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NEGR1 Modulates Mouse Affective Discrimination by Regulating Adult Olfactory Neurogenesis

Kwang Hwan Kim, Kyungchul Noh, Jaesung Lee, Soojin Lee, and Sung Joong Lee

ABSTRACT

BACKGROUND: Affective recognition and sensory processing are impaired in people with autism. However, no mouse model of autism comanifesting these symptoms is available, thereby limiting the exploration of the relationship between affective recognition and sensory processing in autism and the molecular mechanisms involved.

METHODS: With *Negr1*$^{-/-}$ mice, we conducted the affective state discrimination test and an odor habituation/dishabituation test. Data were analyzed using the *k*-means clustering method. We also employed a whole-cell patch clamp and bromodeoxyuridine incorporation assay to investigate underlying mechanisms.

RESULTS: When encountering mice exposed to restraint stress or chronic pain, wild-type mice discriminated between them by either approaching the stressed mouse or avoiding the painful mouse, whereas *Negr1*$^{-/-}$ mice showed unbiased social interactions with them. Next, we demonstrated that both wild-type and *Negr1*$^{-/-}$ mice used their olfaction for social interaction in the experimental context, but *Negr1*$^{-/-}$ mice showed aberrant olfactory habituation and dishabituation against social odors. In electrophysiological studies, inhibitory inputs to the mitral cells in the olfactory bulb were increased in *Negr1*$^{-/-}$ mice compared with wild-type mice, and subsequently their excitability was decreased. As a potential underlying mechanism, we found that adult neurogenesis in the subventricular zone was diminished in *Negr1*$^{-/-}$ mice, which resulted in decreased integration of newly generated inhibitory neurons in the olfactory bulb.

CONCLUSIONS: NEGR1 contributes to mouse affective recognition, possibly by regulating olfactory neurogenesis and subsequent olfactory sensory processing. We propose a novel neurobiological mechanism of autism-related behaviors based on disrupted adult olfactory neurogenesis.

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Autism is characterized by impaired affective recognition and atypical sensory perception. Most animals, including humans, use olfaction to explore, identify, and interact with their conspecifics (1–6). Notably, people with autism have difficulty with olfactory processing (7,8), and their atypical affective response is associated with their olfactory function (9). Therefore, aberrant olfactory processing may be involved in the inability of individuals with autism to discriminate different affective states in others. Nevertheless, the lack of animal models manifesting impairment in both affective discrimination and olfactory processing has hindered studies of any putative link between affective recognition and olfactory sensory processing.

In rodent studies, researchers have evoked positive and negative affective states through manipulations such as watering after deprivation, restraint stress, and electric footshock. Recent updates of the affective state discrimination test (ADT) allow more precise investigation of affective recognition and neural underpinnings in a mouse model (10,11). In these studies, neuronal manipulation modulated affective discrimination without influencing general sociability, revealing that emotional recognition can be differentiated from general sociability. Thus, the ADT now allows researchers to investigate affective perception per se systematically. In the meantime, studies using rodent models have reported inconsistent results regarding the involvement of olfactory impairment in autism. Some rodent models of autism indicate olfactory dysfunction (12–14), while other models do not (15,16). These discrepancies may be due in part to the cursory methods applied to study olfactory dysfunction, merely identifying the presence or absence of olfactory abnormalities. However, olfactory function is composed of subskills such as detection, identification, and discrimination of olfactory stimuli. Therefore, examining each subskill separately is required to dissect olfactory processing in more detail, which allows for a clearer understanding of olfactory dysfunction in rodent models of autism.

NEGR1 has been reported as an autism spectrum disorder risk gene in genome-wide association studies (17–19). *Negr1*$^{-/-}$ mice have been shown to display autism-like behaviors such as social impairment and repetitive behavior (18,19). Concurrently, NEGR1 has been associated with autism-related neurobiological features such as neuronal migration,

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dendritic spine maturation, and axon elongation (18–20). However, the involvement of NEGR1 in affective recognition and sensory processing has not been investigated. In the current study, we investigated the role of NEGR1 in affective discrimination and olfactory sensory processing using *Negr1*$^{-/-}$ mice. We found that NEGR1 was involved in impaired affective discrimination and olfactory sensory processing, which have relevance to autism symptoms. We present data elucidating the role of NEGR1 in adult mouse olfactory bulb (OB) neurogenesis, indicating that it acts as a novel neurobiological mechanism in autism-relevant behaviors.

METHODS AND MATERIALS

Animals

Male *Negr1*$^{-/-}$ (20) and C57BL/6N (DooYeol Biotech) mice from 9 to 15 weeks old were used. All animal care and experiments conformed to the guidelines provided by the Institutional Animal Care and Use Committee of Seoul National University. For additional details, see the Supplement.

Behavioral Tests

All test mice were handled for 3 days (5 min/day) 1 week before the test during the daytime (12 PM–6 PM). For additional details for all behavioral experiments below, see the Supplement.

Affective State Discrimination Test. The ADT was performed as previously described (10,11). After 3 consecutive habituation days, the observer and 2 affective demonstrator mice (i.e., stress and pain) were placed in the testing chamber for 6 minutes. Stress and pain were provoked by tube restraint and sciatic nerve transection (SNT) surgery (21), respectively. The observer's sniffing time was measured.

von Frey Test. The von Frey test was performed to measure mechanical allodynia before and after SNT surgery, as previously described (21). After scoring pain responses (e.g., paw withdrawal) by using von Frey filaments (Stoelting), we calculated the scores using the up-down method (22).

Three-Chamber Social Test. The observer mouse habituated in the 3-chamber social apparatus for 3 consecutive days. On the test day, after the observer moved freely in the chamber for 10 minutes, the first strange mouse was placed on one side of the chamber for 10 minutes, followed by the second strange mouse being put on the other side of the chamber for 10 minutes.

Sensory-Controlled Social Interaction Test. The cylindrical cups used in this test were designed as previously described (1) with minor modifications. This test followed the same experimental procedure as the ADT, but the observer mouse interacted with 3 different strangers in different sensory-controlling cylindrical cups every 3 days.

Buried Food Test. After being fasted for a day (approximately 24 hours), a mouse was placed in the home cage where the food was buried under fresh bedding. The latency to find the food was measured.

Odor Discrimination Learning Test. The odor discrimination learning test was performed as previously described (23,24) with minor modifications. While mice were restricted on food, sugar (Pastorale) and an odor (Sigma-Aldrich) were associated for 4 days. From the fifth day, the mice were tested to determine whether they preferred the sugar-associated odor and whether the odor was associated with the sugar based on their digging behavior.

Odor Habituation and Dishabituation Test. After being habituated in the test cage, water and 2 different social odors were presented 3 times (for 2 minutes with 1-minute intervals) sequentially. The sniffing behavior was scored and analyzed, as previously described (25).

Immunohistochemistry and Image Analysis

Brains were coronally sectioned at −21 °C, and then the sections were stained for Ki67, BrdU (bromodeoxyuridine), and GAD67 (glutamic acid decarboxylase 67). For the BrdU incorporation assay, the BrdU was intraperitoneally injected into 9-week-old mice, and the brains were isolated 8 days after the injection (26,27). The brain slices of the OB, anterior subventricular zone (SVZ), and posterior SVZ were imaged on a Zeiss LSM800 confocal microscope (Carl Zeiss Meditec). Images were analyzed with ImageJ (National Institutes of Health). For additional details, see the Supplement.

Electrophysiology

Transverse acute slices of the OB were prepared for the patch clamp experiment. We selected mitral cells based on their morphology and location in the OB, and then we confirmed the cell type based on the presence of spontaneous firing, which is an electrophysiological characteristic of mitral cells. Spontaneous excitatory postsynaptic currents and spontaneous inhibitory postsynaptic currents were recorded. For additional details, see the Supplement.

Statistical Analysis

Data are presented as mean ± SEM unless otherwise noted. Student's *t* test (or Wilcoxon signed-rank test) and 2-way mixed analysis of variance with post hoc tests were performed according to their statistical assumptions. We applied *k*-means clustering to confirm that *Negr1*$^{-/-}$ and wild-type (WT) mice were distinctively categorized according to behavioral features. For additional details, see the Supplement.

RESULTS

Negr1$^{-/-}$ Mice Are Impaired in Affective Discrimination for Stress and Pain

We employed the ADT to examine the capability of *Negr1*$^{-/-}$ mice to discriminate the affective states of conspecifics (Figure 1A, E). Before the test, we measured locomotion and spatial bias during the habituation phase to take confounding effects on affective discrimination into account. Neither WT nor *Negr1*$^{-/-}$ mice showed spatial bias, but *Negr1*$^{-/-}$ mice exhibited decreased locomotion in the experimental cage (Figure 1B, F). After habituation, we simultaneously presented mice exposed to restraint stress and affectively neutral mice

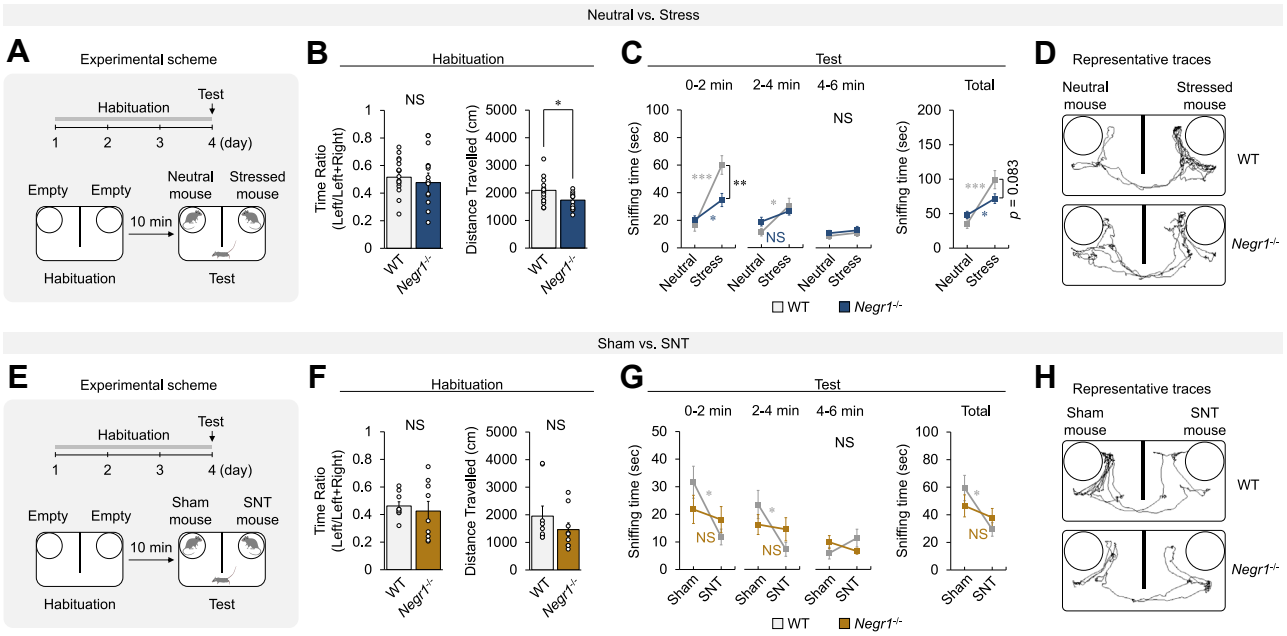
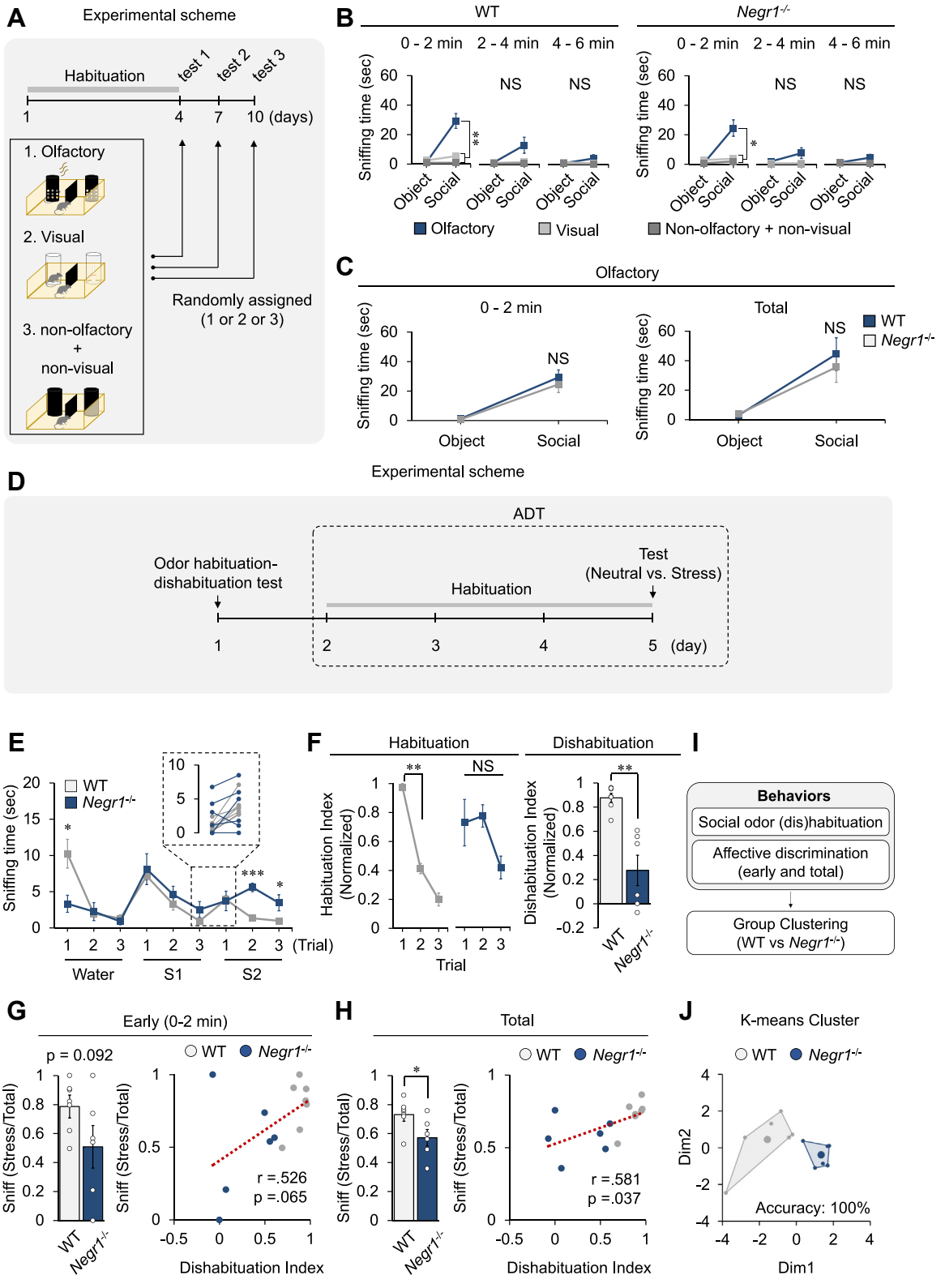


Figure 1. *Negr1*$^{-/-}$ mice demonstrate impaired affective discrimination for stress and pain. **(A, E)** Schematic designs of emotional discrimination test for stressful/neutral (WT, $n = 16$; *Negr1*$^{-/-}$, $n = 15$) and painful/neutral (WT, $n = 17$; *Negr1*$^{-/-}$, $n = 17$) demonstrators. **(B, F)** Left: time ratio between the right and left social areas (1-sample t test, $\mu = 0.5$, stress/neutral: WT, $p = .390$; *Negr1*$^{-/-}$, $p = .812$; painful/neutral: WT, $p = .882$; *Negr1*$^{-/-}$, $p = .839$). Right: total distance traveled in the test chamber (stress/neutral, Student's t test, $^{*}p < .05$; painful/neutral, Wilcoxon signed-rank test, $p = .357$). **(C)** Left: the 6 minutes of the test with stress/neutral demonstrators is divided into 3 sessions (each of 2 minutes), the first 2 minutes (left; 2-way mixed ANOVA, interaction $F_{1,29} = 4.559$, $^{*}p < .05$; Bonferroni post hoc test, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$), the second 2 minutes (middle; 2-way mixed ANOVA, interaction $F_{1,29} = 2.423$, $p = .130$; Bonferroni post hoc test, $^{**}p < .01$), and the last 2 minutes (right; 2-way mixed ANOVA, interaction $F_{1,29} = 0.003$, $p = .960$). Right, the total 6 minutes (2-way mixed ANOVA, interaction $F_{1,29} = 2.768$, $^{*}p < .05$; Bonferroni post hoc test, $^{*}p < .05$, $^{***}p < .001$). The within-group differences are indicated by being color matched. **(G)** Left: the 6 minutes of test with painful/neutral demonstrators is divided into 3 sessions (each of 2 minutes), the first 2 minutes (left; 2-way mixed ANOVA, interaction $F_{1,32} = 4.032$, $p = .053$; Bonferroni post hoc test, $^{*}p < .05$), the second 2 minutes (middle; two-way mixed ANOVA, interaction $F_{1,32} = 2.321$, $p = .137$; Bonferroni post hoc test, $^{*}p < .05$), and the last 2 minutes (right; two-way mixed ANOVA, interaction $F_{1,32} = 0.190$, $p = .666$). Right: the total of 6 minutes (2-way mixed ANOVA, interaction $F_{1,32} = 4.560$, $^{*}p < .05$; Bonferroni post hoc test, $^{**}p < .01$). **(D, H)** The moving traces during the early phase (0–2 minutes) of the tests with both stress/neutral (**D**) and painful/neutral (**H**). ANOVA, analysis of variance; NS, nonsignificant; SNT, sciatic nerve transection; WT, wild-type.

(demonstrators) as social stimuli in the test cage. The WT observers investigated the stress-exposed demonstrator more than the affectively neutral demonstrator in the tests (Figure 1C). Their preferential social investigations were noticeable in the first 2 minutes and for the total duration of the test. However, the preference for the stress-exposed conspecifics was significantly attenuated in the *Negr1*$^{-/-}$ mice, indicating impaired discrimination (Figure 1C). To extend this finding to different types of negative affective states, we presented pain-evoked demonstrators to *Negr1*$^{-/-}$ mice (Figure 1E and Figure S1A). For this, we employed an SNT model, which causes mice to experience chronic neuropathic pain (21). We performed SNT and sham surgery on pain-exposed mice and neutral demonstrators, respectively (Figure S1A) and confirmed pain induction with the von Frey test before and after surgery (Figure S1B). Seven days after surgery, we introduced mice exposed to pain-evoked stress and affectively neutral demonstrators into the ADT (Figure 1E). This time, WT mice preferred neutral mice over pain-evoked demonstrators, demonstrating a significant social preference in the first 2 minutes and for the total duration of the test. However, *Negr1*$^{-/-}$ mice did not show a social preference for either neutral or pain-exposed demonstrators (Figure 1G). To exclude the putative contribution of hypoactivity of *Negr1*$^{-/-}$ mice observed during the habituation phase (Figure 1B) and in our previous findings (20), we further analyzed travel distance in all time bins on the test day (Figure S2A, C) and found no difference between the WT and *Negr1*$^{-/-}$ groups. Additionally, we normalized and evaluated sniffing time by dividing it by travel distance and found that the observed behavioral tendency was maintained (Figure S2B, D). This suggests that hypoactivity of *Negr1*$^{-/-}$ mice did not have a significant effect on affective discrimination. These data indicate that *Negr1*$^{-/-}$ mice were impaired in discriminating affective states among conspecifics.

To determine whether impaired affective discrimination of *Negr1*$^{-/-}$ mice was accompanied by alterations in any other social behaviors, we assessed sociability and social novelty of *Negr1*$^{-/-}$ mice using the 3-chamber social test (Figure S3A, B). *Negr1*$^{-/-}$ mice preferred social stimuli over nonsocial stimuli comparable to WT mice in the sociability testing phase (Figure S3C). However, they were slightly impaired in recognizing novel conspecifics in the social novelty phase, although the effect was not statistically significant (Figure S3D). Taken


A
Experimental scheme
Habituation
test 1
test 2
test 3
1
4
7
10 (days)
1. Olfactory
2. Visual
3. non-olfactory + non-visual
Randomly assigned (1 or 2 or 3)
B
WT
Negr1-/-
0 - 2 min
2 - 4 min
4 - 6 min
Sniffing time (sec)
NS
Object
Social
Olfactory
Visual
Non-olfactory + non-visual
C
Olfactory
0 - 2 min
Total
WT
Negr1-/-
D
Experimental scheme
ADT
Odor habituation-dishabituation test
Habituation
Test (Neutral vs. Stress)
1
2
3
4
5 (day)
E
Sniffing time (sec)
WT
Negr1-/-
(Trial)
Water
S1
S2
F
Habituation
Dishabituation
Habituation Index (Normalized)
Dishabituation Index (Normalized)
Trial
I
Behaviors
Social odor (dis)habituation
Affective discrimination (early and total)
Group Clustering (WT vs Negr1-/-)
G
Early (0-2 min)
p = 0.092
Sniff (Stress/Total)
r =.526
p =.065
Dishabituation Index
H
Total
r =.581
p =.037
J
K-means Cluster
Dim2
Dim1
Accuracy: 100%

together, these results demonstrate that although *Negr1*$^{-/-}$ mice showed normal sociability upon encountering social stimulation, they had difficulty discriminating different social stimuli and affective states of conspecifics.

Dysregulated Olfactory Function of *Negr1*$^{-/-}$ Mice

Affective and social recognition among rodents is mediated by sensory stimulation, such as visual detection of facial expression and olfactory detection of social chemicals (1,10,28,29). To identify which type of sensory modality is involved in the ADT, we adopted sensory-controlling acryl cups used to differentiate the effects of each sensory modality in a social test (Figure 2A) (1). While both *Negr1*$^{-/-}$ and WT mice were able to discern and investigate social stimulation in the olfactory cue-only accessible cups (Figure 2B, C), they did not discriminate conspecifics versus objects without olfactory cues (visual cue only or nonvisual and nonolfactory cues) (Figure 2B). These data indicate that both WT and *Negr1*$^{-/-}$ mice primarily used their olfactory function for social recognition (Figure 2B, C). To further characterize olfactory processing of social odors in *Negr1*$^{-/-}$ mice, we performed an odor habituation and dishabituation test by sequentially presenting social odors to mice (Figure 2D). WT mice showed odor habituation and dishabituation with repeated exposure to social odors, whereas *Negr1*$^{-/-}$ mice were impaired in habituation and dishabituation to social odors (Figure 2E, F), indicating that *Negr1*$^{-/-}$ mice had aberrant olfactory processing for social odors. Next, we conducted the ADT after the odor habituation and dishabituation test to explore whether impaired affective discrimination was associated with social odor processing (Figure 2D). In these mice, affective discrimination was positively correlated with odor dishabituation during the early stage (0–2 minutes) and for the total duration of the test (Figure 2G, H). In the correlation plot between odor dishabituation and affective discrimination (Figure 2G, H), *Negr1*$^{-/-}$ mice were plotted mostly on the bottom left, while WT mice were on the top right of the graph. This indicated that the 2 groups were distinguished by olfactory dishabituation and affective discrimination features. We confirmed this finding via *k*-means clustering analysis, an unsupervised machine learning algorithm that groups observations into *k* clusters based on input data. In this analysis, *Negr1*$^{-/-}$ mice were distinctively categorized by odor (dis)habituation and affective discrimination measures (Figure 2I, J). These data demonstrate that affective discrimination and odor (dis)habituation were not only associated with each other, but also specify that *Negr1*$^{-/-}$ was the atypical group.

To test whether olfactory detection for odors was intact in *Negr1*$^{-/-}$ mice, we assessed *Negr1*$^{-/-}$ mice using a buried food test (Figure S4A). *Negr1*$^{-/-}$ mice foraged hidden food as fast as WT mice by relying on olfaction (Figure S4B), suggesting that they detected odors comparable to WT mice. Therefore, we hypothesized that although *Negr1*$^{-/-}$ mice had an intact ability to detect odors, they did not process social odors as properly as WT mice did. Because the odor-reward association accounts for odor discrimination (30,31), we tested whether *Negr1*$^{-/-}$ mice had impaired odor-reward associations using the odor discrimination learning test (Figure 3A). We trained both *Negr1*$^{-/-}$ and WT mice for 4 days to selectively associate S-carvone with a reward, crystal sugar, while R-carvone was not associated with a reward. On the fifth day, after burying S- and R-carvone under the bedding, we tested whether mice showed preferential foraging behavior (i.e., digging) for the reward-associated odor (S-carvone) over the neutral odor (R-carvone). Consistent with the findings reported above (Figure 2E, F), we found that *Negr1*$^{-/-}$ mice did not show preferences for S-carvone, indicating that they failed to discriminate reward-associated and neutral odors (Figure 3B). However, it was unclear whether *Negr1*$^{-/-}$ mice failed to associate odors with rewards, which led to no response to the reward-associated odor, or whether they simply could not discriminate the different odors. To address this issue, we tested whether *Negr1*$^{-/-}$ mice successfully associated S-carvone with reward by presenting S-carvone only on the sixth day. Interestingly, while WT mice dug the S-carvone side preferentially, *Negr1*$^{-/-}$ mice did not dig in the bedding on the S-carvone–buried side (Figure 3B). These data indicate that *Negr1*$^{-/-}$ mice were also impaired in associating odors with rewards.

Inhibitory Inputs in the Mitral Cells of *Negr1*$^{-/-}$ Mice Increased Along With Decreased Excitability

Our findings indicate that *Negr1*$^{-/-}$ mice had impaired social odor–processing functions. Odorants activate olfactory sensory neurons, which transmit signals to glomeruli in the OB. In glomeruli, periglomerular cells process olfactory information, which is detected and transmitted to the higher brain regions

Figure 2. Olfactory processing for social odors is dysregulated in *Negr1*$^{-/-}$ mice. **(A)** Schematic design of the sensory-controlled social test (WT, $n = 8$; *Negr1*$^{-/-}$, $n = 7$). **(B)** Sniffing time during the sensory-controlled social test (each 2 minutes; upper: WT, bottom: *Negr1*$^{-/-}$). Left: the first 2 minutes (2-way RM ANOVA; WT, interaction $F_{2,14} = 27.6$, $p < .0001$; *Negr1*$^{-/-}$, interaction $F_{1.07,6.42} = 16.2$, $p = .006$; Bonferroni post hoc test, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$). Middle: the second 2 minutes (2-way RM ANOVA; WT, interaction $F_{1.03,7.19} = 3.70$, $p = .094$; *Negr1*$^{-/-}$, interaction $F_{1.04,6.42} = 2.61$, $p = .156$). Right: the last 2 minutes (2-way RM ANOVA; WT, interaction $F_{1.03,7.18} = 2.18$, $p = .183$; *Negr1*$^{-/-}$, interaction $F_{1.09,6.52} = 1.88$, $p = .218$). **(C)** Sniffing time for the olfaction-only condition for the comparison between WT and *Negr1*$^{-/-}$. Top: 0 to 2 minutes (2-way mixed ANOVA, interaction $F_{1,13} = 0.315$, $p = .584$; Bonferroni post hoc test, $^{**}p < .01$, $^{***}p < .001$). Bottom: total 6 minutes (2-way mixed ANOVA, interaction $F_{1,13} = 0.381$, $p = .548$; Bonferroni post hoc test, $^{**}p < .01$, $^{***}p < .001$). **(D)** The schematic design of the experimental schedule (WT, $n = 7$; *Negr1*$^{-/-}$, $n = 6$). **(E)** Raw sniffing time during the odor habituation-dishabituation test (2-way mixed ANOVA, interaction $F_{8,88} = 5.318$, $p < .0001$, Bonferroni post hoc test, $^{*}p < .05$, $^{***}p < .001$). **(F)** Left: olfactory habituation index for social odors (2-way mixed ANOVA, interaction $F_{2,22} = 8.603$, $p = .008$; Bonferroni post hoc test, $^{***}p < .001$). Right: olfactory dishabituation index for social odors (Welch's *t* test, $^{**}p = .004$). **(G, H)** The ADT in the early stage and total duration. Left: sniffing ratio for the stress demonstrator (Student's *t* test, early, $p = .092$; total, $^{*}p = .034$). Right: scatter plot with olfactory dishabituation index on the x-axis and sniffing ratio on the y-axis (Pearson correlation, early vs. sniff, $r = 0.526$, $p = .065$; total vs. sniff, $r = 0.512$, $p = .037$). **(I)** The input behavioral measures used for *k*-means clustering. **(J)** The scatter plot representing clusters on the x-y plane of the reduced dimensionality by the principal component analysis (Dim1 = 54.3%, Dim2 = 22.3%). Enlarged plots indicate the computed means of each group. ADT, affective state discrimination test; Dim, dimension; NS, nonsignificant; RM ANOVA, repeated-measures analysis of variance; WT, wild-type.

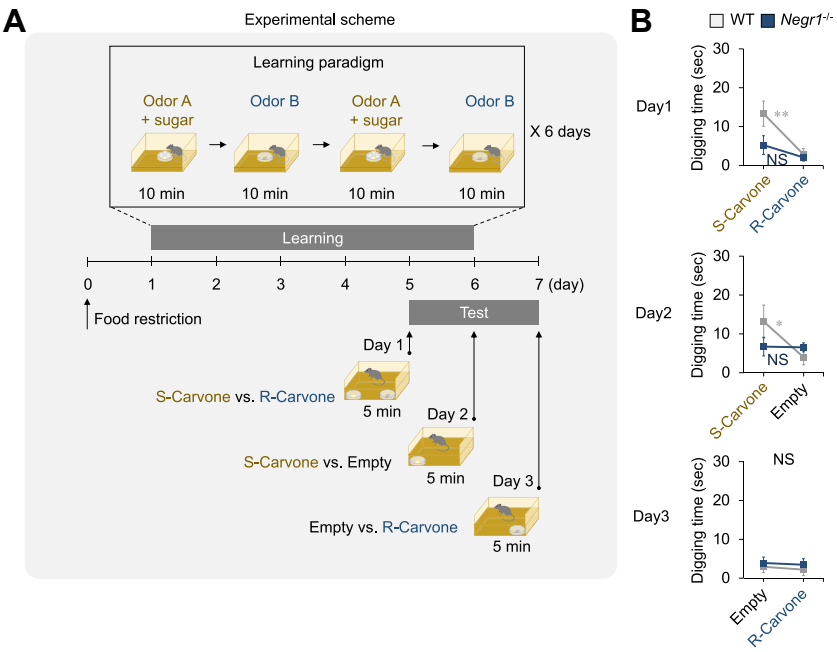


Figure 3. *Negr1*$^{-/-}$ mice fail to associate a novel odor with a reward. **(A)** The schematic design of the odor discrimination learning test (WT, $n = 8$; *Negr1*$^{-/-}$, $n = 8$). **(B)** Digging time for the area under which odors are buried. Top: the first-day test for odor discrimination (2-way mixed ANOVA, interaction $F_{1,14} = 5.740$, $p = .031$; Bonferroni post hoc test, $^{**}p < .01$). Middle: the second-day test for reward-odor association (2-way mixed ANOVA, interaction $F_{1,14} = 7.309$, $p = .017$; Bonferroni post hoc test, $^{*}p < .05$). Bottom, the last-day test with the neutral odor (2-way mixed ANOVA, interaction $F_{1,14} = 0.043$, $p = .839$). The within-group differences are indicated by being color matched. ANOVA, analysis of variance; NS, nonsignificant; WT, wild-type.

by mitral cells as the principal neurons of the OB. Odor information, such as value and identity, is represented by mitral cell activities (32–35), which are also involved in social recognition (36) and social learning (37). Accordingly, to explore functional alterations in mitral cells of *Negr1*$^{-/-}$ mice, we characterized the synaptic activity of mitral cells by whole-cell recording in acute OB slices and recorded spontaneous excitatory and inhibitory postsynaptic currents as well as excitability (Figure 4A). Although we did not observe alterations in spontaneous excitatory postsynaptic currents (Figure 4B), spontaneous inhibitory postsynaptic currents were more frequently transmitted to mitral cells in *Negr1*$^{-/-}$ mice (Figure 4C), suggesting an altered balance of excitatory and inhibitory synaptic transmission (E/I balance) in mitral cells of *Negr1*$^{-/-}$ mice. In addition, the excitability of mitral cells was significantly decreased in *Negr1*$^{-/-}$ mice (Figure 4D). These data indicate that the reduced E/I balance of mitral cells in *Negr1*$^{-/-}$ mice may lead to reduced mitral cell firing.

Adult SVZ Neurogenesis and Subsequent Newly Generated Inhibitory Cell Integration in the OB Were Reduced in *Negr1*$^{-/-}$ Mice

To characterize the relationship between NEGR1 and mitral cell excitability, we focused on our previous finding that NEGR1 plays a critical role in adult neurogenesis (20). Given that adult neurogenesis in the hippocampus and the OB share several molecular mechanisms (38), we suspected that *Negr1* would also be involved in modulating olfactory adult neurogenesis. In addition, considering that adult olfactory neurogenesis has been shown to affect olfactory discrimination (39), reward association (31), and social recognition (40), we examined whether impaired olfactory neurogenesis in *Negr1*$^{-/-}$ mice underlies the neural mechanism of olfactory dysfunction and impaired affective discrimination. We measured neural progenitor cells in the SVZ, where adult olfactory neurogenesis begins, and in the OB, the final destination of olfactory progenitor cells (Figure 5A). The number of neural progenitor cells immunopositive for Ki67, a marker for progenitor cells, was significantly reduced in the posterior part of the SVZ, and we found a decreased tendency in the anterior part of the SVZ and in the OB of *Negr1*$^{-/-}$ mice compared with WT mice (Figure 5B), indicating that adult olfactory neurogenesis of *Negr1*$^{-/-}$ mice was impaired in a particular region of the SVZ.

Olfactory neurogenesis is spatially patterned between the SVZ and the OB (31,32). Thus, we performed the BrdU incorporation assay to measure the number and location of newly generated neurons in the OB 8 days after BrdU administration (Figure 5C). We chose the granule cell layer and glomerular layer as regions of interest because newly generated olfactory neurons are integrated in these layers (41,42) (Figure 5D, E and Figure S5A). Compared with WT mice, the number of newly generated inhibitory cells (BrdU^{+} and GAD67^{+}) was decreased in the glomerular layer, but not in the granule cell layer, of *Negr1*$^{-/-}$ mice (Figure 5F, G and Figure S5B). Taken together, these data indicate that adult SVZ neurogenesis and subsequent integration of newly generated neurons into the glomerular layer are reduced in *Negr1*$^{-/-}$ mice, which may contribute to aberrant olfactory processing and impaired affective discrimination.

DISCUSSION

While the deficit in affective recognition is one of the key characteristics of people with autism (43), little is known about how mouse models of autism respond to affective states of conspecifics. Here, we revealed that *Negr1* deficiency led to impairment in affective discrimination along with a deficit in

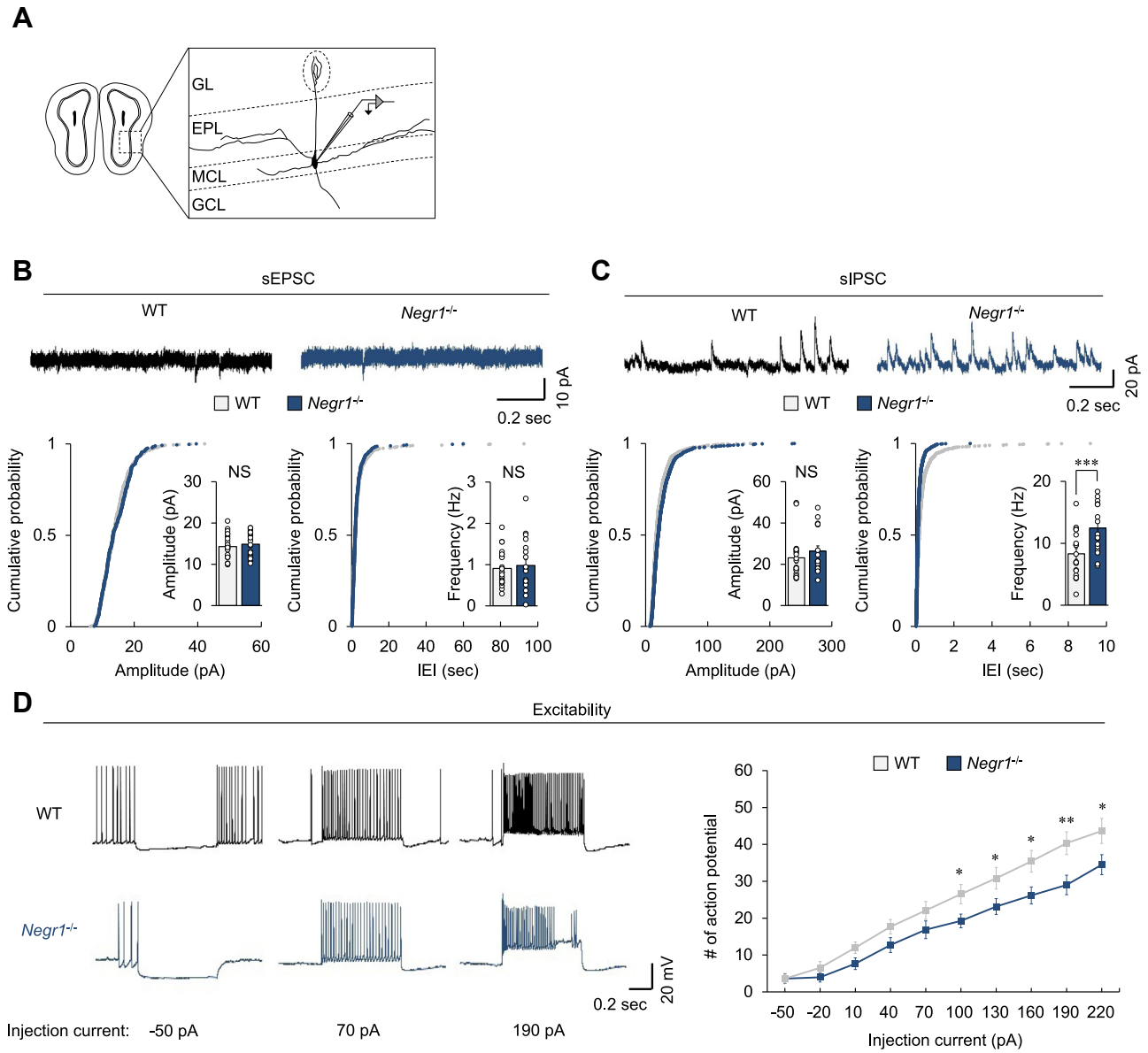


Figure 4. Inhibitory inputs are increased in the mitral cells of *Negr1*$^{-/-}$ mice with a decrease in excitability. **(A)** The schematic design of the whole-cell recording experiment. **(B)** The amplitude and frequency of sEPSCs (24 cells from 4 WT mice and 20 cells from 3 *Negr1*$^{-/-}$ mice; Student's *t* test, $p = .508$ for amplitude and $p = .647$ for frequency). **(C)** The amplitude and frequency of sIPSCs (22 cells from 4 WT mice and 20 cells from 3 *Negr1*$^{-/-}$ mice; Student's *t* test, $p = .452$ for amplitude and $p = .00088$ for frequency). **(D)** Representative voltage traces and quantified graphs of excitability (22 cells from 4 WT mice and 20 cells from 3 *Negr1*$^{-/-}$ mice; 2-way mixed analysis of variance, $F_{9,288} = 2.526$, $p = .008$, Bonferroni post hoc test, $^{*}p < .05$). EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; IEI, interevent interval; MCL, mitral cell layer; NS, nonsignificant; sEPSC, spontaneous excitatory postsynaptic current; sIPSC, spontaneous inhibitory postsynaptic current; WT, wild-type.

olfactory processing in a mouse model. The comanifestation of these deficits in *Negr1*$^{-/-}$ mice allowed us to test whether neural alterations in olfactory brain regions play a role in the impaired affective recognition. We demonstrated that the excitability of olfactory mitral cells was decreased in *Negr1*$^{-/-}$ mice, with increased inhibitory synaptic transmissions. As a potential underlying mechanism, we found that adult olfactory neurogenesis was downregulated in *Negr1*$^{-/-}$ mice, indicating that defective neuronal integration into the OB was associated with impaired olfactory processing and may contribute to reduced affective discrimination in *Negr1*$^{-/-}$ mice.

We demonstrated that *Negr1* deficiency impaired affective discrimination, resulting in unbiased social investigation regardless of the affective states of conspecifics. This suggests that *Negr1* deficiency can disrupt the ability to decide whether to approach or avoid affectively evoked conspecifics. In previous studies (10,11), normal mice either approached or avoided their conspecifics depending on the affective states of

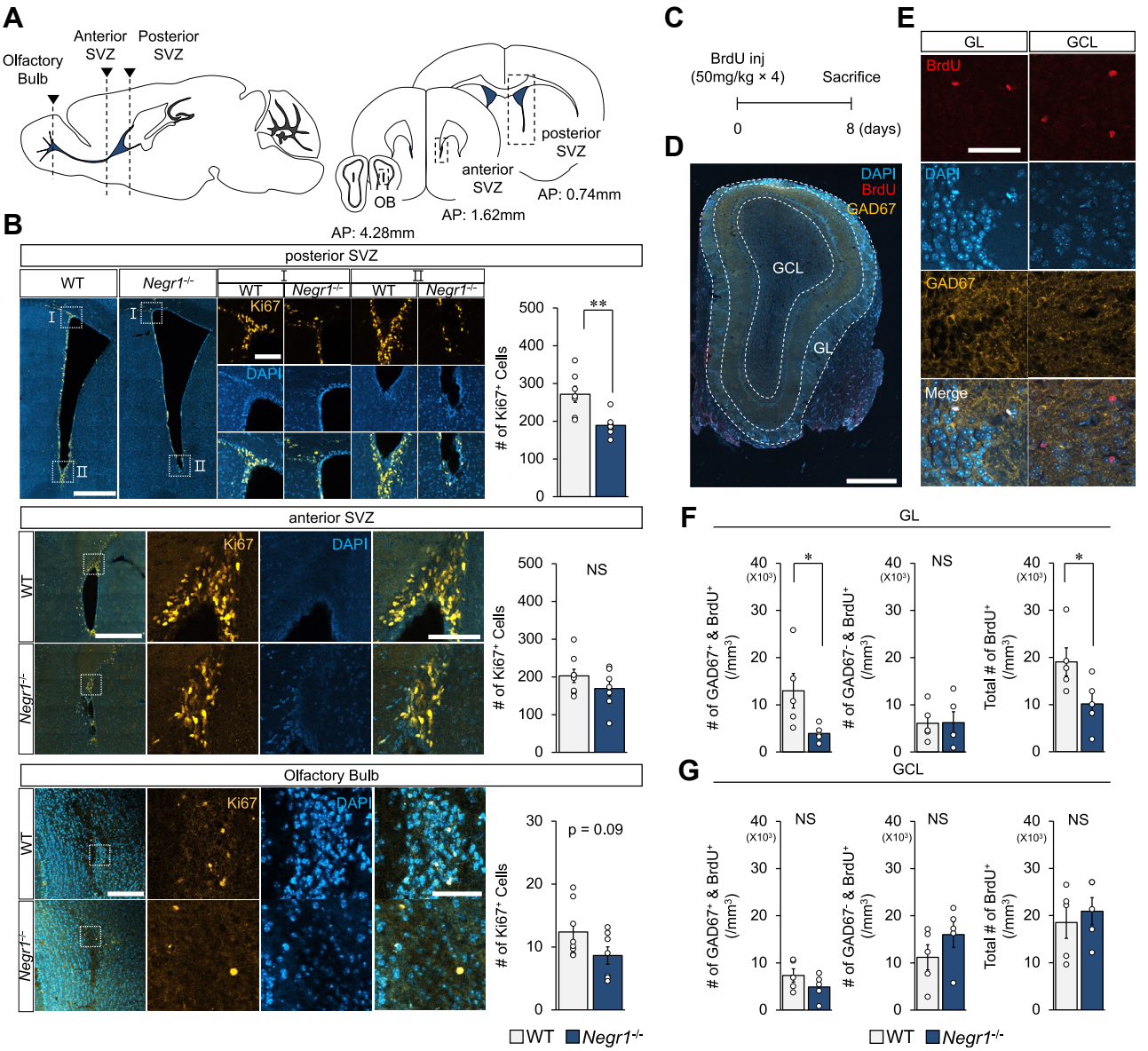


Figure 5. The number of neural progenitor cells in the SVZ and newly generated olfactory neurons in the GL are reduced in *Negr1*$^{-/-}$ mice. **(A)** Along the rostral migratory pathway, anterior SVZ, posterior SVZ, and OB were chosen (WT, $n = 8$; *Negr1*$^{-/-}$, $n = 7$). Dashed lines on the brain sagittal image indicate collected sections, and dashed line squares show regions of interest. **(B)** Immunostaining showing Ki67 (orange) in the posterior SVZ (top; scale bar = 500 μm, 100 μm), anterior SVZ (middle; scale bar = 500 μm, 100 μm), and OB (bottom; scale bar = 200 μm, 50 μm) and the number of Ki67^{+} cells in the posterior SVZ (top; Student's *t* test, $^{**}p = .005$), anterior SVZ (middle; Student's *t* test, $p = .227$) and OB (bottom; Student's *t* test, $p = .915$). **(C)** The schematic design of the BrdU incorporation assay. **(D)** The whole image of the OB delineating GCL and GL. Scale bar = 500 μm. **(E)** Representative immunohistochemistry images showing BrdU (red), DAPI (blue), and GAD67 (orange). Scale bars = 50 μm. **(F, G)** The number of GAD67^{+} and/or BrdU^{+} cells in the GL **(F)** and GCL **(G)**. In panel **(F)** (GL; WT, $n = 5$; *Negr1*$^{-/-}$, $n = 5$), GAD67 and BrdU double-positive cells (left; Student's *t* test, $^{*}p = .421$), GAD67-negative but BrdU-positive cells (middle; Student's *t* test, $p = .963$), and total number of BrdU-positive cells (right; Student's *t* test, $^{*}p = .048$). In panel **(G)** (GCL; WT, $n = 5$; *Negr1*$^{-/-}$, $n = 5$), GAD67 and BrdU double-positive cells (left; Student's *t* test, $p = .222$), GAD67-negative but BrdU-positive cells (middle; Student's *t* test, $p = .243$), and total number of BrdU-positive cells (right; Student's *t* test, $p = .605$). AP, anteroposterior; BrdU, bromodeoxyuridine; GCL, granule cell layer; GL, glomerular layer; OB, olfactory bulb; SCZ, subventricular zone; WT, wild-type.

their conspecifics, such as stress (10,11) or pain (44,45). This approach-avoidance discrepancy could be explained by whether mice were able to process olfactory cues emitted by conspecifics (10,45,46) that would typically affect their level of vigilance (47). In addition, according to the theory of social motivation, people with autism do not have interest in others due to atypical processing of social stimuli that is related to the generation of motivation in the brain circuit (48). Consistent with this, our data indicate that NEGR1 is involved in processing affective information, which may in turn disrupt social information transmission to the motivation-related upstream pathway, such as the basal nucleus of the stria terminalis,

cortical amygdala, and medial preoptic area, known to be involved in social preference and consummatory social behaviors (49–52). Thus, our findings suggest that NEGR1-deficient mice aberrantly process social odors, which may prevent them from receiving intact social chemical signals in motivation-related brain areas. Therefore, it is conceivable that *Negr1*$^{-/-}$ mice are not motivated to interact with or avoid affective conspecifics due to the failure to evoke social motivation by impaired olfactory information processing of affective states of their conspecifics.

In this study, we demonstrated that *Negr1*$^{-/-}$ mice were distinct from WT mice in olfactory processing and affective discrimination, and we distinguished those groups using a machine learning method. Thus, *Negr1*-deficient mice are a useful autism-relevant mouse model to study the association between olfactory processing and affective recognition. Olfactory function and social-affect interaction are associated in people with autism (7–9). For example, children with autism responded differently to (un)pleasant odors relative to typically developed children, and this atypicality was associated with the social affect score of the Autism Diagnostic Observation Schedule (7). Likewise, children with autism showed an abnormal autonomic response to fear-related body odor, which was also correlated with Autism Diagnostic Observation Schedule scores (9). This indicates that autistic symptoms are linked to olfactory function. Therefore, we suggest that *Negr1*-deficient mice are an appropriate animal model to dissect the underlying mechanism of autism involving defects in olfactory sensory processing and affective recognition.

As for cellular mechanisms, we detected increased inhibitory synaptic inputs into and diminished excitability of olfactory mitral cells in *Negr1*$^{-/-}$ mice. The increase in inhibitory inputs to the mitral cells can reduce their excitability, which in turn impairs olfactory processing in *Negr1*$^{-/-}$ mice. As the main output signaling for olfactory information, mitral cell temporal activity patterns are required to identify and discriminate odors accurately (53–55). In this regard, inhibitory inputs to the mitral cell population may play roles in odor identification by tuning output properties (54,56). Specifically, a subset of mitral cells, which fire spontaneously in the absence of odor stimulation, is inhibited when an odor is presented (57), and such temporal inhibition is critical for olfactory discrimination because it enhances signaling contrasts in response to odor stimuli (35). Considering that mitral cell firing was suppressed at the basal level in *Negr1*$^{-/-}$ mice, olfactory signaling contrasts before and after odor presentation might have been impaired, thereby resulting in the dysfunction of odor recognition.

Together with functional alteration in mitral cells, we showed that *Negr1* deficiency decreased neurogenesis in the adult OB. The decreased olfactory neurogenesis in *Negr1*$^{-/-}$ mice explains altered inhibitory inputs to the mitral cell population and the impaired olfactory processing of *Negr1*$^{-/-}$ mice. It has been reported that adult olfactory neurogenesis influences olfactory functions, such as odor discrimination and identification (31,38,58,59). Consistent with our findings, inhibiting adult-born olfactory neurons suppressed mitral cell activation and impaired odor discrimination (39). The distinctive characteristic of adult-born interneurons is their increased excitability with promiscuous connectivity within the olfactory microcircuit (60), and this heightened basal excitability is maintained even after they are fully matured (61). Considering that olfactory interneurons inhibit other inhibitory neurons (i.e., lateral inhibition), lowered activation of adult-born interneurons can transmit fewer inhibitory outputs to preexisting inhibitory neurons, which in turn increases the level of inhibitory inputs to other cell types, such as mitral cells. As a result, the basal and odor-evoked mitral cell activities were unable to show clear contrasts in electrical signals in response to olfactory stimuli (39). Based on these results taken together, we argue that the impairment of olfactory information processing observed in *Negr1*$^{-/-}$ mice is at least partly due to the decrease in adult olfactory neurogenesis, with inefficient signal transmission in the OB.

In this study, we did not identify the mechanisms through which NEGR1 deficiency affects olfactory neurogenesis. In a previous study, we demonstrated that NEGR1 modulated adult hippocampal neurogenesis by interacting with LIF (leukemia inhibitory factor) receptor and inducing Lcn2 expression (20). In contrast, however, the LIF receptor in the SVZ has been reported to downregulate neurogenesis in the presence of LIF (62). Thus, it is unlikely that NEGR1 contributes to olfactory neurogenesis by interacting with the LIF receptor. Instead, NEGR1 forms a heterodimer with FGFR2 (fibroblast growth factor receptor 2), which supports the downstream signaling of FGFR2 (19). FGFR2 is known to be expressed and promotes adult neurogenesis within the SVZ (63,64). Therefore, it is conceivable that NEGR1 plays a role in adult olfactory neurogenesis by interacting with FGFR2, a relationship that needs to be tested in future studies.

Additionally, although we found that adult neurogenesis was reduced in the glomerular layer in *Negr1*$^{-/-}$ mice, we did not determine which type of adult-born periglomerular neurons were involved in olfactory processing for social chemosensory cues and affective discrimination. Adult-born periglomerular neurons are mainly immunopositive for tyrosine hydroxylase (i.e., dopaminergic) or calretinin (65), both of which are GAD67 immunopositive (66) and have GABAergic (gamma-aminobutyric acidergic) synapses on mitral cells (67). Thus, the subtypes of adult-born periglomerular neurons that are regulated by *Negr1* need to be established to further investigate how *Negr1* deficiency influences E/I balance in the OB. In addition, a previous report (68) showed that NEGR1 was required to cluster GAD65 to form inhibitory synapses. Thus, our data do not exclude the possibility that NEGR1 may affect E/I balance by modulating inhibitory inputs directly at synapses, which needs to be tested in future research.

Conclusions

In summary, we showed that *Negr1* deficiency, a risk gene for autism spectrum disorder, led to deficits in social-affective discrimination and abnormal olfactory processing with reduced OB neurogenesis. Our data reveal an unexpected link between affective recognition and olfactory processing mediated by NEGR1, and we propose a novel neurobiological mechanism of autism-related behaviors based on disrupted adult olfactory neurogenesis and processing.

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ARTICLE INFORMATION

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