives rise to postnatal, excitatory neuron-specific recombination[24]. Careful

examination throughout the mouse brains indicated that sparsely expressed *Tlx* cells are found in regions overwhelmingly positive for *CaMKIIα-Cre*-mediated recombination (Supplementary Fig. 12). As expected[24], this strategy gives rise to efficient recombination of *Tlx* in regions expressing *CaMKIIα-Cre*, such as dentate gyrus, CA1 and cortex (see Supplementary Fig. 15). Furthermore, proliferation of NSCs is not affected (data not shown), suggesting that the neuronal function of TLX is not required for NSC maintenance. Interestingly, the performance of $Tlx^{f/f;CamKIIa\text{-}Cre}$ mice was the same as their littermate controls in the water maze under the same challenging training protocol (Fig. 4i,j). These data indicate that most, if not all, neuronal 'non-neurogenic' TLX is not required for spatial learning and memory.

Together, these results provide direct genetic support that NSCs can contribute to the spatial learning and memory circuits[4]. Notably, our results do not support several other studies showing that adult neurogenesis does not have a role in spatial learning[15,25,26]. The cause of such a discrepancy may originate from differences in behavioural testing protocols, the animal species, or genetic backgrounds[27]. Regarding studies specifically using mice[15,28], it is intriguing that, besides the longer duration per trial and/or increased trial numbers per day, both of these previous studies trained the mice on the visible platform before testing with the hidden platform. To test whether such a training paradigm makes a difference on the water maze, we trained a new cohort of mice after inducible deletion of *Tlx* using the protocol described[15,29]. The results clearly demonstrated that under such a training protocol the contribution of adult neurogenesis to spatial learning and memory in the water maze is no longer detectable (Supplementary Fig. 16).

Our development of a new, inducible knockout mouse model provides a new and powerful tool to understand better the role of adult neurogenesis in normal behaviour and disease, and should deepen our insight into which of the many facets of brain function are impacted by this progressive and dynamic cell population.

METHODS SUMMARY

The strategy for generating a conditional allele of *Tlx* using the Cre/*loxP* system is schematically shown in Supplementary Fig. 1. Three correctly targeted embryonic stem cell clones were used to generate mutant mice, which were then backcrossed to C57BL/6J for at least two generations. The resulting Cre transgenic mice were used for crossing to the conditional allele of *Tlx* ($Tlx^{f/f}$): *pNestin-Cre* (ref. 17), $pCAGG\text{-}CreER^{TM}$ (ref. 10) and *pCamKIIα-Cre* (ref. 24), and the resulting strains, were respectively designated $Tlx^{f/f;NCre}$, $Tlx^{f/f;CreER}$ and $Tlx^{f/f;CamKIIα\text{-}Cre}$. Age- and gender-matched littermates were used for behavioural studies. Adult NSCs were isolated from whole-mouse brains as described[7]. Single-factor ANOVA was used for morphological data. ANOVA with repeated measures over time was applied to the behavioural data. Planned comparison tests were used for post-hoc analysis. $P \leq 0.05$ was considered as significant. All values were expressed as mean ± s.e.m.

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

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Animals. The mice were age- and gender-matched littermates that had been handled daily for 1 week before behavioural studies. They were housed under standard 12 h light/dark cycles and controlled temperature conditions and had free access to food and water. Behavioural studies, except for light/dark transitions and circadian rhythms, were conducted at light cycle with dim light. Experimental protocols were approved by the Institutional Animal Care and Use Committee at The Salk Institute for Biological Studies.

Tamoxifen treatment and *in vivo* BrdU labelling. A 20 mg ml^{-1} tamoxifen (Sigma) stock solution was dissolved in corn oil by bursts of sonication and stored at 4 °C for a maximum of 8 days. Because our initial pilot studies by intraperitoneal injection of corn oil or corn-oil-dissolved tamoxifen under tight physical holding led to loss of mice irrespective of the treatment, which was caused by needle damage to internal organs, all the following injections were done under anaesthesia to reduce the injection stress. Eight-week-old mice were injected intraperitoneally once daily with 4 mg tamoxifen per 40 g body weight or sterile corn oil (vehicle) for 8 consecutive days. Dividing cells were labelled by intraperitoneal injection of BrdU twice daily 10 h apart at 50 mg kg^{-1} body weight over a 6-day period. Mice were perfused 1 day (for proliferation assay) or 4 weeks (for survival and phenotype analysis) after the last injection and brains were processed for immunostaining as described[30].

Adult NSC culture. NSCs were prepared from 8-week-old *Tlx* mutant mice carrying a *lacZ* marker knocked into the endogenous *Tlx* locus as described by β-gal-based sorting[31]. Cells on four-chamber slides were infected 12 h after plating with adenovirus expressing GFP or Cre-IRES-GFP for 10 h, washed three times with growth medium, and cultured for another 36 h with BrdU (10 μM), which was added for 4 h at the end of the culture to label dividing cells. Alternatively, twofold more non-infected wild-type cells were added to the chambers after the post-virus infection washing and were cultured for another 36 h.

Gene expression profiling and quantitative RT–PCR analysis. Total RNA samples in duplicate were isolated at the indicated time point after vehicle or tamoxifen treatment using TRIzol reagents (Invitrogen). Expression profiling using Affymetrix mouse genome 430A 2.0 arrays was performed as described[32]. Data with an average fold change of 1.39 at $P \leq 0.05$ for each comparison (total of 4) were considered for gene ontology (GO) analysis. Quantitative PCR was performed as previously described[32].

Histology and quantification. We carried out β-galactosidase staining or immunostaining on 40-μm free-floating sections or cultured NSCs as described[30,31]. For cultured NSCs, we counted GFP-, BrdU- or phospho-H3-positive cells under a ×40 objective using the StereoInvestigator software (MicroBrightfield) and a fluorescent microscope. At least 500 GFP-positive cells were counted for each sample. All quantitative studies were based on four or more independent replicates.

Behavioural analyses. Behavioural studies were conducted in the following order: Morris water maze, visual cliff, light/dark transition, circadian rhythms, rotarod, open field and fear conditioning using 2-month-old mice (3.5-month-old for tamoxifen-treated mice). A separate cohort of mice was used for fear conditioning or water maze tests as indicated in the text.

Morris water maze. The Morris water maze was conducted as described with modifications[33]. A white plastic tank 120 cm in diameter was filled with room temperature water, which was made opaque with white non-toxic Crayola washable paint. A transparent platform (8 cm × 13 cm) was located in the centre of one of the four virtually divided quadrants during training and was submerged 0.5 cm below the surface of opaque water. Distal cues, such as a computer desk and painted cardboard on the white wall, were provided as spatial references. No local or proximal cues were present during hidden platform training. Mice were handled daily for 1 week before the training. We trained mice with two consecutive trials per block and two blocks per day with 3–4 h between the blocks. Each trial was started from a randomly chosen point away from the hidden platform and lasted either until the mouse had found the platform or for a maximum of 40 s. Afterwards, the mouse was allowed to stay on or was put onto the platform for 15 s. During probe trials, the mouse was allowed to swim 40 s without the platform. For reversal learning, the hidden platform was transferred to a new location opposite to its previous site without changing any distal visual cues. For the visible version of water maze testing, the training was conducted in a different room with totally different cues and the platform was made clearly visible to the mouse; its location was changed for each trial, which started from a random point away from the platform. We used a video tracking system (Ethovision; Noldus Information Techonology) to record and analyse the swimming path, velocity and time taken to reach the platform (latency) or in each zone.

For the water maze paradigm using extensive trainings, mice were handled daily for a week before the behavioural testing. Testing was conducted in three phases as described[34,35] and consisted of 2 days of pre-training, 2 days of visible platform in a fixed location, and 7 days of hidden platform in a new fixed position. Pre-trainings were conducted using a plastic water container of 49 × 38 × 32 cm filled with 17 cm of water at room temperature. A platform (10 × 14 cm) was submerged under the opaque water in the centre of the container. The mouse was released in the corner and was allowed to stay on the hidden platform for 20 s once they found it. Each mouse was pre-trained for two trials per day for 2 days with an inter-trial interval of ~5 min. After pre-training, the mice were further tested using a visible platform in a plastic water tank of 120 cm diameter filled with opaque water under room temperature. A submerged (0.5 cm below the water) transparent platform (8 × 13 cm) was located in a fixed position in the centre of one of the four virtually divided quadrants. Platform location was indicated by a multicolour plastic rod, which was 24 cm above the water and was attached to the platform. Mice were trained with visible platform for four trials per day for 2 days with an inter-trial interval of about 15 min. Each trial started with a pseudo-random point away from the visible platform and lasted either until the mouse had found the platform or for a maximum of 120 s. Afterwards, the mouse was allowed to stay on or was put onto the platform for 15 s. Hidden platform trainings were conducted immediately after the visible version in the same pool within the same room. The platform was submerged 0.5 cm below the opaque water in a new fixed position without any visible local cues. Training procedure was identical to that of the visible platform. Memory was examined by using a probe trial that was administered 6 h after the last training. During the probe trial, the mouse was allowed to swim 60 s without the platform in the tank.

Visual cliff, light/dark, locomotion and rotarod. Visual cliff and light/dark transition paradigms were carried out as described previously[36]. For diurnal activities, we monitored the mice using computer-assisted running wheels for two continuous weeks or using metabolic cages equipped with far-red beams for three successive days. Locomotor activities were measured using rotarod and open field paradigms as described[36].

Fear conditioning. We used a video tracking system for the fear conditioning (Med Associates). Mice were handled daily for 1 week and three times 1 day before the test and were transferred to a holding place adjacent to the procedure room for 20 min to 1 h on the day of test. We then put individual mice into the testing chamber and scored freezing behaviour for 3 min as baseline. Each mouse then received three training sessions with each session consisting of 20-s tone, 1-s trace, 1-s foot shock and 1-min of monitoring for freezing behaviour after the shock (trace protocol). After 24 h, each mouse was returned to the same chamber with the exact contextual settings but without tone and foot shock and monitored for 3 min for contextual memory.

For fear conditioning using delayed protocol, we trained mice as described[34,35]. Two-month-old mice were treated daily with tamoxifen or vehicle for 8 days. Fear conditioning was conducted 6 weeks after the last treatment. Each mouse was handled daily for a week before the behavioural testing. Testing was conducted in three phases as described[34,35], which consisted of four trainings with a delayed protocol on day 1, tone-cued conditioning on day 2, and contextual fear conditioning on day 3. Training on day 1 was as follows: each mouse was individually and gently put into the conditioning chamber with a ventilation fan providing background noise (Med Associates). After 120 s, each mouse received four pairings of tone (20 s, 80 dB, 2 kHz) and a co-terminating shock (1 s, 0.7 mA) with a variable inter-trial interval of about 125 s. After the last shock, the mouse was further monitored for 60 s. Chambers were cleaned with isopropanol between each set of mice. The tone-cued fear conditioning protocol on day 2 was as follows: the ventilation fan was turned off. The conditioning chamber was significantly altered by covering the grid floor and the three sides with patterned plastic boards, and the top with a patterned paper. New visual cues were provided on the inside wall of the insulating box and on the wall of the room. The conditioning chamber was further scented with Windex and vanilla solution. Windex was used for cleaning after each testing. The experimenter wore a different style of gloves and a different colour laboratory coat from day 1. Each mouse was gently put into the chamber for 120 s and a 20-s tone was administered after. Freezing behaviour was monitored during the first 120 s and during the 20 s of tone. For contextual fear conditioning on day 3, the testing procedure and environment were identical to those used on day 1 except the tone was not presented. Each mouse was gently put into the conditioning chamber and monitored for 4 min for freezing.

Hippocampal cell culture and staining. Hippocampal neurons from postnatal day 0 $Tlx^{+/lacZ}$ pups were cultured using standard procedures on astrocyte-coated coverslips. After 16 days culturing *in vitro*, cells were fixed using a fixation solution containing 2% paraformaldehyde and 0.1% glutaraldehyde for 10 min on ice and washed three times in ice-cold 1× PBS. Because of the discontinuation of an antibody that can recognize the β-galactosidase product of the *lacZ* gene that is knocked into the *Tlx* locus, we used a colour reaction to detect the

expression of the *lacZ* gene using 1× PBS solution containing X-gal (1 mg ml^{-1}), $K_3Fe(CN)_6$ (4 mM), $K_4Fe(CN)_6$ (4 mM) and $MgCl_2$ (2 mM) at room temperature. The cells were washed with 1× PBS and were further stained for neuronal type markers by standard immunocytochemistry. The following antibodies were used: rabbit anti-glutamatergic receptors 1, 2, 3 and 4 (Chemicon) for excitatory neurons and rabbit anti-calretinin/calbindin D29K (Novus) for inhibitory interneurons.

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