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Sustained Notch activation depletes adult neural stem cells and impairs cognitive function

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Abstract

Notch signaling is essential for maintenance of neural stem cells (NSCs) and proper brain function. To investigate the functional consequences of sustained Notch activation, we generated a spatiotemporally controlled, tetracycline-inducible transgenic mouse model *Nestin-CreER, ROSA26-rtTA, TRE-loxP-DsRed2-loxP-c-Myc-NICD*. In this model, sustained *NICD* overexpression promotes rapid consuming cell division that drives NSCs to terminally differentiate rather than undergo self-renewal. This mechanism led to severe depletion of the NSC pool in both the subependymal zone (SEZ) and subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). In the SEZ, NSC depletion was paradoxically accompanied by an increased supply of newborn neurons to the olfactory bulb, but these cells did not contribute to better function. *NICD* overexpression mice showed significant impairments in spatial and emotional memory, and social discrimination. Collectively, sustained Notch activation in Nestin-lineage cells induces an imbalance in NSC maintenance, resulting in exhaustion of NSC pools and integration of non-functional cells, ultimately altering both olfactory and hippocampal-dependent behaviors.

Keywords: Notch signaling, adult neurogenesis, self-renewal, olfactory bulb, dentate gyrus

Introduction

Notch signaling regulates multiple physiological functions, including stem cell self-renewal, cell fate specification, proliferation, and apoptosis. Four Notch receptors are present in mammals, but Notch1 is the primary mediator of stem cell maintenance. The canonical transduction cascade is strictly regulated through ligand-induced proteolytic cleavage of Notch receptors that promotes release of the Notch intracellular domain (NICD). This active fragment translocates into the nucleus to form a complex with RBP-J and drives expression of downstream target genes like those in the *Hes* and *Hey* families^{1,2}. This pathway involving NICD is reported to be essential for preventing premature differentiation in the pancreas where it controls multiple lineage steps³, but aberrant “gain-of-function” activities are frequently associated with distinct pathologies. For example, sustained Notch activation can be a potent driver of tumorigenesis in hepatocellular carcinoma⁴ and chronic lymphocytic leukaemia⁵. In the central nervous system, Notch signaling acts as a master regulator of adult neurogenesis⁶. Precise regulation is critical for maintaining neural stem cell (NSC) pools as evidenced by the finding that forced upregulation of Notch signaling through genetic deletion of the Notch inhibitor Rnd3 promotes aberrant NSC proliferation and hydrocephalus⁷. These diverse findings underscore the critical, context-dependent need to investigate sustained Notch activity in adult neural precursors.

In the adult mammalian brain, NSCs persist primarily in two neurogenic niches: the subependymal zone (SEZ) of tissue surrounding the lateral ventricles and the subgranular zone (SGZ) of the hippocampal DG⁸. Maintaining a proper balance between NSC quiescence, self-renewal, and differentiation is essential not only for niche homeostasis but also for olfactory discrimination and hippocampal-dependent spatial learning. Notch signaling acts as a gatekeeper in these niches. High levels of NICD activity are generally required to maintain NSCs in a quiescent, undifferentiated state through the suppression of proneural genes⁹. Conversely,

downregulation of Notch signaling is a prerequisite for the transition of quiescent NSCs to vigorously proliferating transit amplifying cells (TACs), and then to their subsequent neuron progeny^{10,11}. The function of Notch in the adult brain extends beyond structural plasticity as accumulating evidence implicates the pathway in higher-order brain functions. Active Notch signaling has been shown to modulate synaptic plasticity and long-term potentiation (LTP), suggesting that it plays a significant role in cognitive processes^{12,13}.

Despite these previous findings, the specific functional consequences of sustained Notch activation on adult NSC dynamics and behavior remain poorly defined. Previous studies predominantly investigated embryonic stages or involved Notch loss-of-function models^{9,14}. In this study, we used an inducible Cre-loxP system that allows specific and targeted Notch1 overexpression in the brain of adult mice. Using this mouse model with inducible *NICD* overexpression, we explored the underlying cellular mechanisms with a neurosphere assay and lineage tracing of quiescent NSCs. We also characterized behavioral outcomes to determine whether sustained Notch activation disrupts learning and memory processes.

Results

Generation of mice with neural precursor cell-specific *NICD* overexpression

DsRed2 cDNA (Clontech, #6973-1) with a SV40 polyadenylation sequence flanked by two loxP sequences and followed by c-Myc-tagged *Notch1* intracellular domain (*NICD*) cDNA¹⁵ was inserted into the pTRE-Tight vector multiple cloning site (Clontech, #6263-1). The plasmid was linearized, and the vector sequence was removed. Then the entire transgene was injected into the pronuclei of fertilized one-cell eggs from C57BL/6 mice. Potential founder mice were screened by PCR, using a set of primers described in Table S1. Among the three lines we established and validated by crossing with *Nestin-Cre*, *α CamKII-tTA* tg mice, we selected line #54 (hereafter termed

TRE-loxP-*DsRed2*-loxP-*NICD*), which showed no apparent leaky transgene expression (Fig. S1).

We crossed TRE-loxP-*DsRed2*-loxP-*NICD* mice with *Nestin-CreER*tg and *R26-rtTA* KI mice to generate *Nestin-CreER*, *R26-rtTA*, TRE-loxP-*DsRed2*-loxP-*NICD* (hereafter termed *NICD* OE mice) (Fig. 1A and S2). We examined transgene expression by first making serial intraperitoneal injections with 4-hydroxy-tamoxifen (4-OHT) into dams at E13.5 to excise *DsRed2* cDNA between the two loxP sequences, followed by intraperitoneal injection with 9-tert-butyl doxycycline (9-TB Dox) at E14.5 (Fig. S2). We analyzed E15.5 embryos and found c-Myc immunoreactivity in the ganglionic eminence of *NICD* OE embryos (Fig. S2). We next determined transgene expression in adult brains. Mice were given 10 mg tamoxifen p.o. twice at 6 weeks, followed by ad libitum access to chow supplemented with doxycycline to induce transgene expression (Fig. 1B). We detected c-Myc⁺ cells in two neurogenic niches, the SEZ and the DG of the hippocampus, in *NICD* OE mice (Fig. 1C). Furthermore, prominent c-Myc expression was observed in the olfactory bulb (OB) (Fig. 1D), confirming the accumulation of progeny of SEZ-resident c-Myc-*NICD*-expressing NSCs. Collectively, these results validate the efficacy of the inducible *NICD* OE system.

NICD* overexpression enhances NSC self-renewal *in vitro

Based on loss-of-function studies of Notch pathway molecules^{6,14,16,17} that show varying degrees of depletion of NSC self-renewal, activation of Notch signaling is generally thought to enhance NSC self-renewal. We first tested this possibility in *in vitro* experiments. Cells isolated from the SEZ of *NICD* OE mice were cultured in the neurosphere-forming cultures (Fig. 2A). Treatment of these cultures with both 4-hydroxy-tamoxifen (4-OHT) and doxycycline significantly increased expression of *NICD* ($P = 0.0021$ by Kruskal-Wallis test; No Tx vs. 4-OHT/Dox, $P = 0.0064$ by Dunn's *post hoc* multiple comparisons test) and its target gene, *Hes1* ($P = 0.0312$ by Wilcoxon matched-pairs signed rank test), in primary neurospheres (Fig. 2B). The self-renewal capability of NSCs was evaluated by passaging, since the number of

secondary neurospheres derived from single primary neurospheres reflects the extent to which symmetric expansive divisions of NSCs occur during formation of primary neurospheres. We observed comparable numbers of primary neurospheres between *NICD* OE and littermate control mice regardless of treatment, but the number of secondary neurospheres formed was significantly increased in the cultures with 4-OHT/Dox treatment of *NICD* OE mouse-derived primary neurospheres ($t_{(12)} = 4.205$, $P = 0.0012$ by paired t -test) (Fig. 2C and 2D respectively). These results suggest that activation of Notch signaling enhances symmetric expansive division of NSCs *in vitro*.

Increase of symmetric expansive division of NSCs can result in decrease of the production of vigorously dividing neural progenitor cells. We tested this possibility by measuring diameters of primary neurospheres and found that *NICD* OE mouse-derived primary neurospheres cultured in the presence of 4-OHT/Dox treatment were significantly smaller than those cultured without treatment ($t_{(2)} = 6.274$, $P = 0.0245$ by paired t -test) (Fig. 2E). We further examined if *NICD* overexpression suppressed the differentiation of NSCs. Single primary neurospheres were plated onto cover glasses coated with polyethyleneimine and Matrigel basement membrane matrix and were induced to differentiate in 1% fetal bovine serum (FBS)/serum-free media (SFM) (Fig. 2F). We observed that significant decrease of GFAP⁺ and β III tubulin⁺ cells differentiated from *NICD* OE mouse-derived primary neurospheres cultured with 4-OHT/Dox than those from wild-type neurospheres [$F_{(2, 6)} = 12.39$, $P = 0.0074$; *NICD* OE 4-OHT/Dox vs. wild-type No Tx, $P = 0.0070$ for GFAP; $F_{(2, 6)} = 6.556$, $P = 0.0309$; *NICD* OE 4-OHT/Dox vs. wild-type No Tx, $P = 0.0312$ for β III tubulin by one-way ANOVA, followed by Tukey's multiple comparisons test]. This decrease was abolished over passaging (Fig. S3). These results suggest that *NICD* overexpression suppresses the differentiation of NSCs to neural progenitor cells and subsequently into neurons and glia.

NICD* overexpression reduces the NSC pool size *in vivo

We next investigated if *NICD* overexpression affected maintenance of neurosphere-forming NSCs in the brain and if it expanded the NSC population. We first examined effects of genotypes and tamoxifen treatment. We performed the neurosphere assay when the mice were 10 weeks old. We found no significant differences in the total numbers of NSCs in the brains of wild-type, TRE-loxP-*DsRed2*-loxP-*NICD*, or *Nestin-CreER* mice or *Nestin-CreER* mice that had been given two 10 mg doses of tamoxifen at 6-week-old (Fig. S4A). Therefore, we used *Nestin-CreER* and *NICD* OE mice in this experiment and administered two 10 mg doses of tamoxifen to all of them at 6-week-old. We next tested if chronic Dox treatment affects NSC pool size using *Nestin-CreER* mice that consumed normal or Dox-containing chow between 6 and 18 weeks of age. No apparent effects were observed (Fig. S4B). Then, we provided chow containing Dox for 4-20 weeks before carrying out the neurosphere assay (Fig. 3A-D). Unexpectedly, *NICD* OE mice consuming Dox showed significantly smaller NSC pool size compared to *NICD* OE mice consuming normal chow or *Nestin-CreER* mice when the mice were 26 weeks old [$F_{(2, 13)} = 8.947$, $P = 0.0224$, *Nestin-CreER* Dox (-) vs *NICD* OE Dox (+), $P = 0.0469$ and *NICD* OE Dox (-) vs *NICD* OE Dox (+), $P = 0.0304$ using one-way ANOVA, followed by Tukey's multiple comparisons test] (Fig. 3D). The number of NSCs in *NICD* OE consuming Dox mice decreased at a faster rate relative to *Nestin-CreER* control mice such that the number of NSCs was significantly reduced in 18-, and 26-week-old *NICD* OE mice compared to control mice ($t_{(9)} = 2.378$, $P = 0.0414$ for 18-week-old and $t_{(9)} = 2.349$, $P = 0.0434$ for 26-week-old by unpaired t -test) (Fig. 3B-D). We previously reported that the number of NSCs in wild-type mice decreases at relatively constant rates: the probability of NSC consumption per week $P_{\text{total NSC}}$ was 0.0189 in CD1 mice and 0.01907 in C57 BL/6 mice^{18,19}. In this study, the $P_{\text{total NSC}}$ in control mice during the 10-26 week period was calculated as 0.01836 (slope value: -0.008049 ± 0.001477 , $10^{-0.008049} = 0.981637$). Meanwhile, for *NICD* OE mice the $P_{\text{total NSC}}$ during the same period was calculated as 0.02911 (slope value: -0.01283 ± 0.001477 , $10^{-0.01283} =$

0.970890), suggesting a 1.6-fold faster NSC consumption rate. Taken together, the *in vitro* data indicate that *NICD* overexpression increases the likelihood of symmetric expansive division in *in vitro* neurosphere cultures, but *in vivo* there appears to be a progressive consumption of NSCs in the brain.

We also determined if *NICD* overexpression altered multipotential capability of NSCs. Primary neurospheres generated from *NICD* OE mice consuming Dox or *Nestin-CreER* mice were evaluated by a differentiation assay (Fig. S4C). We observed that neuronal differentiation was decreased in primary neurospheres generated from *NICD* OE mice as compared to those from *Nestin-CreER* mice ($t_{(4)} = 4.522$, $P = 0.0106$ by unpaired *t*-test) (Fig. S4C). We next performed a TUNEL assay to examine whether cell death was the cause of NSC depletion. We observed no significant increase in apoptosis in the OB, SEZ and DG of *NICD* OE mice compared to controls (Fig. S5). These results suggest that NSCs in the *NICD* OE brain were suppressed for their differentiation potential and that the accelerated loss of NSCs is not due to apoptosis but likely instead stems from increased consumption (i.e., activation and differentiation) of quiescent NSCs.

If *NICD* overexpression indeed drives premature consumption of the quiescent NSC pool, the number of label-retaining cells should be reduced. To test this possibility, we used long-term BrdU incorporation to observe neural precursor cells. BrdU was administered to 15-week-old animals, and the number of BrdU-positive cells in the SEZ and DG was quantified 1 month later when the mice were 19 weeks old. During this interval, BrdU-labeled TACs in the SEZ vigorously proliferated, resulting in dilution of the label and reflecting either cellular differentiation or migration to the OB or undergoing apoptosis, leading to a decline in detectable BrdU signal²⁰. Consequently, BrdU⁺ cells persisting in the SEZ beyond four weeks are typically interpreted as quiescent NSCs. In the DG, BrdU-labeled quiescent NSCs are retained in the SGZ, while their progeny typically migrate radially to the GCL and differentiate into mature neurons. Consistent with the neurosphere assay

results, *NICD* OE mice showed a significant reduction in label-retaining BrdU⁺ cells in both the SEZ and SGZ of the DG compared to controls ($t_{(4)} = 2.860$, $P = 0.0459$ for the SEZ; $t_{(4)} = 0.9244$, $P = 0.4076$ for the SGZ by unpaired t -test) (Fig. 3E-H). These BrdU⁺ cells were positive for GFAP but negative for S100 β (Fig. 3E and 3G), consistent with quiescent NSCs (type B cells) in the adult brain²¹.

To examine proliferative activity, BrdU was administered using the same injection protocol, but the brains were collected 1 hour after the last injection (total labeling time = 13 hours) (Fig. S6A and S6C). The total numbers of BrdU⁺ cells in both the SEZ and SGZ were comparable between *NICD* OE and control mice (Fig. S6B and S6D). Most BrdU⁺ cells labeled during this short interval are likely rapidly dividing TACs originating from NSCs as NSCs generally remain in a relatively quiescent state and exhibit much longer cell cycle durations than TACs²². Thus, the reserve pool of quiescent NSCs is likely being reduced, such that the overall output of dividing progenitors remains temporarily stable at this point. Consistent with this observation, the number of label-retaining BrdU⁺ cells in the dentate gyrus of *NICD* OE mice that differentiated and expressed a mature neuron marker, NeuN, were comparable to those in *Nestin-CreER* mice (Fig. 3I and 3J).

***NICD* overexpression impairs spatial memory retention and flexibility**

To evaluate the functional consequences of *NICD* overexpression on behavior, we subjected mice to a battery of tests carried out at one-week intervals (Fig. 4A). To ensure that the cognitive task would not be confounded by motor deficits or anxiety-like behaviors, we first assessed locomotor activity using an open field test (Fig. 4B). No significant difference was observed between *NICD* OE and control mice in the total distance travelled or the time spent in the center region, indicating that *NICD* OE mice had normal locomotor functions and anxiety levels.

We next assessed hippocampus-dependent spatial learning and memory using the Barnes maze test (Fig. 4C). All mice underwent nine

consecutive training sessions, with two sessions per day. The target goal was placed at 0°. All groups of mice learned to locate the target hole during training, as indicated by gradual reductions in the escape latency ($F_{(1,25)} = 1.598$; $P = 0.2178$). However, in the probe test, *NICD* OE mice spent significantly less time at the target goal ($F_{(15,656)} = 10.25$; $P < 0.001$), suggesting deficits in hippocampus-mediated spatial memory retention. To further assess cognitive flexibility and the ability to update spatial memory, we conducted a reverse training session in which the target was moved to a position opposite of the original target. We found no significant differences in escape latency to the reversed target goal ($F_{(1,25)} = 2.873$; $P = 0.1025$). Similar to the probe test, *NICD* OE mice did not remember the new target goal in the reversal probe test, suggesting that these animals had difficulty in updating and retaining novel spatial memory ($F_{(15,656)} = 17.00$; $P < 0.05$). Collectively, these results suggest that *NICD* OE mice retain the ability to learn a task but exhibit significant impairments in the retention and updating of precise spatial memory.

***NICD* overexpression reduces emotional memory and social novelty discrimination**

We assessed sensorimotor gating and auditory processing using the prepulse inhibition (PPI) test (Fig. S5). We found no significant differences between *NICD* OE and control mice, confirming that the sensorimotor gating and auditory acuity of *NICD* OE mice are intact.

Based on the observed spatial memory deficit in the Barnes maze, we further investigated whether *NICD* overexpression affects associative fear learning that involves environmental context and aversive cues in contextual fear conditioning. During the conditioning phase, mice learned to associate a specific environment with a mild foot shock (Fig. 5A). Both *NICD* OE and control mice exhibited comparable freezing responses to the paired unconditioned stimulus ($F_{(1,40)} = 0.467$; $P = 0.4980$), indicating that fear acquisition was intact. Mice then underwent a contextual test to assess retention of the environment at 1 day and 1 month after fear memory

acquisition. When tested 24 hours post-conditioning, *NICD* OE mice had a lower percentage of freezing compared to controls (Fig. 5B), which marked significantly low total freezing percentage ($P = 0.040$ by two-way ANOVA followed by Bonferroni's *post hoc* multiple comparisons) (Fig. 5D), which together suggest a potential deficit in short-term contextual fear memory. Over time, as memory transitions to cortical regions, the remote memory becomes more stable, and hippocampus-independent^{23,24}, resulting in a non-significant difference in freezing responses ($F_{(1,40)} = 0.496$; $P = 0.4855$) (Fig. 5C). Taken together with the Barnes maze data, these results suggest that *NICD* overexpression disrupts hippocampus-dependent memory processes.

We also examined the social ability of mice in a three-chamber social interaction test (Fig. 5E). In the sociability phase, both groups displayed a significant preference to spend more interaction time with stranger mice ($P < 0.0001$). Interestingly, in a subsequent social novelty phase, control mice showed a significant preference for a newly introduced, novel stranger mouse relative to the familiar mouse ($P = 0.0016$), whereas *NICD* OE mice failed to discriminate between the novel stranger and familiar mice, and showed no significant preference between the animals. This deficit could be related to the involvement of hippocampal and olfactory bulb circuits that might be affected by altered NSC dynamics and neuronal integration^{25,26} although further study is needed to prove a causative link between them.

Lineage-tracing of quiescent NSCs by lentiviral integration sites

To explore why quiescent NSCs were depleted in adult *NICD* OE mice over time and how the accelerated reduction of the NSC pool affected its output to the OB through the rostral migration stream (RMS), we performed a lineage-tracing study of NSCs with a lentivirus-mediated clonal labeling technique. We injected 1.0×10^5 TU of GFP-2LoxP lentivirus (Fig. 6A) into the right ventricle of postnatal day (P) 0 *NICD* OE or control mouse neonates as described previously^{18,19}. The mice were administered with tamoxifen, followed by Dox treatment to induce expression of the transgene. The 18-26-week-old mice were then used for the neurosphere assay to generate primary

neurospheres. GFP⁺ primary neurospheres were individually selected under a fluorescent microscope and amplified in passaging cultures. We could isolate 101 GFP⁺ neurospheres from five *NICD* OE mice and 102 GFP⁺ neurospheres from five littermate control mice. The DNA from these neurospheres was clonally extracted and subjected to inverse PCR to determine clone-specific lentiviral genome-integration sites. DNA was also extracted from the OB and frontal cortex including the RMS on the injected side of each mouse, from which GFP⁺ neurospheres were isolated. Using the LV-A26 and LTR-B1 primers (Fig. 6A), we first examined the integrated lentiviral genome and found that 287/296 clones (overall clones including the neurospheres mentioned above) had the lentiviral genome without the sequence between the loxP sites, suggesting that the Cre-mediated recombination efficiency of our protocol was ~97%.

Among the 101 GFP⁺ neurosphere clones derived from *NICD* OE mice and 102 GFP⁺ clones from control mice, the integration site of the lentiviral genome was identified in 64 and 68 clones, respectively. Among the 64 clones derived from the *NICD* OE mice, 29 clones contributed progeny cells to the OB (OB_{total} NSCs, 45.31%), and of these, 6 clones also contributed cells to the RMS (RMS/OB NSCs) (representative examples are shown in Fig. 6B, Clone #19-14-28 was OB_{only} NSC and Clone #19-14-26 was RMS/OB NSC). In addition, cells from one clone were present only in the RMS (RMS NSCs). Among the 68 clones derived from control mice, 19 clones were OB_{total} NSCs (27.94%), and of these, 4 clones were RMS/OB NSCs (Fig. 6C). Our analysis indicated that NSCs in the SEZ of *NICD* OE mice provided new neurons to the OB significantly more frequently than did those of control mice ($P = 0.047$ by Fisher's exact test).

We found that 5 pairs of clones in the *NICD* OE brain (15.63%) (for example, Clones #21-5-8/19 in Fig. 6B) and 3 pairs of clones in brains from control mice (8.82%) shared the same integration site (Duplicate NSCs). Although not statistically significant ($P = 0.290$ by Fisher's exact test), this increase in the frequency of Duplicate NSCs in *NICD* OE mice could be

explained in at least two ways. First, *NICD* overexpression could enhance symmetric expansive divisions of NSCs. However, we think that this possibility is unlikely given that the number of NSCs in *NICD* OE mice was significantly lower than that in control mice (Fig. 3A), and that 4 or more clones, resulting from at least two symmetric divisions, never shared the same integration site in the over 300 neurosphere clones analyzed in previous and current studies^{18,19}. Alternatively, *NICD*-overexpressing NSCs could undergo asymmetric division more frequently than control NSCs. After asymmetric division, both daughter cells can still form neurospheres *in vitro* for a while (hence, Duplicate NSCs). Then, one daughter cell remains undifferentiated as a neurosphere-forming NSC, while the other differentiated via TACs and provides progeny cells to the RMS only (RMS_{only} NSCs at this stage), and then, to the OB (RMS/OB NSCs) (Fig. 6D). After cessation of the cell supply to the RMS/OB, the daughter NSCs were labeled as OB_{only} NSCs. We found that the percentages of Duplicate and OB NSCs were relatively constant over time (Fig. 6E and 6F). Accordingly, the latter possibility is more likely because the percentages of Duplicate NSCs, RMS_{total} NSCs, and OB_{total} NSCs in the brains of *NICD* OE mice were 1.77-fold, 1.86-fold and 1.62-fold, respectively, higher than those in the control brains (Fig. 6C).

The above results suggest that the cell supply to the OB is enhanced in *NICD* OE mice. To test this possibility, we first estimated cell numbers provided by each NSC clone using quantitative nested PCR with primers targeting the clone integration site¹⁸. *NICD*-overexpressing OB NSCs and control OB NSCs provided $2,461 \pm 959$ and 725 ± 307 (mean $\pm$ SEM) progeny cells, respectively, to the OB ($P = 0.1833$ by unpaired Student's *t*-test) (Fig. 6G). We next measured DNA content and calculated the number of cells in each OB. The OB in *NICD* OE mice contained significantly more cells than did OB from control mice ($P = 0.0243$ by unpaired Student's *t*-test) (Fig. 6H). This lineage tracing data support a model in which *NICD* overexpression drives NSC cell division and differentiation more actively towards the OB

lineage, thus increasing OB neuronal output at the expense of accelerated NSC depletion.

Altered olfactory function of *NICD* OE mice

To determine whether *NICD* overexpression affects basic olfactory function, we conducted a hidden cookie test (Fig. 7A). Both *NICD* OE and control mice demonstrated comparable latencies in locating and consuming a hidden butter cookie ($P = 0.5833$ by Mann-Whitney test). We next examined sensitivity to non-social odorants (vanilla extract with vanillin as the primary ingredient and banana extract with isoamyl acetate) in a cotton-tip olfactory threshold test (Fig. 7B). We compared sniffing time in each trial of each concentration session (Fig. 7C and Fig. 7D). Both *NICD* OE and control mice showed significant sniffing response to vanilla and banana at the dilution range of 1:2,500 and 1:500 (Fig. 7C and 7D). Based on these results, we performed a habituation/dishabituation assay using two non-social odors (vanilla and banana at 1:1,000 dilution) and two social odors (urine from the opposite and same genders) (Fig. 7E-G). Habituation is olfactory adaptation defined by a progressive decrease in olfactory investigation towards a repeated presentation of the same odor stimuli, whereas dishabituation is olfactory discrimination defined by a reinstatement of investigation when a novel odor is presented²⁷. For the non-social odors, *NICD* OE and control mice exhibited effective habituation across all odor sessions (Fig. 7F and 7G). In the dishabituation session of non-social odors, control mice showed significant increase of sniffing time to odor transitions (Fig. 7F) (water T3 vs vanilla T1, $P=0.0022$; vanilla T3 vs banana T1, $P < 0.0001$ by one-way RM-ANOVA, followed by Tukey's post hoc test). Contrary to this observation, *NICD* OE mice showed little difference only in the transition from water T3 to vanilla T1 ($P=0.1040$ by one-way RM-ANOVA). For the social odors, both mice showed significant dishabituation (Fig. 7G), indicating that the response to the social smell was preserved in *NICD* OE mice. Thus, these results suggest that *NICD* OE mice showed no apparent deficit as for the sensitivity

to non-social and social odors but that *NICD OE* mice exhibited less dishabituation response than control mice.

Discussion

Consistent with results of previous studies regarding Notch signaling in neural precursor cells, our *in vitro* experiments suggest that activation of Notch signaling enhances NSC self-renewal in the adult brain. On the other hand, our *in vivo* analysis indicates that the number of quiescent NSCs in the SEZ of *NICD OE* mice decreases more rapidly over the animal's lifespan than that of control mice. This apparent inconsistency could be explained by the ability of *NICD* overexpression to induce a transition of NSCs from a dormant/quiescent state to an active state while suppressing further differentiation supposing the consumption of most, if not all, active NSCs after divisions. Indeed, our lentiviral integration site-mediated clonal analysis showing the 1.77-fold and 1.86-fold increase of transient Duplicate NSC and RMS_{total} NSC populations, respectively, support this idea. Activation of Notch signaling drives neural precursor cell proliferation in culture and in the mouse postnatal brain^{28,29}, further supporting the former possibility. The fate of active NSCs is not well understood. After divisions (hence, active NSCs), daughter cells may have one of at least three fates: (i) two or more quiescent NSCs, (ii) one quiescent NSC and one or more TACs, and (iii) two or more TACs. The lentivirus-mediated lineage-tracing studies suggest that the first scenario is unlikely because clonal expansion of NSCs in the adult brain is extremely rare, and perhaps non-existent^{18,19}. We think that the second scenario is also unlikely because the number of OB-NSCs is relatively stable, suggesting that quiescent NSCs that provide neurons to the OB do not accumulate over the lifespan of the animal. Our previous study suggests that most, if not all, NSCs that had undergone asymmetric division were destined to be consumed within ~7 weeks¹⁹. We therefore speculate that sustained *NICD OE* forces quiescent NSCs into an active state without replenishing the dormant pool. Such accelerated activation would transiently increase

precursor output but progressively reduce the long-term neurogenic reservoir. This model aligns with studies demonstrating that excessive stem cell activation can lead to premature exhaustion in multiple tissues³⁰. It is also possible that *NICD* overexpression alters differentiation potential of NSCs as suggested by the *in vitro* and *in vivo* differentiation assay. Whether or not *NICD*-overexpressing NSCs contribute not only to the classical neurogenic regions but also to other brain regions remains to be investigated.

Adult-born neurons in the hippocampal DG are thought to contribute to pattern separation, cognitive flexibility, and memory updating, particularly during their immature, hyperexcitable phase³¹⁻³³. Our behavioral analyses suggest that hippocampus-dependent spatial and emotional memories are impaired in *NICD* OE mice. We observed preservation of remote memory expression but reduction of recent hippocampus-dependent memory expression, suggesting the involvement of differential circuits. As a recent memory relies heavily on hippocampal function³⁴, it may seem counterintuitive that remote memory appears to be more stable than recent memory. However, over time, the process of systems consolidation wherein information is transferred from the hippocampus to the prefrontal cortex^{24,35} may allow the *NICD* OE mice to reach a performance ceiling that is similar to that of the controls, albeit via a less efficient pathway. This suggests that Notch signaling specifically modulates the early, highly plastic stages of memory formation rather than the long-term cortical storage, although this interpretation should be directly tested.

As the neural precursor cell population in the SGZ was significantly reduced in *NICD* OE mice, it is possible that the functional impact may stem from an impairment in the maturation of these newborn neurons. In this context, exhaustion of the NSC reservoir could compromise cognitive flexibility and the ability to update contextual representations. However, our findings should be interpreted with caution because chronic Dox treatment affects neurogenesis and function of the adult hippocampus³⁶. Moreover,

NICD overexpression in other cell types than new-born neurons may contribute to the altered spatial and emotional memories. Astrocytes and oligodendrocyte precursor cells in the cortex and other regions respond to external stimuli and express *Nestin*^{37,38}. Our study cannot exclude a possibility that those cells transiently upregulated *Nestin* when the mice received tamoxifen injection and that their function was affected by the *NICD* overexpression. In addition, activation of Notch signaling could affect dendritic arborization and synaptic plasticity of mature neurons^{29,39,40}. These possibilities are not mutually exclusive, and distinguishing among them will require additional approaches.

On the other hand, the OB of *NICD* OE mice contains more than double the number of cells as the control OB, possibly due to the accumulation of an enhanced cell supply from the NSCs in the SEZ. Although *NICD* OE mice exhibited impaired discrimination between familiar and novel conspecifics supported by our three-chamber social interaction data, their normal sniffing duration toward conspecific urine indicates that their basic olfactory detection remained intact. This apparent discrepancy suggests that the deficit does not arise from a primary sensory impairment, but rather from altered higher-order processing within the olfactory bulb circuitry⁴¹. Adult-born neurons in the OB have been implicated in fine odor discrimination and pattern separation, which are critical processes for distinguishing between similar social odours^{42,43}. While the observed attenuation in non-social odor dishabituation was moderate and characteristic common in dishabituation assays it remains highly consistent with previous literature linking altered OB neurogenesis to subtle habituation/dishabituation deficits^{41,44}. Therefore, *NICD* overexpression may impair the maturation or functional integration of these neurons, leading to reduced discrimination, specifically in social interaction and non-social odor dishabituation, despite preserved odor detection. Alternatively, *NICD* overexpression in other cell types than OB neurons may contribute to the altered olfaction because NSCs in the SEZ of postnatal brains provide a few astrocytes to the cortex⁴⁵. While our findings

are in accordance with a model in which NSC depletion is associated with the observed behavioral phenotypes, the possibility that these phenotypes arise in parallel from broader effects of sustained Notch activation cannot be excluded. Furthermore, the behavioral phenotype may additionally reflect changes in social memory or associative learning rather than purely olfactory dysfunction, as social recognition requires the integration of both odor encoding and memory retrieval⁴⁶⁻⁴⁸. Further investigation is necessary to clarify how prolonged Notch activity alters the fate and functional integration of adult-born neurons, to evaluate the dendrite and axon structures and synaptic formation of adult-born neurons in the OB and GCL of *NICD* OE mice, and to determine whether restoring normal Notch dynamics can rescue the observed behavioral alterations.

Conclusion

Collectively, these findings reveal that sustained activation of the Notch signaling pathway profoundly disrupts the finely tuned balance between quiescent and activated states of adult NSCs. Persistent *NICD* overexpression initially drives aberrant NSC activation but ultimately exhausts the stem cell reservoir, leading to impaired adult neurogenesis in both the SEZ and DG of the hippocampus. The results highlight the critical importance of appropriate regulation of Notch signaling rather than its sustained activation for maintaining long-term NSC homeostasis and supporting the continual neuronal turnover that is essential for cognitive and social functions. Chronic Notch activation in *Nestin*-lineage cells also results in selective deficits in hippocampal-dependent spatial and contextual memory, as well as compromised social recognition.

Methods

Animals and transgenic models

The Animal Care and Use Committee of Shiga University of Medical Science approved the use and care of the animals in this study (permission No. 2020-

1-3, 2022-1-1, and 2023-12-2), which was performed in accordance with ARRIVE guidelines. All animals were maintained at 24°C with a 12-h light-dark cycle and free access to water and food. The animals were housed in cages lined with wood bedding with a maximum of 5 animals per cage. *Nestin-Cre* transgenic (tg) mice⁴⁹, *Nestin-CreER* tg mice⁵⁰, *R26-rtTA* knockin (KI) mice (R26-M2rtTA; TetOP-H2B-GFP, #016836, Jackson Lab), *αCamKII-tTA* tg mice⁵¹ (#007004, Jackson Lab), and TRE-loxP-*DsRed2-loxP-c-Myc-NICD* tg mice on a C57BL/6J genetic background were maintained by mating with C57BL/6J mice (Japan SLC, Japan). To induce recombination, 6-week-old mice received two 10 mg doses, administered via oral gavage every other day, of 50 mg/mL tamoxifen (T006000, Nacalai Tesque, Japan) dissolved in sesame oil (25620-65, Nacalai Tesque). Subsequently, for *NICD* induction, mice were given normal chow supplemented with doxycycline [2 g doxycycline/kg chow (2,000 ppm), 5ST2-5001, TestDiet] continuously until sacrifice. DNA was obtained from the tip of the tail and *Nestin-CreER* tg mice were genotyped by PCR as previously described⁴⁹. Genotypes of *αCamKII-tTA* tg and *R26-rtTA* KI mice were determined according to the manufacturer's protocol. TRE-loxP-*DsRed2-loxP-c-Myc-NICD* tg mice were genotyped by PCR using TRE-A1 and DsRed-B1 primers. Primers used for genotyping are summarized in Table S1. Littermate wild-type mice were used in this study unless otherwise described.

Immunohistochemistry

Mice were deeply anesthetized with 100 mg/kg ketamine (1119400A2038, Daiichisankyo, 10 mg/mL in PBS) and 10 mg/kg xylazine (80504001, Bayer, 1 mg/mL in PBS) in PBS i.p. and then perfused transcardially before fixation with 4% paraformaldehyde (162-16065, FUJIFILM Wako). Brains were post-fixed with the same fixative solution overnight followed by 15% and 30% sucrose in PBS at 4°C. Coronal cryosections (20 μm thick) of adult brains made using a Leica cryostat (CM3050S) were used for immunochemistry analyses. Briefly, cryosections were boiled in 10 mM citrate buffer, pH 6.0 for 5 min. Brain sections were permeabilized with 0.3% Triton X-100 (25987-

85, Nacalai) for 5 min at room temperature and then blocked with Blocking-One (25987-85, Nacalai) for 1 hour. The primary antibodies used were anti-BrdU rat polyclonal antibody (1:1000, OBT0030CX, Bio-Rad), anti-NeuN mouse monoclonal antibody (1:500, Clone A60, MAB377, Millipore), anti-GFAP rabbit polyclonal antibody (1:1000, 16825-1, Proteintech), anti-c-Myc mouse monoclonal antibody (1:500, Clone 9E10, SC-40, Santa Cruz) and anti-S100 β rabbit polyclonal antibody (1:500, ab41548, Abcam) that were incubated with the sections overnight at 4°C. On the next day, after extensive washing with PBS and PBS-T, the sections were incubated with Alexa Fluor 488-, 594-, or 647-conjugated secondary antibodies (Invitrogen), followed by counterstaining of cell nuclei with Hoechst 33342 (1 μ g/mL; Sigma-Aldrich). Apoptotic cell death was assessed by TUNEL assay (G3250, Promega) according to the manufacturer's protocol. TUNEL⁺ cells were identified based on nuclear-localized fluorescence. As TUNEL⁺ cells were rarely observed, we manually counted in defined regions (olfactory bulb, lateral ventricle, and dentate gyrus). Data are presented as the number of TUNEL-positive cells per section. Images were obtained with an Olympus microscope (Olympus BX51; Olympus) and digital camera system (Olympus DP70; Olympus) or a confocal laser-scanning microscope (TCS SP-8; Leica) with 10x, 20x or 40x objective lenses.

RT-qPCR

Total RNA (0.5-1.0 μ g) isolated using a RNeasy Mini kit (74104, QIAGEN) was reverse-transcribed with an oligo(dT) primer (18418012, Invitrogen) and ReverTra Ace (TRT-101, Toyobo) to synthesize cDNA. Quantitative RT-PCR analysis using a Light-Cycler system (Roche) was carried out according to the manufacturer's protocol. The primers used in this study are summarized in Table S1.

Neurosphere assay

The detailed protocol for generating neurospheres *in vitro* from adult mouse brains was described previously^{52,53}. Briefly, mice were deeply anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine i.p., followed by cervical

dislocation. Brains were aseptically excised and each brain was placed into oxygenated artificial cerebrospinal fluid. The lateral and medial portion of the subependymal zone (SEZ) surrounding the lateral ventricles was dissected and the tissue was treated with an enzyme solution [1.33 mg/mL trypsin, 0.67 mg/mL hyaluronidase and 0.2 mg/mL kynurenic acid (all from Sigma)] for 1 hour at 37°C to facilitate tissue dissociation. The tissue was then triturated in SFM containing trypsin inhibitor (Roche) with a fire-polished Pasteur pipette. Single cells were cultured in 24-well and 6-well plates at a density of 5 cells/ μ L in SFM containing 10 ng/mL fibroblast growth factor-2 (FGF-2), 2 μ g/mL heparin, and 20 ng/mL epidermal growth factor (EGF) (all from Sigma-Aldrich). After culturing for 6 days *in vitro*, the number of floating spherical colonies (neurospheres) having a diameter greater than 0.1 mm as measured with a scale equipped in the eyepiece lens was counted. The total number of neurosphere-forming NSCs in the adult brain was calculated using the formula [number of resultant neurospheres in the 24-well plate culture] x [volume in which SEZ cells were suspended (usually 600 μ L)] / [volume applied in the culture]⁵⁴.

To assess self-renewal, primary single neurospheres were mechanically dissociated into single cells in 0.2 mL of SFM containing FGF2, EGF and heparin, and then subcloned in 96-well (0.2 mL/well) plates (Falcon). NSC self-renewal *in vitro* was assessed by identifying new neurospheres after further culture for 7 days. For lentiviral infectants, primary single neurospheres expressing enhanced green fluorescent protein (GFP) were picked up under a fluorescent microscope, mechanically dissociated into single cells, and then subcloned in 48-well (0.3 mL/well) plates (Falcon). The resulting GFP⁺ neurospheres were serially passaged and amplified in larger dishes until a sufficient number of neurospheres was obtained (usually 3 passages). Genomic DNA was extracted by proteinase K treatment.

To assess multipotential capability, single neurospheres were transferred to individual cover slips, which had been serially coated with 0.1% polyethyleneimine (Sigma) in borate buffer (pH 8.4) and then with

Matrigel basement membrane matrix (0.6 mg/ml in SFM; BD Biosciences), and cultured in SFM containing 1% FBS (Life Technologies). Cells were processed 6 days later using immunocytochemistry as described previously¹⁴. We used anti- β III tubulin mouse monoclonal (IgG) (1:1000; T8660, Sigma), anti-GFAP rabbit polyclonal (IgG), and O4 mouse monoclonal (IgM) (1:10; a kind gift of Dr. K. Ono) antibodies as primary, then followed by appropriate Alexa-conjugated secondary antibodies (Invitrogen). Cultures were counter labeled with the nuclear stain Hoechst 33258 (1 μ g/ml; 94403, Sigma).

Here quiescent NSCs were defined as cells in the SEZ that respond to FGF-2 and EGF and proliferate to form neurospheres *in vitro*. Dormant NSCs were defined as cells that retained the potential to convert to quiescent NSCs but cannot be detected in the neurosphere assay.

5-Bromo-2'-deoxyuridine short-term and long-term labeling

Mice were given 50 mg/kg 5-bromo-2'-deoxyuridine (BrdU; 05650-24, Nacalai Tesque) dissolved in PBS and delivered i.p. A total of five injections were delivered at 3 hour intervals. For long term labelling, mice received injections beginning at 15 weeks of age and were sacrificed 4 weeks later. For short term labelling, mice received injections at 19 weeks of age and were sacrificed 1 hour later. All animals were sacrificed at 19 weeks old. The total number of BrdU⁺ cells in the SEZ and DG was estimated using the optical dissector method⁵⁵. We determined the total number of BrdU⁺ cells in the rostro-caudal axis of SEZ within an area encompassing from the genu of corpus callosum and extending caudally to the rostral tip of the crossing of the anterior commissure. For the DG, the number of BrdU⁺ cells in the subgranular zone (SGZ) and granule cell layer (GCL) ranging from the first emergence of DG to the corpus callosum separation, was determined and corresponds to 5-7 sections of positive cells within the regions of interest.

Behavioral assessments

Behavioral tests began when the mice were 10 weeks old. The open field, startle response and prepulse inhibition response, three-chamber social interaction, Barnes maze, and contextual fear conditioning tests were

performed sequentially with a one-week interval between each test⁵⁶. These routine behavioral batteries were performed across 8 independent littermate groups. All apparatus and software were set up and the tests conducted in accordance with the manufacturer's instructions (O'Hara & Co., Ltd., Tokyo, Japan). Tests were conducted during the daytime in a soundproof behavioral room. All behavioral testing including the subsequent olfactory bulb behavior assessments utilized a control group consisting of wild-type, *Nestin-CreER*, and TRE-loxP-*DsRed2*-loxP-*NICD* mice and *NICD* OE mice without doxycycline treatment. Both male (n=48) and female (n=25) mice were used to ensure generalizability of findings. Preliminary analysis revealed no significant sex-dependent variation in behavioral performance. Therefore, data from both sexes were pooled for statistical analyses. This approach is supported by previous studies reporting minimal or no significant sex differences in these behavioral assays⁵⁷⁻⁶⁰. To ensure consistency in animal handling, the same investigators performed all tasks including husbandry, group allocation, and execution of behavioral assays. Consequently, the investigators were not blinded to the treatment groups during the experimental phase.

Open field test

The open field apparatus comprised a 40 cm-square gray arena enclosed by 30 cm-high walls housed in a soundproof chamber. The center of the floor was illuminated at 70 lux. Ten-week-old mice were individually placed in the center of the arena and allowed to explore freely for 10 min. Their behavior was recorded using a video tracking system and infrared photobeam sensors. Horizontal activity (total distance), vertical activity (number of rearing behaviors measured by counting the number of photobeam interruptions), and time spent in the central region were analyzed. Total distance was used as an index of locomotor activity in a novel environment, and the percentage of time spent in the central area was used as an index of anxiety.

Three-chamber social interaction

Sociability and social novelty preferences assessments were conducted in a gray experimental cage that was divided into a three-chambered box (61 x 40 x 22 cm). Each chamber was 20 x 40 x 22 cm, and the dividing walls were transparent Plexiglas with small square openings (5 x 3 cm) that allowed access into each chamber. Before the actual test, the mice were placed in the center chamber and allowed to habituate freely in all three empty chambers for 10 minutes. To assess sociability, an unfamiliar C57BL/67 mouse (Stranger 1) that had had no prior contact with the subject mouse was enclosed in a wire cage that was placed in the left chamber, while the chamber on the right side remained empty. In the next test session representing the social novelty phase, a second unfamiliar mouse (Stranger 2) of the same strain was placed in the chamber on the right side. Each test session was performed by placing the subject mouse in the center chamber and then allowing it to explore and choose between the now familiar mouse (Stranger 1) and the newly introduced unfamiliar mouse (Stranger 2). To focus on social affiliation rather than mating behavior, mice of the same sex and similar age were used as strangers in both sessions. Time spent in proximity to each cage over a 5-min session was recorded and used as an indicator of sociability and social preference. The location of the stranger was exchanged between trials.

Startle response and prepulse inhibition response

To measure sensorimotor gating, startle response and prepulse inhibition tests were conducted. An individual mouse was placed into a plexiglass cylinder chamber and put on an acceleration sensor $\square$ inside a sound attenuating box. The experiment began with 1-minute acclimation period with a background white noise of 60 dB. After acclimation, mice were exposed to two blocks of trials. Block 1 was a startle-pulse alone (115 dB) and Block 2 involved PPI trials consisting of randomized prepulse intensities (70 dB, 74 dB, 78 dB and 82 dB) and with/without a startle pulse alone for 20 msec. The prepulse inhibition percentage (%PPI) was calculated as %PPI = (mean

startle of pulse alone mean startle of prepulse / mean startle of pulse alone) x 100.

Barnes maze

To assess spatial learning and memory, a Barnes maze test was conducted over a two-week period. The test was begun when mice were 13 weeks old and conducted before the fear conditioning test. The apparatus consisted of a white circular platform (1 m in diameter) with 16 holes equally distributed around the perimeter; one hole was equipped with an escape box. Mice underwent habituation with a maximum escape latency of 15 minutes to familiarize themselves with the apparatus. Following habituation, each mouse was placed at the center of the platform, and the latency time to find and enter the escape box was recorded. The acquisition phase included nine training sessions conducted twice daily (with a 2 h inter-session interval), in which each training session comprised three trials. To evaluate spatial memory using distal environmental room cues, a 3-min probe trial without the escape box was performed exactly 4 hours after the final training session. Two days after completion of the previous training, the reversal learning task was conducted to assess cognitive flexibility. The target escape box was relocated to a new position opposite the original target hole. This was followed by another nine training sessions and a subsequent probe trial, conducted according to the same procedures as the previous paradigm.

Contextual fear conditioning test

The chamber used for the conditioning training and contextual test was a rectangular transparent Plexiglas chamber (width × depth × height: 34 × 26 × 29 cm, 200 lux). The chamber floor consisted of 46 stainless steel rods with a diameter of 2 mm that were placed 4 mm apart and equipped with a ceiling-mounted CCD camera connected to a video monitor. A shock generator was connected to the rods via a cable harness. The training (conditioning) session consisted of three tone-shock pairings. Fifteen-week-old mice were placed into the bright rectangular chamber and allowed to explore freely for 2 min. A tone stimulus (auditory cue; 55 dB white noise) was presented as the

conditioned stimulus (CS) for 30 sec, and was followed by a mild foot shock (aversive stimulus, 2 sec, 0.5 mA) as the unconditioned stimulus (US). Two subsequent CS-US pairings were administered at 2-min intervals. The contextual fear conditioning test was evaluated at 1 day (recent memory) and 28 days (remote memory) after the training. During the contextual test, the same mice were re-exposed to the same conditioning chamber for 5 min in the absence of CS-US. Freezing behavior was defined as a lack of movement for >2 sec as measured by video tracking software. The percentage of time spent freezing was used as an index of fear memory during all sessions.

Olfactory behavior - hidden cookie test

Distinct from the previous order of behavioral paradigms, the olfactory behavior battery comprising hidden cookie, threshold olfactory cotton tip tests and habituation/dishabituation tests was conducted when the mice were between 10 and 14 weeks of age. These additional tests were prompted by the observation of substantial cellular accumulation in the olfactory bulb after lineage tracing analysis, resulting in a different sample size from the other tasks. Experiments were performed with two independent littermate groups. During the hidden cookie test, mice were first habituated to butter cookies in the home cage overnight. The next day, the mice were deprived of food for 18-24 hours. The test was conducted in a new cage. A piece of butter cookie (0.8 g) was placed in a random corner of the cage, and then the entire cage floor was covered with wood chip bedding to a depth of 4 cm. The subject mouse was then placed into the cage, and the latency to find the cookie was recorded with a maximum time limit of 10 min. We defined finding the cookie as when the mouse ate the cookie.

Olfactory behavior - cotton-tip olfactory threshold test

This test was performed with varying concentrations of non-social odors (vanilla and banana). Odorants were freshly diluted with Milli-Q water to 1:500, 1:2,500, 1:5,000, 1:10,000, and 1:50,000. The assessment was conducted over consecutive days, with a 24-hour interval between each concentration, as adapted from a previous protocol⁶¹. The daily testing

procedure followed the described habituation/dishabituation paradigm, in which the mice were exposed to a cotton swab saturated with 50 μ L of a specific odor dilution for three consecutive trials, separated by an inter-session interval of 1-2 minutes.

Olfactory behavior - habituation/dishabituation test

The protocols were adapted from previously published studies^{27,62}. Prior to the test, mice were acclimated to a new cage containing cotton tips for 30 min. Mice were then tested with five olfactory cues: Milli Q water as a baseline; two non-social odors (vanilla and banana); and two social odors (urine from the opposite and same gender). For non-social odors, banana and vanilla extract from McCormick & Company Inc., Hunt Valley and Home Made, Kyoritsu Foods, respectively, were used. The solutions were kept at room temperature and were freshly diluted 1:1000 with Milli Q. Mice were exposed to a cotton swab soaked with 50 μ L water or an odor solution for three trials (T1, T2 and T3) with 2 min per trial. Sniffing behavior was defined as the mouse orienting its nose within approximately 1 cm of the cotton-tip. For each course of an odor exposure, the mouse was given a 2 min inter-session period that was an adequate amount of time to change to a subsequent odor. New clean cotton swabs were used for each trial and each session. For social odors, urine from male and female mice was used. Urine samples were collected twice in the early morning and evening from 10 different mice of each gender. A mixture of fresh urine was collected with a sterile Pasteur pipette into the sterile tube. The collected urine was diluted 1:3 with Milli Q and frozen until further use. Each experimental group was first exposed to urine from the opposite sex (e.g., males exposed to female urine and females exposed to male urine), and then urine from the same sex group was presented.

Preparation of GFP-expressing lentivirus and lentivirus infection

Generation of the lentiviral plasmid vector pCL20c-MSCV-GFP-2LoxP and vesicular stomatitis virus-G protein pseudotyped lentivirus production has been previously described^{18,19}. Newborn *NICD* OE pups and littermate

control mice were anesthetized by brief inhalation of isoflurane (FUJIFILM Wako) using the open-drop method and hypothermia, typically 1 min on ice until no spontaneous movement was observed. Lentivirus solution (1 μ L) containing 1.0×10^5 TU virus and 0.1% Fast Green was injected stereotactically into the right lateral ventricle using a Hamilton syringe with a 30G needle. The pups were gently warmed to restore normal body temperature and returned to the dam. No analgesics or antibiotics were used because only minimal needle holes were made using a 27G needle to the skin and cranium and a 30G needle to the cortex, and neither hemorrhage nor infection was observed during or after the surgery. We monitored the pups daily to assess general movements and body weight gain.

Inverse PCR

The protocol for inverse PCR has been previously described¹⁹. Briefly, genomic DNA from clonally amplified GFP⁺ neurospheres was digested with 4-base cutter restriction enzymes. DNA fragments were self-ligated with T4 DNA ligase (Takara Bio) and the resulting DNA mixture was subjected to nested PCR. We used a pair of primers, LTR-A1 and LTR-B2, for the first amplification and another pair of primers, LTR-A2 and LTR-B1, for the second PCR (Table S1). Amplified DNA fragments were sequenced to determine the lentiviral integration site. Two integration site-specific primers from the mouse genome were synthesized, and nested PCR was carried out with the appropriate primers within the lentiviral DNA to detect the presence of lentiviral DNA at the integration site. Deletion of *DsRed2* from the TRE-loxP-*DsRed2*-loxP-*NICD* transgene were determined by PCR using TRE-A1 and NICD-B4 primers.

Calculation of NSC consumption probability by age

Means of total numbers of neurosphere-forming NSCs in hemispheres from 10-week- to 26-week-old mice were converted to common logarithm numbers. Subsequently, a reduction rate per week was calculated by a simple linear regression model (Prism 8 for Mac OS, ver. 8.4.3). The slope value was converted to a probability of the reduction in NSC numbers per week.

Data analysis

Statistical analysis was performed in GraphPad Prism 10 using one-way or two-way ANOVA followed by Tukey's, Bonferroni's, or Dunn's *post hoc* comparisons. If applicable, we used an unpaired two-tailed Mann-Whitney test, Wilcoxon matched-pairs signed rank test, Student's *t*-test or Fisher's exact test. Normality of the data was analyzed by Shapiro-Wilk test. Data represent mean $\pm$ SEM. The level of significance was set at $*P < 0.05$.

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Data availability

This study did not generate new unique reagents. Further information and requests for resources, analysis and methodology should be directed to and will be fulfilled by the corresponding author.

Code availability

All data reported in this paper will be shared by the corresponding author upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the corresponding author upon request.

Abbreviations

ANOVA, analysis of variance; BrdU, 5-bromo-2'-deoxyuridine; Dox, doxycycline; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; KI, knockin; LTR, long terminal repeat; NICD, Notch1 intracellular domain;

NSCs, neural stem cells; OB, olfactory bulb; 4-OHT, 4-hydroxytamoxifen; RMS, rostral migratory stream; rtTA, reverse tetracycline transactivator; SEZ, subependymal zone; SFM: serum-free media; SGZ, subgranular zone; TAC, transit amplifying cell; tg, transgenic; TU, titer unit.

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Author contributions

SH designed the research. NAFMA, NM, KD and SH performed the research. NAFMA, NM and SH analyzed the data and prepared the manuscript.

Competing interests

The authors declare no competing interests.

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Availability of data and materials

Data can be obtained upon request to the corresponding author.

Figure Legends

Fig. 1. Generation of *NICD* overexpression mice.

(A) Mice with *NICD* overexpression (*NICD* OE) were generated by mating *Nestin-CreER* transgenic, *R26-rtTA* knockin, and TRE-loxP-*DsRed2-loxP-NICD* transgenic mice. After Cre excision from the transgene, the reverse tetracycline transactivator (rtTA) binds to the tetracycline responsive element (TRE) in the presence of doxycycline (Dox) to drive *NICD* expression. (B) Schematic diagram of the study protocol. c-Myc-NICD was induced by

consumption of chow supplemented with Dox. (C) Coronal cryosections of brains from *NICD* OE and control *Nestin-CreER* mice taken at the level of the lateral ventricle (top) and dentate gyrus (bottom) were immunostained for c-Myc. Dashed red boxes in the illustrations at the left highlight areas shown at higher magnification in the panels on the right. White arrows indicate representative c-Myc⁺ cells. Scale bar, 100 μ m (top panels in the subependymal zone and dentate gyrus) and 50 μ m (bottom panels in the subependymal zone). (D) Coronal cryosections of the olfactory bulb from *NICD* OE and control mice immunostained for c-Myc. Dashed white boxes in the lower magnification images (left) highlight areas shown at higher magnification in the panels on the right. Scale bars, 500 μ m (lower magnification) and 100 μ m (higher magnification).

Fig. 2. NICD overexpression enhances NSC self-renewal *in vitro*.

(A) Schematic diagram of a floating colony-forming neurosphere assay. (B) Expression levels of *NICD* and *Hes1* determined by RT-qPCR in secondary neurospheres derived from the same primary neurospheres with and without doxycycline (Dox) treatment. Values were normalized to those of β -actin. (C) Number of primary neurospheres generated from 2,500 cells in the subependymal zone (SEZ) of *NICD* OE and wild-type mice. (D) The number of secondary neurospheres generated from single primary neurospheres derived from the same mice cultured in the absence and presence of 4-hydroxytamoxifen (4-OHT) and Dox. (E) Diameters of primary neurospheres derived from *NICD* OE mice cultured in the absence or presence of 4-OHT/Dox treatment were measured. Vertical bars mean SD. (F) Primary neurospheres were induced to differentiate for 6 days and immunolabeled for astrocytes (GFAP), neurons (β III tubulin), and oligodendrocytes (O4, arrowheads). Scale bars, 50 μ m. (G) Marker-positive cells were counted in three random optical areas at the periphery of individual neurospheres. The left axis represents percentages for GFAP⁺ cells, and the right axis represents percentages for β III tubulin⁺ or O4⁺ cells. Data represent the mean $\pm$ SEM

(**B-D, G**) or the mean $\pm$ SD (**E**). * $P < 0.05$ and ** $P < 0.01$ by one-way ANOVA, followed by Dunnett's post hoc test or by paired t -test.

Fig. 3. Reduced NSC pool size and neurogenesis in brains of adult mice with NICD overexpression.

(**A**) Schematic diagram of the study protocol. (**B-D**) Total numbers of neurosphere-forming NSCs in brains from *Nestin-CreER* and *NICD* overexpression (OE) mice with and without doxycycline (Dox) treatment as indicated were determined when the mice were 10 (**B**), 18 (**C**), and 26 (**D**) weeks old. The total NSC numbers shown as *Nestin-CreER* Dox (-) mice at 10 and 18 weeks old are re-used from the same data sets shown in Figs S4A and S4B, respectively. (**E-J**) Mice received five intraperitoneal injections of 5-bromo-2'-deoxyuridine (BrdU) (50 mg/kg) at 3-hour intervals and tissues were analyzed by histochemistry 4 weeks after the last injection. (**E, G, I**) Coronal cryosections taken at the level of the lateral ventricles (**E**) and the dentate gyrus (DG) (**G, I**) were immunostained for BrdU and cell markers as indicated. The areas within the dashed box are shown to the right at higher magnification (**E**). The merged images are shown as confocal orthogonal images (**E, G**). White arrowheads indicate BrdU⁺ cells in the DG (**G, I**). Scale bars are 200 μ m (**E**, lower magnification), 20 μ m (**E**, higher magnification), and 50 μ m (**G, I**). (**F, H, J**) The total number of BrdU⁺ cells in the SEZ (**F**) and the SGZ (**H**) and BrdU⁺NeuN⁺ cells in the DG (**J**) were counted. Data represent the mean $\pm$ SEM. * $P < 0.05$ by one-way ANOVA, followed by Tukey's post hoc test or unpaired t -test.

Fig. 4. Impairment of spatial memory in *NICD* OE mice.

(**A**) Behavioral tests were performed with a one-week interval between each paradigm. (**B**) Locomotor activity and anxiety levels were assessed with an Open field test. Representative trace trajectories for *NICD* OE and Control mice are shown on the left. There was no difference between *NICD* OE and Control mice for the total distance traveled and time spent in the center region. Circles represent results for individual mice. (**C**) Spatial memory assessed with the Barnes Maze. Escape latency was defined as the time taken

to locate and enter the target hole, and was used as an index of spatial learning performance across the training. In the probe test, the time spent around each hole was measured in relation to angle of the target goal. $n = 26$ (15 males and 11 females) and 17 (8 males and 9 females) for Control and *NICD* OE mice, respectively. Data represent the mean $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ by 2-way ANOVA.

Fig. 5. Reduced emotional memory and social discrimination in *NICD* OE mice.

(A) Emotional memory was evaluated with a fear conditioning test involving an electrical foot shock (unconditioned stimulus). (B, C) The percentage of mice that froze (freezing percentage) was analyzed as an index of fear memory during contextual testing after 24 hours (B) and 1 month (C) as recent and remote memory, respectively. (D) Total freezing time during the conditioning and recent and remote memory sessions was calculated. Circles indicate results for individual mice. $n = 26$ (15 males and 11 females) and 17 (8 males and 9 females) for Control and *NICD* OE mice, respectively. (E) Three-chamber social interaction test began when the mice were 12 weeks old. Mice were first habituated to the chamber (left), followed by social interaction (middle) and social novelty testing (right). The time spent in each chamber was recorded over a 5-minute period. $n = 25$ (14 males and 11 females) and 17 (8 males and 9 females) for Control and *NICD* OE mice, respectively. Data represent the mean $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ by 2-way ANOVA. Outliers were omitted using Grubs' test.

Fig. 6. Lineage-tracing analysis of NSCs by identification of lentiviral genome integration sites.

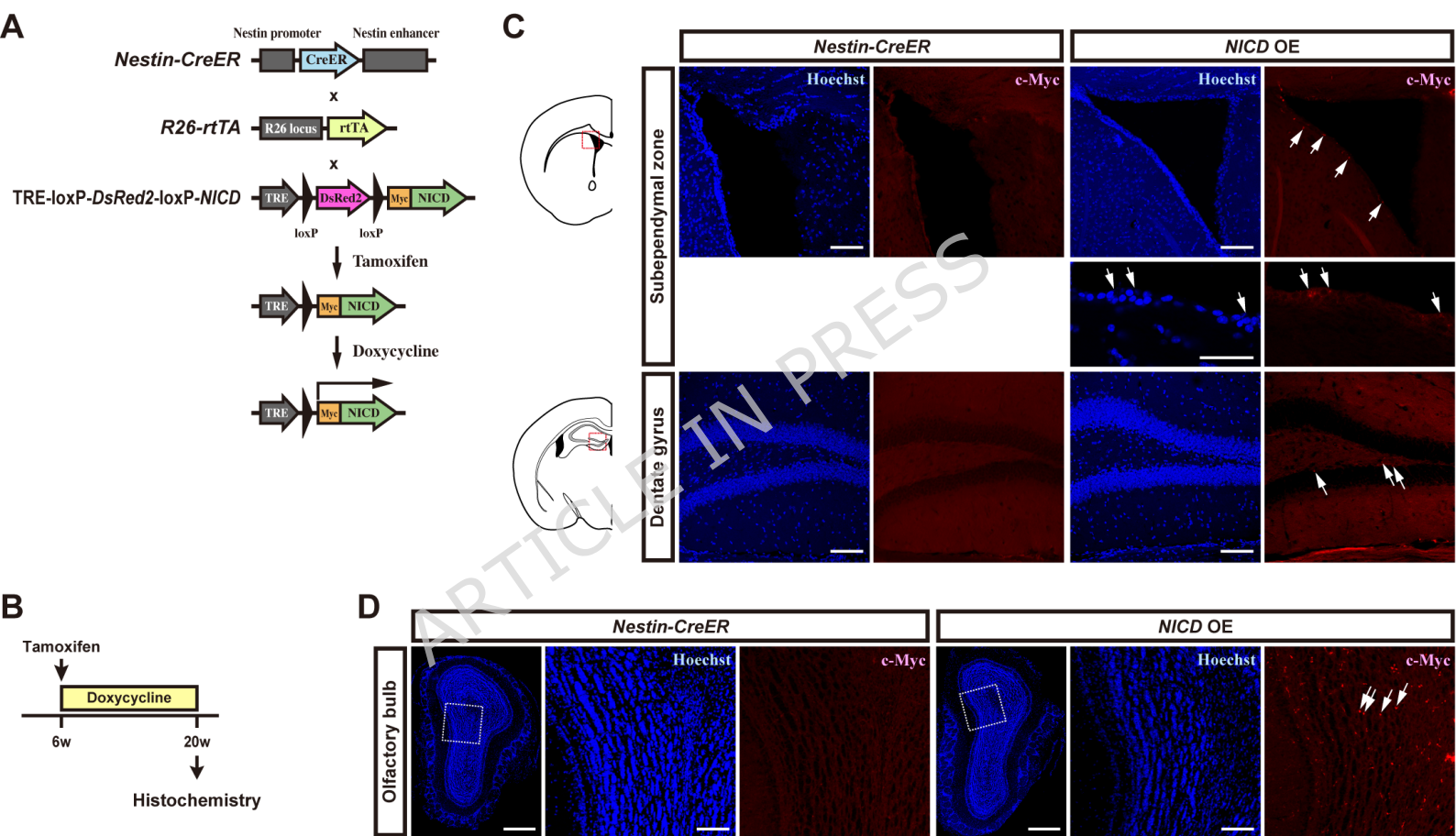
(A) Schematic diagram of lentiviral infection in neonatal mouse brains and analysis of adult brains. (B) DNA from each neurosphere clone, the olfactory bulb (OB), the frontal cortex containing the rostral migratory stream (RMS), and the remaining cortex (Ctx) derived from the same brain were subjected to integration site-specific nested PCR. Representative PCR results from a Negative NSC (Clone #19-14-21), OB_{only} NSC (Clone #19-14-28), RMS/OB

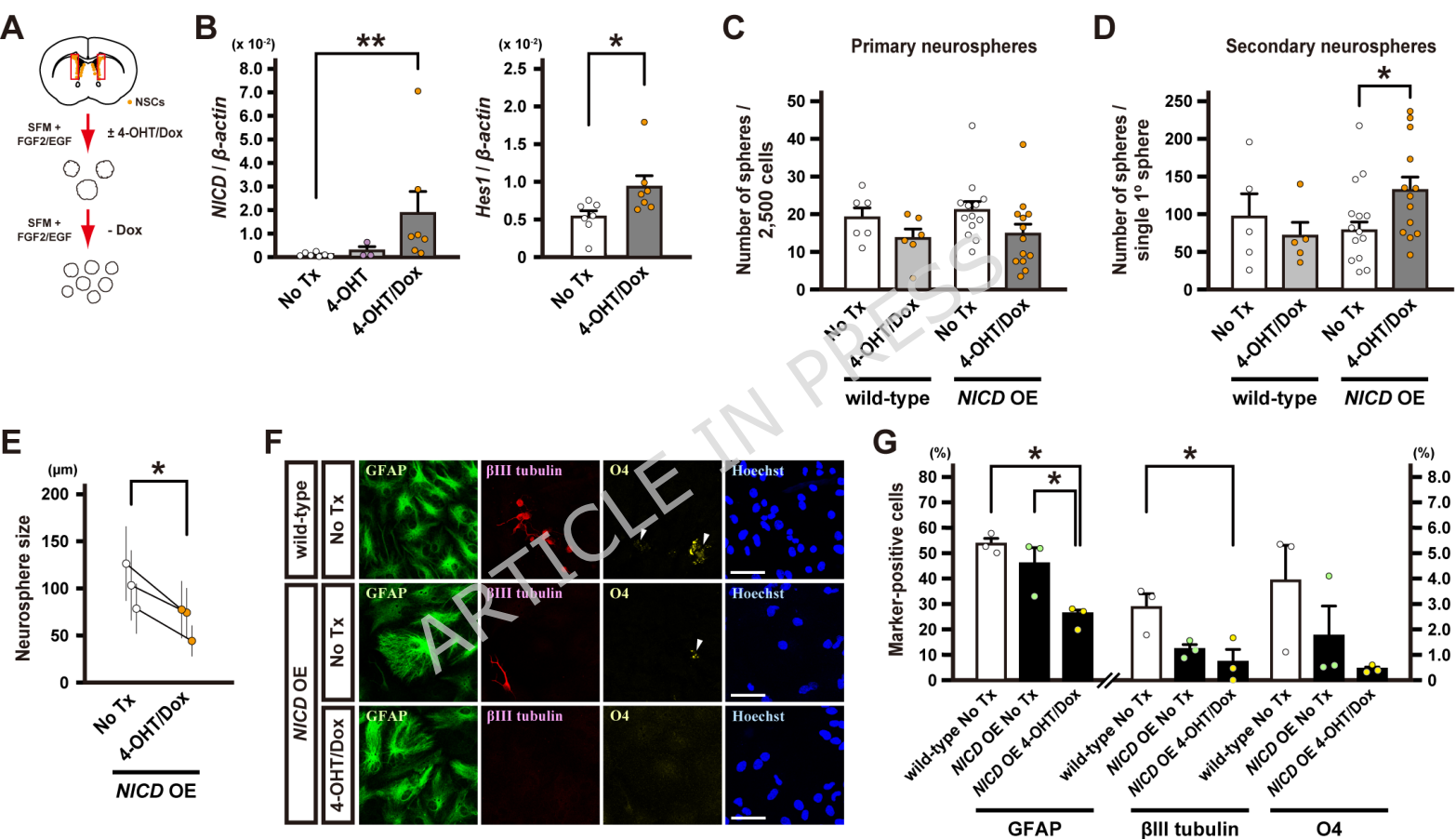
NSC (Clone #19-14-26), and Duplicate NSC (Clones #21-5-8 and #21-5-19) that also sent cells to the OB from the brains of *NICD* OE mice (#19-14 and #21-5) are shown. Φ , Φ X174/HaeIII DNA marker, genomic DNA of the Φ X174 bacteriophage digested with the HaeIII restriction enzyme. **(C)** Analysis of 68 and 64 independent GFP⁺ neural stem cell clones derived from *Nestin-CreER* and *NICD* OE mice ($n = 5-6$ for each group), respectively, is shown. **(D)** Schematic diagram of our model for NSC lineages. Negative NSCs that distribute no cells to regions other than the subependymal zone (SEZ) undergo symmetric division to produce two daughter NSCs, which can be detected as Duplicate NSCs until one or both progeny cells either differentiate into transit amplifying cells (TACs) or die. After one daughter cell is consumed to provide OB neurons through the RMS, the other NSC can be detected as an RMS_{only}, RMS/OB, or OB_{only} NSC until it is ultimately lost. **(E, F)** Percentage of Duplicate NSCs **(E)** and OB_{total} NSCs **(F)** over the animal's lifespan. **(G)** Lentiviral DNA copy numbers in the olfactory bulb were determined by quantitative PCR using clone-specific primers. **(H)** Amount of DNA in the right OB was measured, and the results were converted to cell numbers based on one cell containing 6pg DNA. Data represent the mean $\pm$ SEM. * $P < 0.05$, by unpaired t -test.

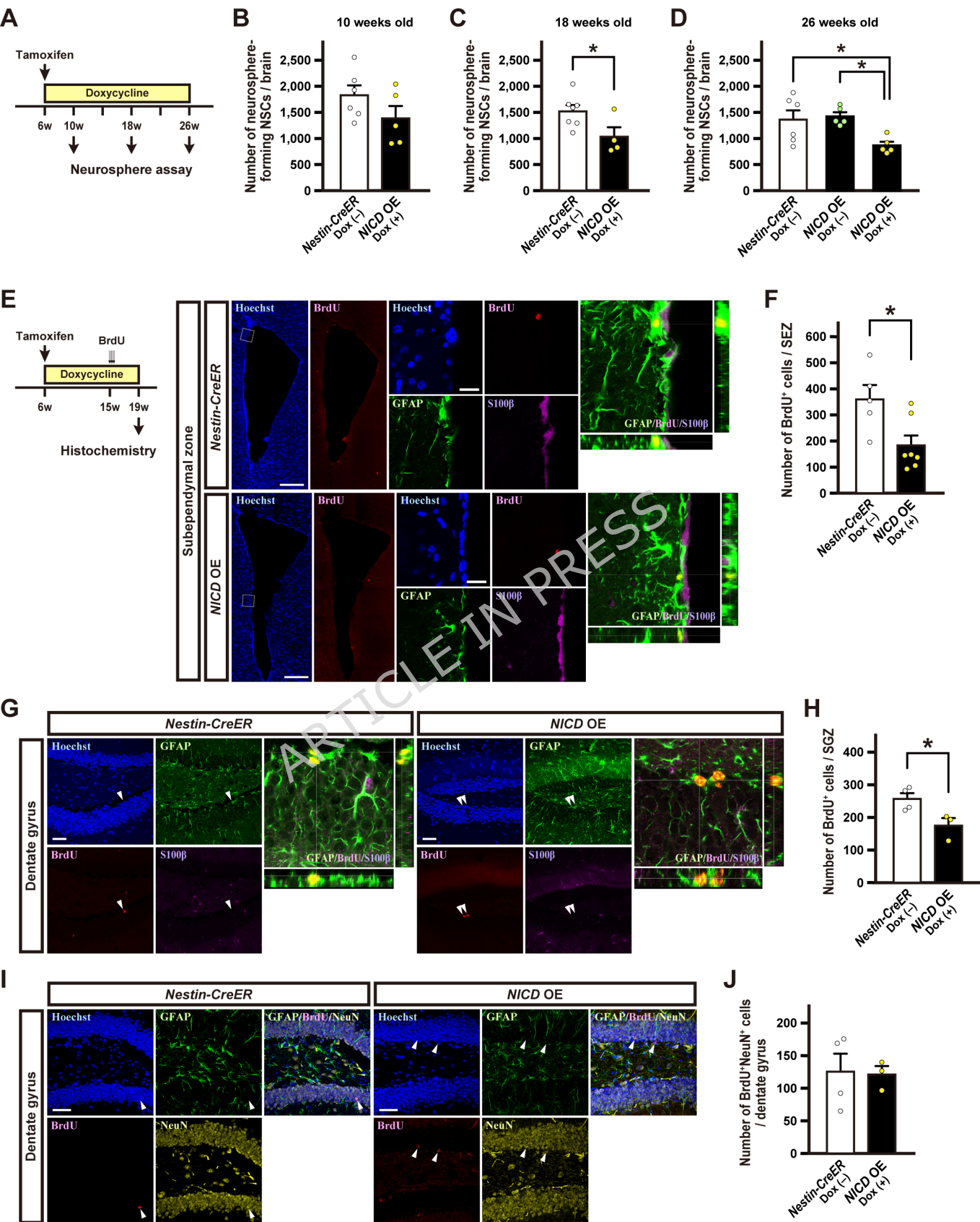
Fig. 7. Altered odor behavior in *NICD* OE mice.

(A) Schematic representation of the hidden cookie test. Mice were assessed for latency in locating and consuming a butter cookie hidden under 4 cm of bedding. $n = 14$ (6 males and 8 females) and 8 (3 males and 5 females) for Control and *NICD* OE mice, respectively. **(B)** Schematic representation of a cotton-tip olfactory threshold test. The test was conducted at one concentration per day over 5 consecutive days, in order of increasing concentration (i.e., decreasing dilutions: 1:50,000, 1:10,000, 1:5,000, 1:2,500, and 1:500). Odor presentation in each session consisted of three trials (T1-T3). **(C, D)** Sniffing time during each trial across different dilutions for vanilla **(C)** and banana **(D)** in the cotton-tip olfactory threshold test. Data were analyzed by one-way repeated ANOVA between trials or two-way ANOVA

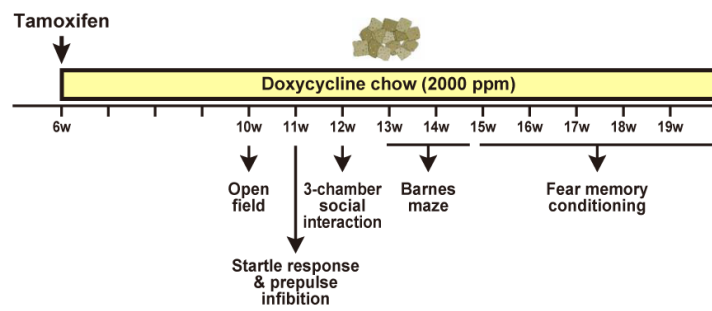
between groups, followed by Tukey's post hoc test. $n = 8$ per group, except at 1:50,000 dilution ($n = 5$ per group). **(E)** Experimental setup for the habituation and dishabituation test. Mice were sequentially presented with water (baseline), non-social odors (vanilla and banana at 1:1,000 dilution), and social odors (urine from opposite and same gender). Each odor was presented for 2 minutes over three trials (T1, T2, and T3). **(F, G)** Sniffing time of each trial and total sniffing time for non-social **(F)** and social **(G)** odors were measured. Data were analyzed by one-way repeated ANOVA, followed by Tukey's post hoc multiple comparison test. Data represent the mean $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. ns, not statistical.



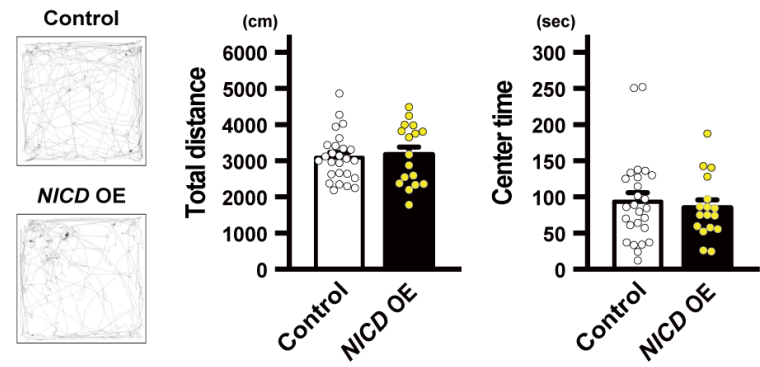




A



B



C

