Dlk1 dosage regulates hippocampal neurogenesis and cognition

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Neurogenesis in the adult brain gives rise to functional neurons, which integrate into neuronal circuits and modulate neural plasticity. Sustained neurogenesis throughout life occurs in the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and is hypothesized to be involved in behavioral/cognitive processes such as memory and in diseases. Genomic imprinting is of critical importance to brain development and normal behavior, and exemplifies how epigenetic states regulate genome function and gene dosage. While most genes are expressed from both alleles, imprinted genes are usually expressed from either the maternally or the paternally inherited chromosome. Here, we show that in contrast to its canonical imprinting in nonneurogenic regions, Delta-like homolog 1 (Dlk1) is expressed biallelically in the SGZ, and both parental alleles are required for stem cell behavior and normal adult neurogenesis in the hippocampus. To evaluate the effects of maternally, paternally, and biallelically inherited mutations within the Dlk1 gene in specific behavioral domains, we subjected Dlk1-mutant mice to a battery of tests that dissociate and evaluate the effects of Dlk1 dosage on spatial learning ability and on anxiety traits. Importantly, reduction in Dlk1 levels triggers specific cognitive abnormalities that affect aspects of discriminative differences in environmental stimuli, emphasizing the importance of selective absence of imprinting in this neurogenic niche.

Generating new neurons occurs normally in the adult brain in two locations: the subventricular zone (SVZ) in the walls of the lateral ventricles (1) and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (2, 3). Neurogenesis is supported by multipotent neural stem cells (NSCs), astrocyte-like cells that are relatively quiescent and express the stemness-related transcription factor SOX2 (sex determining region Y [SRY]-box 2) (4, 5). In the SGZ, two types of lineage related SOX2+ cells that contribute to neuronal progeny have been identified: the SOX2+ cells with radial processes spanning the granule cell layer and that express the glial fibrillary acidic protein GFAP and Nestin (type one cells) and the nonradial SOX2+ cells (type two) that have short processes, express TBR2, and arise from the radial population (6, 7). Radial SOX2+ cells are the source of stem cells in the DG and rarely divide, whereas nonradial cells cycle more often, being suggested as the intermediate precursor (8). Both the horizontal and radial progenitors respond differently to neurogenic stimuli, suggesting that in the SGZ, there are different populations of progenitors with different properties (9). Type two cells generate in turn type three cells, which are neuronal precursors that express markers of immature migrating neurons, such as doublecortin (DCX) and the polysialylated neural cell adhesion molecule (PSA-NCAM). Once formed, new neurons migrate to the granule cell layer (GCL) of the DG, where they develop morphological and functional properties of granule neurons and integrate neuronal circuits into the hippocampal CA3 region, forming dendrites and spreading their axons, modifying the existing circuitry (7, 10). In addition to the production of granular neurons, a low percentage of activated NSCs divide asymmetrically to give rise to astrocytes. The latter migrate into the hilus and the molecular layer, where they lose their stem cell potential (11). Hippocampal NSCs can be isolated from the DG and cultured in vitro in the presence of the epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) mitogens, forming neurogenesis | genomic imprinting | behavior | gene dosage | hippocampus

Generation of new neurons occurs normally in the adult brain in two locations: the subventricular zone (SVZ) in the walls of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus. Neurogenesis in the adult hippocampus has been implicated in cognitive functions such as learning, memory, and recovery of stress response. Imprinted genes are highly prevalent in the brain and have adult and developmental important functions. Genetic deletion of the imprinted gene Dlk1 from either parental allele shows that Dlk1 is a key mediator of quiescence in adult hippocampal NSCs. Additionally, Dlk1 is exquisitely dosage sensitive in the brain with perturbations in levels resulting in impaired NSCs function and cognitive phenotypes.

Significance

Generation of new neurons occurs normally in the adult brain in two locations: the subventricular zone (SVZ) in the walls of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus. Neurogenesis in the adult hippocampus has been implicated in cognitive functions such as learning, memory, and recovery of stress response. Imprinted genes are highly prevalent in the brain and have adult and developmental important functions. Genetic deletion of the imprinted gene Dlk1 from either parental allele shows that Dlk1 is a key mediator of quiescence in adult hippocampal NSCs. Additionally, Dlk1 is exquisitely dosage sensitive in the brain with perturbations in levels resulting in impaired NSCs function and cognitive phenotypes.

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free-floating aggregates called “neuroospheres” (12, 13). Self-renewal and multipotency characteristics of NSCs are assessed in vitro by clonal analysis in which single cells give rise to neurospheres (14, 15).

Neurogenesis in the adult hippocampus has been implicated in cognitive functions such as learning, memory, and recovery of stress response (16–19) as well as contributing significantly to hippocampal plasticity throughout the life span (20, 21). However, how adult neurogenesis contributes to hippocampal function is largely unknown. The hippocampus is involved in memory formation as well as spatial navigation. Indeed, promotion or suppression of hippocampal neurogenesis corresponds with improvement or impairment in learning and memory performance, respectively (17, 18, 22, 23). Both specific brain circuits within the hippocampus and adult neurogenesis, have been suggested as modulatory mechanisms for the encoding of spatial/contextual information (23, 24). Notably, the discrimination between memorized similar stimuli (e.g., a particular location) and the new stimulus conditions (e.g., a new location) are fundamental sensory/cognitive mechanisms that pave the way for the reorganization of complex behaviors and cognitive processes. Importantly, these processes are shared across many species, including mice and humans (25). A functional role for hippocampus neurogenesis in spatial pattern separation has also been demonstrated with lesions of the DG circuitry, resulting in impaired pattern separation-dependent memory (17, 26, 27). Furthermore, an excessive pattern separation prevents the integration of multiple information within the environment and results in cognitive inflexibility, anxiety, and excessive attention to details as manifested in autism and obsessive-compulsive disorders (23).

Thus, adult neurogenesis in the hippocampus serves as a normal cellular process for learning and memory consolidation.

Genomic imprinting is an epigenetic process that causes genes to be expressed according to their parental origin, resulting in activation of one of the two alleles of a gene and repression of the other (28). Imprinted genes are highly prevalent in the brain and have adult and developmental important functions (29, 30). The vertebrate-specific atypical NOTCH ligand Delta-like homolog 1 (Dlk1) is an imprinted gene that encodes membrane-bound and secreted isoforms that function in several developmental processes (31–34). The previously shown that mice deficient in Dlk1 have defects in postnatal SVZ neurogenesis, resulting in depletion of mature neurons residing in the olfactory bulb. Dlk1 is secreted by SVZ niche astrocytes, whereas its membrane-bound isoform is present in NSCs and is required for the inductive effect of secreted Dlk1 on self-renewal within this niche (33). In most human and mouse tissues, Dlk1 is expressed only from the paternally inherited chromosome (35, 36). However, in the SVZ, there is a requirement for Dlk1 to be expressed from both maternally and paternally inherited chromosomes (33), suggesting that modulation of imprinting might be specific in different developmental contexts. Nothing is known about the imprinting status of Dlk1 in the adult SGZ and its role in adult hippocampal function. Although most of the work on imprinted genes has centered on fetal and early postnatal growth, growing evidence suggests a fundamental role in learning and memory (30). For instance, deficiencies in contextual memory are observed when Ube3a, Ppyl1δ9a, or Rasgfl are absent (42–44), and Iqg2 has been shown to be necessary for memory consolidation (45). Here, we test the requirement of Dlk1 signaling for the regulation of NSCs and evaluate the effects of maternal and paternal heterozygous mutations and homozygous mutations of the Dlk1 gene in specific behavioral and cognitive domains that involve hippocampal circuits.

Genetic deletion of Dlk1 from either parental allele shows that Dlk1 is a key mediator of quiescence in adult hippocampal NSCs. Additionally, we have subjected Dlk1-deficient mice to spatial learning tasks (geometry test), anxiety-like assessments (elevated plus maze), and discrimination tests (touch screen task). Within this battery of behavioral tests, we dissociated the effects of Dlk1 dosage on learning ability, on pattern separation and completion, and on repetitive/anxiety traits. Taken together, our evidence indicates that Dlk1 is exquisitely dosage sensitive in the brain, with perturbations in levels resulting in impaired NSCs function and cognitive/psychiatric phenotypes.

Results

Biallelic Expression of Dlk1 Is Required for Adult Hippocampal Neurogenesis. Previous studies have suggested that radial glia-like cells, which express GFAP and Nestin and form neurospheres in vitro, are NSCs in the hippocampal SGZ (13, 46). We first examined Dlk1 expression in the DG of the adult mouse hippocampus using qPCR and immunohistochemical analysis. We observed that Dlk1 is present in NSCs derived from the adult SGZ and in double GFAP/SOX2+ cells in the wild-type hippocampus in vivo (Fig. 1 A and B), but on the contrary to the SVZ, differentiated astrocytes from the SGZ show very low levels of expression of this gene (Fig. 1A). Alternatively, spliced transcripts of Dlk1-encoding protein isoforms that are either membrane tethered or proteolytically cleaved and secreted have been described (33, 47). In the adult hippocampus, we found that both the Dlk1 membrane-bound and the secreted isoforms are expressed (SI Appendix, Fig. S1A). However, the secreted isoform represents the predominant type in the SGZ NSCs (SI Appendix, Fig. S1A), also contrary to what it happens in the SVZ NSCs (33), suggesting a different mechanism of action of Dlk1 in this neurogenic niche.

Dlk1 is canonically expressed from the paternally inherited chromosome (35, 48); thus, to study whether the imprinting status of the gene is maintained in the adult hippocampus, we next examined Dlk1 expression from each of the two parental chromosomes. To do so, we determined Dlk1 allele-specific activity in the SGZ of wild-type F1 hybrid offspring from reciprocal crosses of Mus musculus domesticus (C57BL6/J) and Mus musculus castaneus (CAST/EiJ) strains, in which a single-nucleotide polymorphism (SNP) was identified between the two subspecies at the Dlk1 gene (SI Appendix, Fig. S1B). Whereas whole hippocampus tissue from reciprocal hybrids showed the expected paternally inherited imprinted expression of Dlk1, the adult NSCs showed biallelic (nonimprinted) expression of the gene (Fig. 1C) as was previously shown in NSCs derived from the SVZ (33). Importantly, other imprinted genes, such as the adjacent Gtl2 (also known as Meg3) and Snrpn genes, maintained their imprinting state in SGZ NSCs (Fig. 1C and SI Appendix, Fig. S1 B and C) and did not alter their expression levels (SI Appendix, Fig. S1F). This identifies a previously undescribed biallelic expression of specifically Dlk1 in the adult hippocampus neurogenic niche.

In order to investigate the role of Dlk1 in DG neurogenesis in vivo, we analyzed the SGZ of wild-type, maternal (Dlk1mat/+) , or paternal (Dlk1mat+pat) heterozygotes and of Dlk1 homozygous (Dlk1−/−) mice (33) for Dlk1 mutation. We first demonstrated by qPCR and immunocytochemical analysis that Dlk1 was reduced in NSCs derived from the SGZ of Dlk1mat+pat or Dlk1mat+pat heterozygotes (Fig. 1D and SI Appendix, Fig. S1 D and E), confirming absence of imprinting in this niche. To assess any functional relevance of this and the possibility that Dlk1 can regulate proliferation of NSCs in the SGZ, as we had previously described for the SVZ (33), 2-mo-old Dlk1 mutant mice were injected with the nucleotide analog BrdU 3 wk prior to killing (Fig. 2A). Based on proliferation capacities, two major classes of cells are expected to retain the BrdU label in the DG of the hippocampus: slowly dividing SOX2+ NSCs that do not dilute the BrdU through division (named as label retaining cells, LRC-BrdU) and newborn neurons that incorporate BrdU prior to cell division.
cycle exit (SI Appendix, Fig. S2A). Among the cells that retained BrdU, we quantified the proportion of GFAP/SOX2+ undifferentiated cells in Dlk1 mutant mice and observed that the total number of SOX2+ cells was diminished through a reduction in both SOX2+ radial and nonradial cells (Fig. 2 A–D). Additionally, as measured by anti-Ki67, GFAP/SOX2+ proliferation was diminished, indicating a restriction in the proliferative activity in the SGZ in both maternal and paternal Dlk1/+ mice (Fig. 2B). We next counted neuronal progenitors and immature neurons, identified by their positivity for TBR2 and DCX, respectively, and observed significantly less TBR2+ progenitor cells in Dlk1 mutant mice (SI Appendix, Fig. S2 B–D). Dlk1 deletion also resulted in significantly less DCX+ immature neurons compared with controls (Fig. 2E and F), although their proliferation activities were not affected (SI Appendix, Fig. S2C). As a result, the number of BrdU+/+LRC/NeuN+ differentiated progeny was significantly reduced in the granular layer of the SGZ after both maternal and paternal transmission of the mutation (Fig. 2F). Importantly, Dlk1 homozygous mutants showed a more severe reduction in the number of undifferentiated GFAP/SOX2+ and newborn cells in the SGZ, suggesting a dosage-dependent effect of the protein (Fig. 2B, D, and F). We confirmed that these phenotypes were specifically due to a reduction in the available levels of DLK1 by generating paternal transmission Dlk1 mutants that were also hemizygous for a Dlk1-expressing transgene (Dlk1+/pat/Dlk1tg++) (Fig. 2B). The number of GFAP/SOX2/BrdU-LRCs and GFAP/SOX2/Ki67 cells in the transgene-containing mutant SGZ tissue was not significantly different from that obtained in wild-type littermates, indicating that rescue of the mutation by the intact transgene had occurred in vivo (Fig. 2B and D). Interestingly, overexpression of Dlk1 in the brain did not have an effect on the distribution of the different cell populations in the intact SGZ, as indicated by the proportion of GFAP/SOX2 positive cells in the DG of Dlk1 transgenic mice (48) (SI Appendix, Fig. S3 A–C).

These results demonstrated that Dlk1 regulates hippocampal neurogenesis in young mice and that selective absence of imprinting and therefore biallelic expression of the gene in the NSCs in the DG is required for the maintenance of stem cells in the SGZ neurogenic niche.

**Dlk1 Regulates Self-Renewal of Postnatal NSCs in the SGZ.** To investigate the specific role of Dlk1 on the self-renewal capacity of neural progenitors, we assessed whether the same phenotype could be reproduced in vitro under experimentally controlled conditions. In support of the in vivo data, the number of primary neurospheres isolated from the DG at different postnatal ages was reduced in Dlk1-deficient mice compared to controls (Fig. 3A and SI Appendix, Fig. S4 A and B). Again, Dlk1 homozygous mutant cultures showed a more severe reduction in the number of primary spheres than heterozygote, suggesting a dosage-dependent effect of the protein also in vitro (Fig. 3A and SI Appendix, Fig. S4 A and B). Consistently, no effects on the proportion of primary neurospheres obtained from the SGZ were observed in Dlk1-transgenic mice overexpressing Dlk1 (SI Appendix, Fig. S3D). It has been demonstrated that disruption of quiescence leads to loss of stem cell potential and depletion of the NSC pool with age (16, 49). To test whether Dlk1 was required for NSC maintenance in the aged SGZ, we determined the yield of primary neurospheres from 2-, 5-, 8-, and 12-mo-old mutant DG. Aged Dlk1-deficient mice showed a more severe progressive decline in the number of primary neurospheres than wild-type mice from the same age (Fig. 3A). Moreover, this decline was more acute in Dlk1+/− than in heterozygote cultures, confirming that a Dlk1 dose is important for NSC behavior in the SGZ (Fig. 3B–E). The number of in vitro neurospheres obtained from Dlk1+/pat/Dlk1tg double-mutant SGZ tissue was not significantly different from the number obtained from wild-type littermates (Fig. 3A), indicating that rescue of the mutant had.

![Image](https://doi.org/10.1073/pnas.2015505118)
ocurred also in vitro and that accelerated exhaustion of the stem cell pool was due to a reduction of DLK1 levels.

To next characterize the self-renewal capability of Dlk1-deficient cultures, primary neuropheres were individually dissociated into single cells and plated at clonal density (2.5 cells/μl) (SI Appendix, Fig. S4A). Dlk1-deficient NSCs exhibited a significant reduction in the number of secondary neuropheres formed at early passages (Fig. 3B). To determine whether this reduction in the self-renewal capacity defect in Dlk1 deficient cultures could be due to a proliferation or survival defect, we performed a tetrazolium salt (MTS) colorimetric assay, which showed a reduction in the proportion of metabolically active cells in the neurosphere cultures of both maternal and paternal Dlk1 heterozygous SGZ (Fig. 3C). Accordingly, cell cycle analysis by flow cytometry indicated that Dlk1 mutation reduced the proportion of hippocampal NSCs in S-phase (Fig. 3D). This decrease in proliferation was not due to cell death given that the propidium iodide labeled cells were 1.45% ± 0.85% in wild-type cultures versus 1.78% ± 0.64% in Dlk1 mutant cultures. Furthermore, multipotentiality in clonal differentiation assays was decreased in Dlk1-deficient NSCs as indicated by the percentage of clones with the capacity to give rise to the three types of cells from the central nervous system (astrocytes, oligodendrocytes, and neurons) (Fig. 3E). Therefore, the compromised self-renewal and multipotency capacities in Dlk1-deficient NSCs is caused by an effect on the overall proliferation rate and differentiation efficiency upon both maternal or paternal transmission of the mutation. It is worth noting that, in contrast to the SVZ, in which DLK1 acts as a niche-secreted factor (33), NSCs in the SGZ do not respond to exogenously added DLK1 (Fig. 3B).
Moreover, a change in the levels of the NOTCH effector Hes1 was observed in Dlk1-deficient NSCs (Fig. 3F), suggesting that DLK1 acts in an autocrine manner in the adult DG.

**Full Absence of Dlk1 Correlates with Anxiety-Like Phenotypes.** We next examined the differences in behavior of the Dlk1-deficient mice in the elevated plus maze (EPM). This test is used to measure the overall anxiety (50). The overall behavior of mice in the elevated plus maze (EPM) was measured, with the time spent in the open arms of the EPM inversely correlated to their level of anxiety (50). Full absence of Dlk1 leads to the exhaustion of the NSCs pool in the adult SGZ, whereas the Dlk1+/- mice were also analyzed. Representative images of the primary neurosphere cultures obtained from wild type and Dlk1 mutant SGZ (Left). (B) Quantification of the number of secondary neurospheres formed after 6 d in vitro in Dlk1+/- and Dlk1-deficient NSCs treated or not with recombinant Dlk1. (C) MTS colorimetric quantification of viable cells in proliferating NSC-SGZ cultures (Right). (D) Cell cycle analysis of neurosphere cultures expanded from the SGZ in wild type and Dlk1+/-, showing a reduced proliferation capacity in the mutant cultures. (E) Quantification of unipotent (astrocytes, A), bipotent (astrocytes/neurons, A/N), and tripotent (astrocytes/neurons/oligodendrocytes, A/N/O) clones derived from wild type and Dlk1 mutant NSC cultures (Left). Immunocytochemistry for the astrocyte marker GFAP (blue), the neuroblast marker β-tubulin (green), and the oligodendrocyte marker O4 (red) in differentiated neurospheres (Right). (F) qPCR of Hes1 expression in NSCs derived from Dlk1+/-, Dlk1+mat+, Dlk1+pat-, and Dlk1+/- mutants. Dlk1+/- mice showed significantly increased Hes1 expression in NSCs. All error bars show SEM. Simple linear regression was applied in A. One-way ANOVA and Sidak multiple comparison tests were used in B, E, and F. Unpaired t tests were performed in D. P values and number of samples are indicated. ns, nonsignificant. (Scale bars in A: 100 µm; in E: 20 µm.)

**Maternal and Paternal Expression of Dlk1 Regulates Spatial Navigation.** Adult hippocampus neurogenesis is considered to be involved in cognitive functions that are essential for humans (20). For example, spatial navigation has been traditionally ascribed to the functions of the hippocampus. However, in traditional assessments of spatial navigation in rodents, it is difficult to test whether the animal is capable of deriving spatial information from boundary-based “geometry” features or from landmarks (51). Both geometry-like and landmark spatial references are essential properties of the environment, and their use during correct navigation is advantageous, as testified in many species (45, 51, 52). Moreover, the ability to derive spatial references from reduced landmark information allows the capture of spatial learning independently from performance or reinforcement (53).

In an effort to investigate the precise role of maternal and paternal expression of Dlk1 in the regulation of spatial navigation, we used a spatial test in which we compared the spatial ability of mice to navigate across a water tank either in a condition with clear landmarks or in a condition in which the main visible landmarks were reduced. In the latter condition, the animal must rely more on the environmental surface of the tank (52, 54). Dlk1 heterozygotes and homozygous 2-mo-old mutant mice were tested. The assay consisted of a white rectangular tank filled with water mixed with nontoxic white paint to ensure the invisibility of a platform (Fig. 4C). The platform was maintained just below water level and kept during the training phase of the test. Each trial started when the animal was introduced to the tank and lasted until the mouse reached the platform. All animals explored the arena to find the platform. The rationale of this test was to assess the capability of the mice to acquire spatial information from a visible landmark or from prevalently boundary
Fig. 4. Maternal and paternal expression of Dlk1 controls spatial navigation. (A) Schematic drawing of the EPM system. (B) Time spent and number of entries in open arms by the different animals after a 10-min observation period. Dlk1+/− mice showed fewer number of entries and less time spent in open arms than their wild-type littermates. (C) Schematic representation of the tank, landmark, and platform used for the geometric test. The test is performed in a white rectangular tank, convertible in a square, with a platform and a black landmark (Left). Mean latency in the training phase (day 1 to day 3, Right). (D) Schematic representation of the landmark condition. The tank is a square with no platform. A, B, C, and D indicate the four zones in the tank. The time spent in each zone was monitored and represented for each group of mice (Right). (E) Schematic representation of the geometry condition with no landmark or platform (Left). The time in each zone is represented in the graph (Right). Wild-type animals spent significantly more time in the zone “A” where the platform was located both in landmark and geometry conditions. (F) Schematic representation of the competition condition with a rectangular tank and the landmark located in a different zone (Left). The time in each zone is represented again in the graph (Right). All error bars show SEM of at least eight animals per genotype. One-way ANOVA and Sidak multiple comparison tests were used. Two-way ANOVA was applied in C–F. P values and number of samples are indicated. ns., nonsignificant.

information in which the visible landmarks were removed. The training was spread across 3 consecutive days with daily sessions of three trials. After training, we subjected all animals to a series of probe trials in which the platform was removed, and the spatial navigation of the animals was monitored and analyzed. Within the tank, a black landmark was positioned on the short wall above the platform and stayed there for the whole training (Fig. 4C). When testing, in the “Landmark” condition, the tank was converted into a square arena with the landmark located on the same wall as in the training condition (Fig. 4D). For the “Geometry” condition, the tank was maintained in its rectangular shape with no visible landmark (Fig. 4E). Finally, on the last session of the experiment, we subjected all animals to a “Competition” condition in which the landmark was incorrectly located on one of the long walls and the shape of the tank stayed rectangular (Fig. 4F). Here, the animals faced both boundary (based on rectangular shape) and landmark (based on incorrect landmark reference) conditions at once. The “Competition” condition informed us whether genotype differences affected the capability of mice to derive the correct spatial information for navigation independently from the main landmark.

Each group progressively reduced the latency to reach the platform across the daily sessions, which indicated that learning was not impaired in mutants compared to controls (Fig. 4C). In addition, the overall latency to reach the platform and the latency to first entry the zone “A,” where the platform was located, during training were not statistically different among the groups (SI Appendix, Fig. S5 C and D). Nevertheless, the test revealed some remarkable differences concerning the use of boundary-based versus landmark conditions in spatial navigation (51, 55, 56). In particular, both in landmark and boundary-based conditions, the wild-type animals spent significantly more time in the zone “A” (platform) compared to both heterozygous and homozygous mutants (Fig. 4D and E and SI Appendix, Fig. S5E), suggesting that wild-type mice developed proper spatial reference information within the environment while mutants maintained a certain level of uncertainty. Moreover, in the competition condition, only wild-type mice explored more of the correct location (zone “A”) (Fig. 4F and SI Appendix, Fig. S5D), demonstrating that the spatial information previously acquired was sufficient to permit the correct navigation in absence of the visible landmark. Indeed, the time they spent in zone “A” (platform) was significantly higher than the time spent in zone “B” (uninformative landmark) (Fig. 4F and SI Appendix, Fig. S5E). In contrast, heterozygotes and Dlk1 homozygous mutant mice spent a similar time in zone A and in zone B (Fig. 4F and SI Appendix, Fig. S5E), confirming their uncertainty between the two types of information. All together, these results indicate that Dlk1-deficient animals were not able to properly use landmark or geometric information, suggesting that biallelic expression of Dlk1 is required for spatial navigation.

**Dlk1 Does Not Participate in Location Discrimination.** The DG is also thought to contribute to spatial or episodic memory by functioning as a pattern separator through the formation of distinct representations of similar inputs (26, 57). This ability to form and
use memories derived from very similar stimuli that are closely presented in space and/or time depends upon the ability to pattern separate incoming information. In order to determine the potential role of Dlk1 in spatial discrimination, we have used a spatial non-navigable task using a mouse touch screen method (17, 58) in which we analyzed pattern separation-dependent memory by testing whether mice could differentiate between two locations that were presented closely. Dlk1 heterozygotes and Dlk1 homozygous 2-mo-old mutant mice were tested in the touchscreen location discrimination (LD) test. Mice were assessed on two types of condition in separate sessions: the big separation (easiest) condition and small separation (hardest) condition, which heavily taxes pattern separation and which is dependent upon neurogenesis in the DG (17). Mice received 20 sessions in total, 10 of each, in pairs of each separation. In each session, mice had the opportunity to acquire a contingency—which requires them to consistently (seven out of eight consecutive trials) pick, for example, the left location and then reverse it (again, seven out of eight consecutive touches), picking, for example, the right location. Thus, the number of trials that mice require to acquire and reverse can be analyzed separately. Mice with deleted hippocampal neurogenesis show impairments discriminating similar locations with a small separation between them—that is, when there is a high load on pattern separation—but no difficulty discriminating dissimilar locations with a large separation (17). However, Dlk1 heterozygous and homozygous mutant mice with random expression of Dlk1 in hippocampal neurogenesis had no impairment in discriminating either similar or dissimilar locations (SI Appendix, Fig. S6A and B). This suggests that there may be a critical amount of neurogenesis needed to affect this behavior significantly.

Deletion of Dlk1 in the GFAP+ Population Results in Defects in Spatial Memory Consolidation. In the adult brain, Dlk1 is biallelically expressed in the neurogenic niches, but is also expressed in nonneurogenic regions including the ventral tegmental area, the septum, and the ventral striatum, where the gene is imprinted and expressed only from the paternal allele (33, 59, 60). Differentiated astrocytes also expressing GFAP in the SGZ niche showed very low levels of expression of Dlk1 gene (Fig. 1A). Thus, in order to evaluate the regulatory function of paternal and maternal Dlk1 specifically in the neural stem cell population from the adult SGZ, a murine conditional genetic model was generated by crossing mice carrying loxp sites flanking part of the Dlk1 gene (61) with mice expressing the Cre-recombinase under the control of the Gfap promoter (62). Significant expression of Cre-recombinase in Dlk1loxp+/-;Gfapcre+/- (with a deletion of the maternal allele and referred to as Dlk1-GfapMatCre), Dlk1loxp+/-;Gfapcre +/0 (with a deletion of the paternal allele and referred to as Dlk1-GfapPatCre), and Dlk1loxp+/+;Gfapcre+/- (with a deletion of a maternal and paternal alleles and referred to as Dlk1-GfapPat/MatCre) compared to Dlk1loxp+/+;Gfapcre/+ (referred to as Dlk1-Gfapcontrol) control mice was confirmed in SGZ-derived NSCs (Fig. 5A). The removal of Dlk1 messenger RNA was demonstrated specifically in neuroepithelia isolated from Dlk1-GfapMatCre, Dlk1-GfapPatCre, and Dlk1-Gfapcontrol deficient SGZ (Fig. 5A). Importantly, X-gal histochemistry in the adult brain of LacZ/Rosa26R;Gfapcre+/- mice (referred to as Gfap-CRE/LACZ) showed positive staining in the SGZ, of the adult Gfap-CRE/LACZ brains and colociled with GFAP+cells only within the SGZ (Fig. 5B), corroborating the specific deletion of Dlk1 in the adult GFAP+ stem cell population.

To further investigate the role of maternal and paternal Dlk1 in DG neurogenesis, we injected 2-mo-old Dlk1-Gfapcontrol, Dlk1-GfapPatCre, Dlk1-GfapMatCre, and Dlk1-Gfapcontrol conditional mice with the nucleotide analog BrdU 3 wk prior to killing. As previously noted, among the cells that retained BrdU, we quantified the proportion of GFAP/SOX2+ undifferentiated cells in Dlk1 conditional mice and observed that the total number of GFAP/Sox2+ cells was also diminished both in Dlk1-GfapPatCre and Dlk1-GfapMatCre conditional mice (Fig. 5 C and D). As a result, the number of DCX+ neuroblasts was significantly reduced after both maternal and paternal transmission of the mutation (Fig. 5 C and D). Consistently with nonconditional study, a more severe reduction in the GFAP/Sox2+ and newborn DCX+ cells was observed in Dlk1-GfapPatCre conditional mice, confirming that Dlk1 dosage regulates SGZ neurogenesis. Moreover, the number of primary neurospheres isolated from the DG was reduced in Dlk1-GfapMatCre and Dlk1-GfapPatCre conditional mice, which grew less efficiently than control in long-term cultures (Fig. 5 E and F). Dlk1-GfapPatCre cultures showed a more acute growth impairment also in vitro (Fig. 5 E and F). These data demonstrate that Dlk1 controls stem cell maintenance in the adult SGZ and that biallelic expression of the gene in the GFAP+ population regulates hippocampal neurogenesis.

We next examined the behavior of the Dlk1 conditional mice in the EPM. The time spent and the percentage of entries in open arms, estimated during a 10-min observation period, was not affected, indicating that, similarly to wild-type and Dlk1 heterozygous constitutive mutant mice, there was no change in overall ambulatory behavior in Dlk1 conditional mice (Fig. 6A). Moreover, Dlk1-GfapPatCre and Dlk1-GfapMatCre conditional mice were not more anxious than wild type in agreement with our previous results in nonconditional mutants (Fig. 6A). To further determine whether the postnatal down-regulation of Dlk1 in the GFAP+ population within the SGZ niche has an effect in spatial memory consolidation, we performed a Morris water maze (MWM) test in Dlk1-Gfapcontrol, Dlk1-GfapMatCre, and Dlk1-GfapPatCre conditional mice. We used a round water maze filled up with water with a platform submerged below the surface (Fig. 6B) (63). A landmark was positioned close to the wall of zone A where the platform was located. To maximize the potential differences because of altered hippocampal neurogenesis between experimental groups, we used a simplified protocol in which mice entered the maze through the same quadrant (zone D) and in a straight line to the position of both the platform and the landmark (Fig. 6B). Each animal underwent three trials/day for only 3 d so as to acquire the task easily. After the training, we performed a probe trial with the same experimental conditions but without the platform. The time spent in every zone was measured progressively reduced the latency to reach the platform across the training daily sessions, which indicated similar learning in conditional mutants and controls (Fig. 6D). However, when the animals were tested in the probe as a first nonreinforced trial, the difference between experimental groups appeared after they failed to find the platform. Data showed that Dlk1-Gfapcontrol mice spent significantly more time exploring zone A (where the platform was previously located) than in any other zones (Fig. 6E), indicating that they were able to recall a spatial association between the landmark position and the platform. In contrast, maternal and paternal Dlk1 conditional heterozygous mice navigated randomly throughout the maze with no significant preferences between zones showing no spatial navigation-based searching strategies (Fig. 6D), consistent with the behavioral phenotypes observed in Dlk1 constitutive mutant mice (Fig. 4D). These data confirm that biallelic expression of Dlk1 in the neural stem cell population within the SGZ is required for spatial memory in adult mice.

Discussion

This work highlights a positive role for Dlk1 in promoting NSCs self-renewal in the adult hippocampus. Both short-term and long-term (age-related) neurogenesis in the SGZ requires selective absence of imprinting of Dlk1 and hence is sensitive to Dlk1 dosage. We show that defective postnatal neurogenesis in constitutive Dlk1 mutant mice is associated with an increased anxiety-related behavior in unfamiliar environments. Moreover,
Fig. 5. Specific deletion of Dlk1 in GFAP cells causes a neurogenic defect in the adult SGZ. (A) qPCR for Cre-recombinase and Dlk1 in NSCs of Dlk1-Gfap<sup>control</sup>, Dlk1-Gfap<sup>MatCre</sup>, and Dlk1-Gfap<sup>PatCre</sup> conditional mice. (B) X-galactosidase staining (blue) and immunohistochemistry for the astrocyte marker GFAP (brown) in the SGZ of Gfap-CRE/LACZ mice. Gfap-CN/LACZ samples with no Cre-recombinase were used as controls for the staining. (C) Representative images of the primary neurosphere cultures obtained from Dlk1-Gfap<sup>MatCre</sup>, Dlk1-Gfap<sup>PatCre</sup>, and Dlk1-Gfap<sup>MatPatCre</sup> genotypes. (D) Quantification of the number of BrdU-LRCs that are double positive for GFAP and SOX2 (Left) and DCX positive neuroblasts in the SGZ from the different genotypes. (E) Growth curve for Dlk1-Gfap<sup>MatCre</sup>, Dlk1-Gfap<sup>PatCre</sup>, and Dlk1-Gfap<sup>MatPatCre</sup> cultures during passages one to seven. All error bars show SEM of at least four animals per genotype. One-way ANOVA and Sidak multiple comparison tests were used. Simple linear regression analysis was applied in F. P values and number of samples are indicated. (Scale bar in B and C: 100 μm; high magnification images, 60 μm; in E: 60 μm.)

although Dlk1-deficient mice show normal learning, a reduction of Dlk1 dosage triggers specific defects in performing spatial behavioral tasks. Consistently, conditional deletion of the paternal or maternal Dlk1 alleles in the SGZ neural stem cell population confirm that biallelic expression of Dlk1 in the GFAP+ population is necessary for hippocampal neurogenesis and for normal cognitive functions such as spatial memory consolidation.

Loss of Dlk1 compromises the maintenance of the neural stem cell pool in the SGZ, leading to reduced neurogenesis and affecting the tripotent differentiation capacity of neural progenitors both in vivo and in vitro. In contrast to the SVZ, where Dlk1 acts as an astrocyte-secreted niche factor (33), Dlk1 seems to function autonomously in the DG based on the reduction of secondary neurosphere formation and proliferation capacities in SGZ progenitors isolated from Dlk1<sup>−/−</sup> mice. Accordingly, Dlk1 is highly expressed in NSCs but nearly absent in SGZ differentiated astrocytes, in contrast to what happens in the adult SVZ (33). Deletion of Dlk1 also causes a reduction in the number of radial NSCs and nonradial progenitors in the DG in vivo that results in a decrease in the number of DCX+ immature neurons and NeuN+ mature neurons located in the GCL. It is still not known if Dlk1 is required in the SGZ to maintain quiescence at early postnatal phases as happens in the SVZ (33). Thus, these findings raise the question of how Dlk1 affects adult neurogenesis in the different genetic niches. Whereas Dlk1 deletion may decrease hippocampal neurogenesis simply through the repression of NSC proliferation, it is also possible that Dlk1 regulates the cell cycle of populations other than radial NSCs in the DG. However, we did not observe a significant change in the number of proliferating TBR2+ progenitors or DCX+ neuroblasts after Dlk1 deletion; hence, the cell cycle of neural progenitors does not appear to be a major target of Dlk1 in the regulation of hippocampal neurogenesis. It has been previously described that continuous depletion of the neural stem cell pool, as a consequence of their division, may contribute to the age-related decrease in hippocampal neurogenesis (49). Our data also reveal a role of Dlk1 in the regulation of NSC maintenance in the long-term (age-related) modulation of neurogenesis. This is based on the observation that although deletion of Dlk1 initially resulted in a moderate decrease of the proportion of stem cells in the SGZ of the adult hippocampus, it later led to excessive reduction in the NSC pool and impaired neurogenesis. This is consistent with the classical view of stem cell exhaustion, where cells achieve the limit of their self-renewal capacity (64), a process that seems to be accelerated in the Dlk1 mutant SGZ. Thus, adult neurogenesis acts as a base for brain plasticity, allowing modulation of neural circuits in the hippocampus, and we show here that Dlk1 plays a role in determining this rate during the entire life of the organisms.

Dlk1 is canonically expressed from the paternally inherited chromosome and belongs to the Dlk1–Dio3 imprinted gene cluster on mouse chromosome 12. We previously confirmed Dlk1 transcription from the paternal allele in the nonneurogenic regions of the adult brain; however, we have observed neurogenic phenotypes in both the SVZ (33) and the SGZ of heterozygotes upon maternal or paternal transmission of the mutation and in

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Dlk1 dosage regulates hippocampal neurogenesis and cognition
 attenuation some phenotypes (68). And that a compensatory and/or redundant pathway may operate brain functions by subjecting mice with constitutive and GFAP miR-410 that changes in dendritic spine morphology occurring in miR-379/entire microRNA cluster may be less detrimental than down-regulated miR-379/miR-410 homozygotes mutant mice. This indicates that the paternal and the maternal inherited alleles are specifically required for postnatal neurogenesis in both neurogenic niches. This supports the hypothesis that regulation of the imprinting process might be adaptable to the environmental niche in which the stem cells are acting. Indeed, we have previously shown that another imprinted gene, Igf2, acts differentially in the two adult neurogenic niches (65). Igf2 is biallelically expressed in the vascular compartment, influencing the activation of the stem cell pool in the SVZ, whereas it is solely expressed from the paternal allele in the SGZ, where it acts as an autocrine factor (65). However, our data also show that other imprinted genes such as Gtl2 or Snrpn maintain their imprinting state in both the SGZ, consistent with previous data in the SVZ (33). This data confirms that loss of genomic imprinting is not a generalized phenomenon in the ventral striatum, where the gene is imprinted and expressed only from the paternal allele (33, 59, 60). Our data indicate that Biallelic expression of Dlk1 in the GFAP population regulates memory consolidation. (A) Number of entries and time spent in open arms (o.a) after a 10-min observation period in Dlk1-Gfapcontrol, Dlk1-GfapPatCre, Dlk1-GfapMatCre, and Dlk1-GfapMatCre conditional mice. (B) Schematic representation of the MWM test. The test is performed in a white circular tank with a platform and a black landmark. A, B, C, and D indicate the four zones in the tank. Mice were introduced in the water maze always from the same quadrant (zone D). (C) Representative images of the water maze test videos acquired for offline scoring and analyses. (D) Mean latency in the training phase (day 1 to day 3; Right) for the MWM test. (E) Graph representing the time spent in each zone for each group of mice. Control animals spent significantly more time in the zone “A” where the platform was located compared to Dlk1-GfapMatCre and Dlk1-GfapPatCre mice. Dashed line indicates random swim time. All error bars show SEM. One-way ANOVA and Sidak multiple comparison tests were used. Two-way ANOVA was applied in D and E. P values and number of samples are indicated.

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Dlk1 homeostasis and cognitive defects upon conditional deletion of the gene to a battery of cognitive and behavioral tests. Complete lack of Dlk1 expression leads to increased anxiety-related phenotypes as shown by a reduced activity and number of entries in the open arms as well as the increased defecation and urination. However, Dlk1 maternal or paternal heterozygous mice show no indication of anxiety. Importantly, adult Dlk1 mutant mice with deficiency in adult neurogenesis show selective defects in remembering spatial information from reduced landmark conditions. In the spatial navigation test, Dlk1mutat+/ PatCre and Dlk1mutat−/− exhibited an inferior performance when trying to localize the platform in the absence of the main landmark, while wild-type mice succeeded in all conditions. This suggests that wild-type mice can perform in spatial navigation even in the absence of the main landmark information, for example, relying on boundary information and/or other subtle environmental information. However, Dlk1 mutation affects spatial learning retrieval. In our study, we can conclude that the overall learning acquisition process is not compromised in Dlk1-deficient mice, but their performance during memory tests is diminished. In accordance, mice with a specific deletion of Dlk1 alleles in the GFAP+ stem cell population failed to find the platform after training in a MWM test and navigated randomly throughout the maze, suggesting that Dlk1 deletion in the stem cell population has a functional effect in the hippocampus ability to consolidate spatial learning. It is worth noting that given that Dlk1 is expressed in NSCs but nearly absent in terminally differentiated astrocytes in the SGZ, the defects found in Dlk1 deficient mice are unlikely due to alterations of this gene in the astrocytic population. Interestingly, Dlk1 mutants did not differ from wild types in the pattern separation task with the touch screen (17, 58), suggesting that the level of neurogenesis decrease might play a role in this more difficult task.

In the adult brain, Dlk1 is also expressed in nonneurogenic regions including the ventral tegmental area, the septum, and the ventral striatum, where the gene is imprinted and expressed only from the paternal allele (33, 59, 60). Our data indicate that Dlk1 mutation of the canonically nonexpressed maternal allele also causes defects in the proportion of newborn neurons incorporating to the SGZ. These findings have the potential to contribute new
insights into understanding the function of imprinted genes and the evolution of dosage control by selective absence of imprinting in particular stem cell developmental contexts. Together, these findings suggest that genomic imprinting can be used as a sophisticated mechanism of gene dosage control and raises questions about how intrinsic environmental cues modulate the adaptability and flexibility of this epigenetically regulated process. Further studies are needed to determine the role of imprinted genes and the epigenetic mechanisms underlying imprinting dynamics in the adult neurogenic niches.

Methods

Animals and In Vivo Manipulations. The generation and genotyping of Dlk1 mutant and transgenic mice have been described previously (33, 48, 69). To generate the Dlk1 knockout mice, a neomycin resistance cassette was used to replace 3.8 kb of the endogenous allele, including the promoter and first three exons of Dlk1 (33, 69). The generation of transgenic mice was done using bacterial artificial chromosomes (BACs). Dlk1 BAC transgene contains 49.5 kb of sequence upstream of Dlk1 and ends ~18 kb downstream of the Dlk1 transcriptional start site (48). Dlk1 conditional mice were generated by crossing mice carrying loxP sites flanking part of the Dlk1 gene (61) with mice expressing the Cre-recombinase under the control of the Gfap promoter (62). Experiments were done with mice on a C57BL/6 genetic background and their corresponding wild-type littermates. Housing of mice and all experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and with Spanish Royal Decree 1386/2018 guidelines, under appropriate Home Office personal and project licenses.

Immunohistochemistry and BrdU Administration. After 2-mo-old mice were deeply anesthetized, they were perfused transcardially with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Fixed brains were sectioned with a vibratome (Leica VT1000) at 30 μm. Sections were incubated with a vibratome (Leica VT1000) at 30 μm. Sections were incubated in 2% hydrogen peroxide (H2O2) at 37 °C, neutralized in 0.1 M sodium borate (pH 8.5), blocked in 10% fetal bovine serum (FBS) and 0.1% Triton X-100 in phosphate buffer for 1 h, and incubated overnight in primary antibodies (SI Appendix, Table S1a) in the same blocking buffer. Immunofluorescent detections were performed with secondary antibodies (SI Appendix, Table S1b). DAPI (1 μg/mL, 5 min; Molecular Probes) was used for counterstaining. Samples were analyzed with OlympusFV10 confocal microscopes. Labeled cells were counted in every 10th 30-μm section through the entire rostrocaudal length of the DG (∼0.92 mm to ∼3.90 mm from bregma). To count GFAP+ radial cells, we used association of the cell body with a DAPI+ nucleus and a single radial process extending through the granule layer.

Cell Cultures and Cell Cycle Analysis. SGZ-derived neurosphere cultures were obtained by dissecting out DG and digestion in 0.025% trypsin and 0.265 mM ethylenediaminetetraacetic acid (Invitrogen) as previously described (13). Briefly, a single-cell suspension was obtained by gentle trituration and diluted in serum-free medium composed of Dulbecco’s Modified Eagle Medium/F12 with 1% B27 supplement, 10 ng/mL EGF (Invitrogen), and 5 mM β-glycerophosphate buffer for 1 h, and incubated overnight in primary antibodies (SI Appendix, Table S1a) in the same blocking buffer. Immunofluorescent detections were performed with secondary antibodies (SI Appendix, Table S1b). DAPI (1 μg/mL, 5 min; Molecular Probes) was used for counterstaining. Samples were analyzed with OlympusFV10 confocal microscopes. Labeled cells were counted in every 10th 30-μm section through the entire rostrocaudal length of the DG (∼0.92 mm to ∼3.90 mm from bregma). To count GFAP+ radial cells, we used association of the cell body with a DAPI+ nucleus and a single radial process extending through the granule layer.

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Immunostaining. A Dlk1 imprinting assay to analyze parental allele-specific gene expression was based on PCR amplification followed by direct sequencing as previously described (33). To determine the imprinting status of Dlk1 and Gtiz2 in the hippocampus and SGZ-derived NSCs, we used the reciprocal F1 hybrid offspring of Mus musculus domesticus (strain C57BL/6, abbreviated BL6) and Mus musculus castaneus (strain CAST/Ei, abbreviated Cast) subspecies in which an SNP was identified between the two subspecies that could differentiate the parent-of-origin. For these analyses, PCR was performed in the presence of pooled cDNA from heterozygous mice (Dlk1+/-, Gtiz2+/-) before sequencing. The sequences of murine Dlk1, Gtiz2, and Snrpn were obtained from GenBank (accession numbers: NM010052 for Dlk1, Y13832 for Gtiz2, and NM001082961.2 for Snrpn). The Dlk1 polymorphism, located at nucleotide 721 of exon 5, is a “T” in BL6 mice and a “C” in Cast mice. The Dlk1 imprinting assay used primers Dlk1-F and Dlk1-R (SI Appendix, Table S1d) to amplify a fragment between exons 4 and 5, with the thermal cycle conditions as 94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min, and 30 cycles. The primer Dlk1-aqseq was used for direct reverse PCR fragment sequencing. The Gtiz2 polymorphism, located at nucleotide 26 of exon 8, is a “C” in BL6 mice and a “T” in Cast mice. The Gtiz2 imprinting assay used primers Gtiz2-F and Gtiz2-R (SI Appendix, Table S1d) to amplify a fragment between exons 6 and 8, with the thermal cycle conditions as 94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min, and 33 cycles. Primer Gtiz2-R was used for direct sequencing. Genomic DNA sequence traces from BL6 and Cast SVZs were used to identify strain-specific polymorphisms (SI Appendix, Fig. S1b). The Snrpn polymorphism, located at nucleotide 1270 of exon 10, is a “C” in BL6 mice and a “T” in Cast mice. The Snrpn imprinting assay used primers Snrpn-F and Snrpn-R (SI Appendix, Table S1d) to amplify a fragment between exons 9 and 10, with the thermal cycle conditions as 94 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min, and 32 cycles.

Behavioral Tests. Geometry test. We used a white rectangular tank (126 × 66 cm, 50 cm high) convertible into a square arena (66 × 66 cm, 50 cm high) as described (54). The tank was filled with ~9 cm of water mixed with nontoxic white paint to ensure the nonviability of a platform. The platform was maintained just below the level of the water and kept during the training phase of the test. Each trial started when the animal was introduced to the tank and lasted until the mouse reached the platform or after 1 min. Water represented a negative condition, and all animals explored the arena to find the platform. Before each trial, all animals were disoriented by removing them from their home cage and transported into the testing room inside a curtained cage from an adjacent room. Then, the experimenter released them into the center of the maze. The training was spread across 3 consecutive days with daily sessions of three trials. After training, we subjected all animals to a series of probe trials in which the platform was removed, and the spatial navigation of the animals was monitored and analyzed according to specific environmental conditions. Each post-training daily session started with a
probe trial followed by two training trials. These latter training trials were used to avoid extinction of the memory for the platform. The surrounding space along the apparatus was enclosed by white panels to eliminate extramaze cues, stressing the relevance of the geometric and/or landmark information. Within the tank, a black landmark (21 × 50 cm) was positioned on the short wall above the platform and stayed there for the whole training (Fig. 4C). At the end of each trial, the animals were positioned on a heating pad in order to keep them warm. Videos were acquired by a camera mounted above the tank and the ANY-maze video tracking system was used for analysis. For the “landmark” condition, the tank was converted into a square arena with the landmark located on the same wall as in the training condition. For the “geometry” condition, the tank was maintained in its rectangular shape with no landmark. Although we removed the main visible landmark, we could not exclude that animals were able to use subtle references within the experimental setting. The order of presentation of the first test probe was counterbalanced across subjects for each group. On the last session of the experiment, we subjected all animals to a “competition” condition in which the landmark was located on one of the long walls and the shape of the tank stayed rectangular. Here, the animals faced both geometric (of the rectangular shape) and landmark (because of the landmark reference) conditions at once, where the landmark was placed in an incorrect location.

**Spatial discrimination test: Mouse touch screen.** This test was performed in a similar manner to previously described (17, 58). Briefly, the testing apparatus was the in-house style of operant chambers and consisted of a sound-attenuating box containing a standard modular testing chamber fitted with an infrared touch screen, a pellet receptacle with light illumination and head entry detectors, a pellet dispenser, a house light, and a tone generator (71). At the beginning of each session, the touchscreen was reset and the animals were presented with white windows or locations in which white square stimuli could be presented on the screen (SI Appendix, Fig. S6A). Prior to LD training, mice were habituated to the apparatus, and then in a Pavlovian learning stage, they learned to associate a pellet delivery reward with the offset of a single white square stimulus on the screen, the sound of the tone, and the onset of the magazine light. Next, mice learned to nose poke to the stimulus on the screen during each of the 30 trials per session in order to obtain a reward, and then the requirement to initiate trials by nose poking into the illuminated magazine was introduced. In the final training stage, a punishment was introduced for touching one of the empty (plain black) locations instead of the correct location with the white stimulus, therefore introducing a cue signaling incorrect responses: a 10 s “time out” (stimulus disappeared, houselights off, no pellet). Following either type of response, the next trial was presented after a 10 s interval. This final stage was considered complete when at least 76% of the 30 trials were correct on two consecutive sessions, with the final session taking less than 30 min. The LD training sessions comprised 60 trials in up to 60 min (except the first two sessions, which comprised only 30 trials). On each trial, two plain white stimuli were presented in locations two and five (with two gaps between). One of these locations was initially correct and rewarded, while the other was punished. Mice “acquired” the discrimination first in each session when they achieved seven out of eight consecutive trials correct, and then the reward contingency was reversed. If mice reached the seven out of eight consecutive trials correct criterion again, they were said to have achieved “reversal” of the discrimination. The criterion for completion of this LD training was achieving an acquisition and at least one reversal on at least three out of four consecutive sessions. In the 5 d before probe testing began, all mice received five consecutive reminder sessions. Finally, mice received LD probe sessions with two different separations: the small separation used locations one and six (SI Appendix, Fig. S6A). The separation received first and initial correct side (left/right) were counterbalanced across genotype and performance on the LD reminder sessions. Otherwise, the probe sessions were identical to LD training sessions, except that the number of reversals per session was limited to one acquisition and one reversal, and mice were allowed unlimited trials in a 60-min session. All mice completed at least 60 trials in sessions which were not terminated due to reaching reversal criterion. Mice received double alternating sessions of each separation (i.e., big, big, small, small, etc.). On the first session of each pair of probes, the correct side was that assigned during the initial counterbalancing. On the second condition, the correct side was that which was correct at the end of the previous session. Mice received 20 sessions of probes, that is, five pairs of each separation.

**EPM.** The maze consists of four arms arranged in a cross configuration and elevated from the floor. The two opposing “closed” arms have walls (but are open at the top), while the “open” arms do not. Anxious mice would be expected to avoid the open arms. The mouse is placed into the center of the maze, facing an “open” arm, and its movements in the maze are recorded during a 10-min session. At the end of the session, the mouse is removed and the number of urine patches and fecal boli are recorded by an experimenter blind to genotype. Several parameters are measured, such as the percentage of arm entries into open arms, which is the number of entries into open arms × 100 divided by entries into open arms plus entries into closed arms; the percentage of arm time (seconds) spent in the open arms, which is the open arm time × 100 divided by open arm time plus closed arm time; and the percentage of arm activity (distance in cm) occurring in open arms, which is the distance in open arms × 100 divided by distance in open arms plus distance in closed arms. In addition, the total distance (centimeters) traveled in all zones, including the center of the maze, provides a measure of overall activity (which is potentially confounding).

**MWM.** The maze consists of a pale blue, plastic, round water maze very similar in measures to the original MWM (63) (120 cm diameter, 25 cm height). The maze was filled up with ~20 cm high water mixed with nontoxic white colorant in order to camouflage a 10-cm diameter round platform placed in the left superior quadrant (referred as Zone A in Fig. 6). The platform was submerged 1 cm below the surface. Water was maintained at 20 °C and it was cleaned every day. The testing room was clear of any external cues except for a 60 cm high landmark positioned close to the wall of Zone A. The landmark and platform were maintained exactly in the same position every experimental day. Every trial and test was recorded with a webcam hung 2 m over the water maze. The SMART video tracking system (Panlab) was used for offline evaluation and analyses. Mice were nicely introduced (not dropped) in the water maze, always from the same quadrant (Zone D) and in a straight line to the position of both the platform and the landmark (Fig. 6B). Experimental animals were trained with three trials per day during 3 consecutive days. The trial’s time was stopped when the mouse found the target or was limited to 100 (first trial) or 90 (second and third trials and experimental test) s to avoid mice exhaustion. During the first two trials, mice that did not find the target were gently guided to it, as recommended (72). After 3 d of training, we performed the memory test with the same experimental conditions but no present target or platform. The time spent in every zone was evaluated.

**Statistical analysis.** All statistical tests were performed using SPSS software (version 26.0.0 for windows; SPSS, Inc.). The significance of the differences between groups was evaluated using the most appropriate test for each case: t tests, one or two-wayANOVA (using Tukey’s, Dunnett, or Sidak post hoc tests), Kruskal–Wallis tests (using Dunn post hoc test), or linear regression. When comparisons were performed with relative values (percent arms), normalization of data were done using a square root transformation. Data were always presented as the mean ± SEM. Number of experiments (n) performed with independent animals/cultures are indicated. Box and whisker plots show the median (horizontal line in box), mean (±), and minimum and maximum values. Values of P < 0.05 were considered statistically significant.

**Data Availability.** The data supporting the findings of this study are provided with the paper as Dataset S1.

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