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Elimination of Adult-Born Neurons in the Olfactory Bulb Is Promoted during the Postprandial Period

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SUMMARY

Granule cells (GCs) in the mouse olfactory bulb (OB) continue to be generated in adulthood, with nearly half incorporated and the remainder eliminated. Here, we show that elimination of adult-born GCs is promoted during a short time window in the postprandial period. Under restricted feeding, the number of apoptotic GCs specifically increased within a few hours after the start of feeding. This enhanced GC apoptosis occurred in association with postprandial behaviors that included grooming, resting, and sleeping, and was particularly correlated with the length of postprandial sleep. Further, deprivation of olfactory sensory experience in the local OB area potentiated the extent of GC elimination in that area during the postprandial period. Sensory experience-dependent enhancement of GC elimination also occurred during postprandial period under natural feeding condition. These results suggest that extensive structural reorganization of bulbar circuitry occurs during the postprandial period, reflecting sensory experience during preceding waking period.

INTRODUCTION

In mice, granule cells (GCs) in the olfactory bulb (OB) are generated and incorporated into the neuronal circuitry from the embryonic stage right through into adulthood (Lledo et al., 2006; Lois and Alvarez-Buylla, 1994; Luskin, 1993). Among adult-born GCs, approximately half are incorporated into the preexisting neuronal circuitry while the remainder are eliminated (Petreanu and Alvarez-Buylla, 2002; Rochefort et al., 2002; Yamaguchi and Mori, 2005). Adult neurogenesis in the OB therefore resembles embryonic development in that excess neurons are first prepared and then selected to ensure adequate fine tuning of the neuronal circuitry. Pruning of excess cells and synapses is considered crucial to maintaining the number of cells and synapses within an appropriate range, and to adding new functions to the circuitry without disrupting those already present (Buss et al., 2006).

The survival rate of adult-born GCs is regulated by olfactory sensory experience (Petreanu and Alvarez-Buylla, 2002; Rochefort et al., 2002). This in turn suggests that their selection underlies the experience-dependent reorganization of OB circuitry. Selection occurs during a critical period, with survival and death strongly influenced by sensory experience from days 14 to 28 after cell generation (Yamaguchi and Mori, 2005). This time window corresponds to the period when adult-born GCs make synaptic contact with preexisting neurons (Carleton et al., 2003; Kelsch et al., 2008; Petreanu and Alvarez-Buylla, 2002; Whitman and Greer, 2007), suggesting that synaptic input plays a crucial role in the selection of adult-born GCs.

The synaptic plasticity underlying learning and memory is crucially regulated by the wake-sleep cycle. Sensory experience-induced neuronal activity occurs during waking states, while neuronal activity during subsequent sleep is thought to facilitate the consolidation of sensory experience memories and promote the concomitant reorganization of neuronal circuits (Buzsáki, 1989; Diekelmann and Born, 2010).

Given this background, we asked whether the selection of adult-born GCs occurs continuously throughout the day, or in association with specific behavioral states. By combining behavioral analysis with immunohistochemical detection of apoptotic GCs, we found that extensive elimination of adult-born GCs occurs during the postprandial period. In addition, the extent of GC apoptosis during the postprandial period was regulated by olfactory sensory experience. From these observations we propose a two-stage model for the selection of adult-born GCs which states that sensory input during waking and active signals during the subsequent postbehavioral period may work together to direct the sensory experience-dependent elimination or incorporation of adult-born GCs.

RESULTS

Apoptotic Elimination of GCs in the OB Occurs Preferentially during the Feeding and Postprandial Period under Restricted Feeding

We first investigated whether the elimination of adult-born GCs occurs during specific daily time windows in mice housed under

conventional conditions with ad libitum feeding. The number of apoptotic GCs in mice at various circadian times was examined by immunohistochemical detection of activated caspase-3-expressing GCs (Yamaguchi and Mori, 2005; Yuan et al., 2003; Figure 1D). While results showed no statistically significant difference in the average number of caspase-3-activated GCs at different time points, the wide variation in number seen across animals indicated that the control of GC elimination may involve mechanisms other than circadian rhythm.

The initial clue indicating a time window for enhanced GC elimination, namely the postprandial period, came from food restriction experiments. We hypothesized that the apoptotic process of GCs might be associated with olfactory behavior and examined whether GC elimination is associated with feeding, a typical olfaction-dependent behavior (Doty, 1986). Since feeding behavior varies under ad libitum feeding, we controlled behavior using a restricted feeding paradigm (Gooley et al., 2006; Mistlberger, 1994; Figure 1A) in which access to food pellets was limited to 4 hr per day (11:00 to 15:00). After habituation for 9 days, mice were mostly devoted to eating during the initial hour (11:00–12:00) after supply but showed various postprandial behaviors during the following hour (12:00–13:00), such as grooming, resting, and sleeping (see Figure 3A). Mice were sampled at various times (Figure 1A; day 10) and the number of caspase-3-activated GCs was counted (Figures 1B and 1E). The number at 2 hr after the start of supply (13:00) was an average 2.4-fold higher than that before supply (11:00), and then tended to decrease at 4 hr after the start of supply (15:00). In a separate experiment, caspase-3-activated GC number showed no significant increase at 1 hr after the start of feeding (see Figure 3C). Number of caspase-3-activated GCs thus increased in the short time window between 1 and 2 hr after the start of feeding and declined by 4 hr. Outside this feeding time window, the caspase-3-activated GC number was similar to or less than that immediately before supply (Figure 1E). To examine whether an increase in caspase-3-activated GCs also occurs during feeding and postprandial period at a different circadian time, a different feeding time (21:00 to 1:00) was set in a second group of mice (Figure 1F). Results showed an increase in the number of caspase-3-activated GCs during the shifted time window of feeding and postprandial behaviors.

This increase in the number of caspase-3-activated GCs during the eating and postprandial periods suggests that the number of apoptotic GCs would be increased during this time. However, given that activation of caspase-3 is not always associated with cell death (D'Amelio et al., 2010), we also examined the TUNEL method of detecting cell death, which detects DNA fragmentation. Results showed a remarkable increase in TUNEL-positive GCs during the feeding and postprandial period, confirming the increased death of GCs during the time window (Figures 1C and 1H).

This result indicates that caspase-3-activation is an excellent indicator of GC death. In fact, most caspase-3-activated GCs were TUNEL-positive (before feeding, 85.5% ± 4.7%; 2 hr after the start of food supply, 83.5% ± 3.4%; n = 4 mice, average ± SEM) (Figure 1G). In addition, about 96% of caspase-3-activated GCs showed apoptotic chromatin deformities, such as marginalization, fragmentation, and condensation (Clarke, 1990), whereas activated caspase-3-negative GCs showed no nuclear deformity (Figure 1G and see Figure S1A available online).

The increase in the number of caspase-3-activated apoptotic GCs during the feeding and postprandial period occurred throughout the OB, from the rostral to caudal regions (Figure S1B). Similar numbers were seen in the left and right OB of the same animal (Figure S1C). These results indicate that the induction of GC apoptosis during feeding and postprandial period occurs globally in all regions of the OB.

Apoptosis of Newly Generated GCs Increases during Feeding and Postprandial Period

To determine the cellular ages of GCs that showed enhanced apoptosis during the feeding and postprandial period, we first labeled adult-born new GCs by BrdU injection. We classified the new GCs into four subsets with different cellular ages; those aged 7–13 days, 14–20 days, 21–27 days, and 28–34 days, and then examined the apoptosis in each subset (Figures 2A and 2B). Subsets of new GCs within the critical period for the survival and death decision, aged 14–20 days and 21–27 days (Yamaguchi and Mori, 2005), showed enhanced apoptosis during feeding and postprandial period (Figure 2B, green bars). Given that BrdU injection cannot label all proliferating cells (Taupin, 2007), the results indicate that new GCs aged 14–20 days constitutes at least 24.5% of caspase-3-activated GCs and GCs aged 21–27 days constitutes at least 22.6% (Figure 2C). New GCs after the critical period (days 28–34) also showed enhanced apoptosis during the time window, although their contribution to total apoptotic cell ratio was smaller (9.5%). Interestingly, new GCs before the critical period (days 7–13) showed no significant enhancement in apoptosis during the feeding and postprandial period (Figure 2B; see Discussion). Thus caspase-3-activated GCs is comprised of at least 7.8% of new GCs aged 7–13 days, 24.5% of new GCs aged 14–20 days, 22.6% of new GCs aged 21–27 days, and 9.5% of new GCs aged 28–34 days. Rough approximation by summating the percentage of each new GC subset suggests that more than 64% of caspase-3-activated GCs are new GCs aged day 7 to 34, indicating that the majority of the apoptotic GCs were adult-born new GCs. This notion was supported by the coexpression of doublecortin (DCX) in many caspase-3-activated GCs (40%–46%) (Figures 2A and S2A; Brown et al., 2003). The total number of BrdU-labeled GCs per OB did not significantly differ before and at 2 hr after the start of feeding in all periods examined (Figure S2B), indicating that the increase in apoptotic GCs during feeding and postprandial period was not due to any rapid recruitment of new GCs in the OB.

Neonate-born GCs are gradually eliminated in the adult period (Imayoshi et al., 2008). To examine whether preexisting neonate-born GCs also showed increased apoptosis during feeding and postprandial period, they were BrdU-labeled on postnatal days 4-5 and examined in adulthood (Figures 2D and S2C–S2E). Although the number was small, caspase-3-activated GCs with BrdU labeling were observed and increased by 2-fold at 2 hr after food supply. Thus, neonate-born, preexisting GCs also showed increased apoptosis during the feeding and postprandial period.

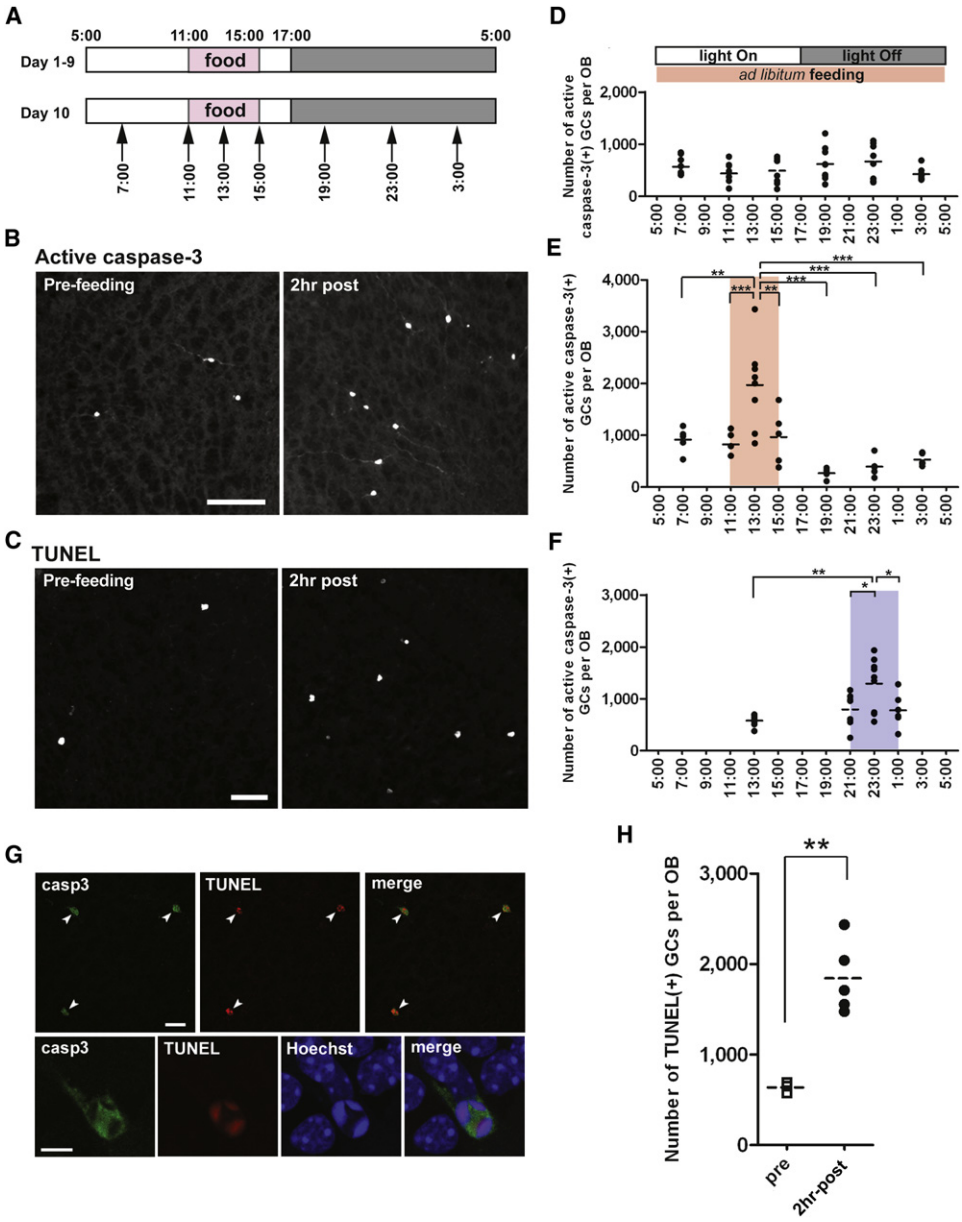

Figure 1. Apoptosis of GCs in the OB Occurs Preferentially during the Feeding and Postprandial Period

(A) Restricted feeding protocol. On days 1–10, food was supplied for only 4 hr (11:00–15:00) per day. On day 10, mice were analyzed at various circadian time points (arrows). Mice were maintained under a 12 hr light-dark cycle (light on, 5:00–17:00, white bars; light off, 17:00–5:00, gray bars).

(B) Caspase-3-activated GCs in the granule cell layer (GCL) of the OB before and 2 hr after the start of food supply. Scale: 100 μm.

(C) TUNEL-positive GCs in the GCL of the OB before and 2 hr after the start of food supply. Scale: 100 μm.

(D–F) Number of caspase-3-activated GCs under different feeding paradigms. Each dot represents the number of caspase-3-activated GCs in one animal (average of left and right OBs), and bars indicate the average number at respective time points. (D) ad libitum feeding. (E) food supply from 11:00 to 15:00. (F) food supply from 21:00 to 1:00.

(G) Codetection of caspase-3 activation and DNA fragmentation (TUNEL) in GCs. Confocal images of apoptotic GCs in low magnification (upper panels, arrowheads) and an apoptotic GC in high magnification (lower panels) 2 hr after the start of food supply. The GC in the lower panels shows a fragmented nucleus. Green, activated caspase-3; red, TUNEL signal; blue, nuclear chromatin staining by Hoechst 33342. Scale: 10 μm (upper) and 5 μm (lower).

(H) Number of TUNEL-positive GCs before and 2 hr after the start of food supply.

*p < 0.05; **p < 0.01; ***p < 0.001; one-way ANOVA (D) (p = 0.49), one-way ANOVA with post hoc Bonferroni test (E and F), and unpaired t test (H). See also Figure S1.

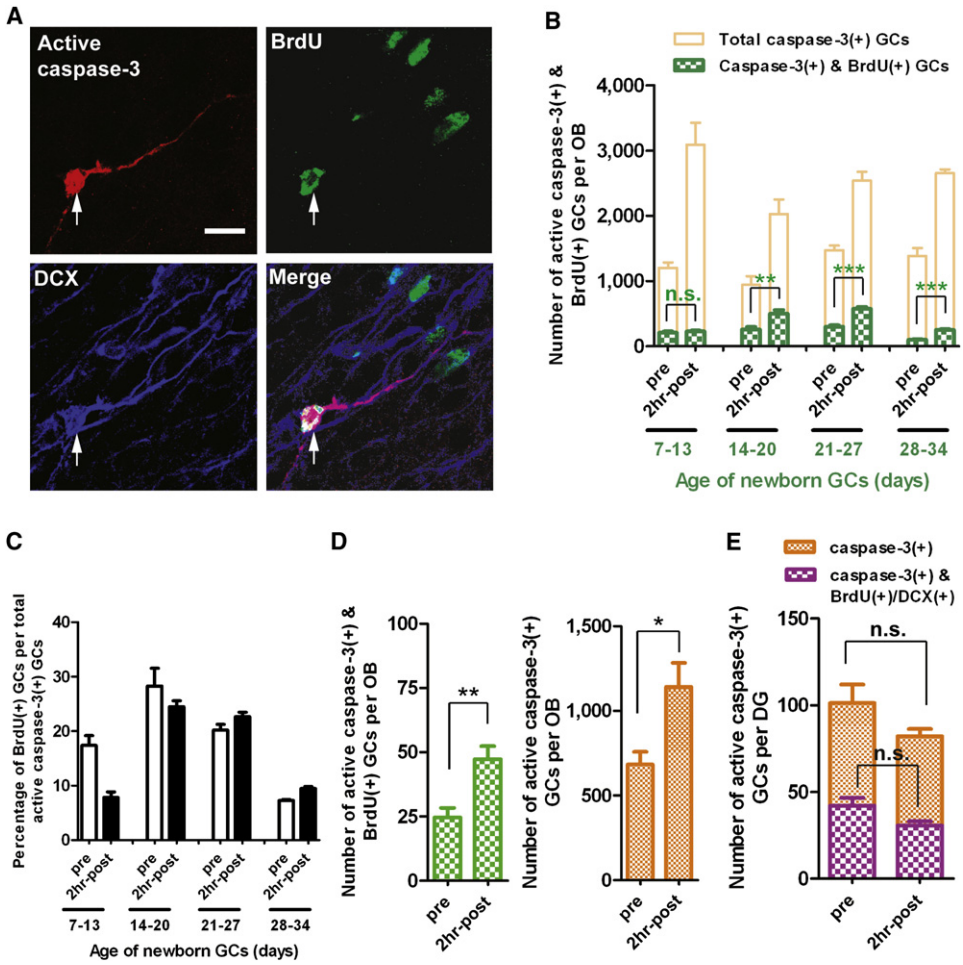

Figure 2. Cell Death of Newly Generated GCs Increases during the Feeding and Postprandial Period

(A) BrdU labeling and DCX expression in caspase-3-activated GCs (arrow). BrdU-labeled cells are at 14–20 days of age. Scale: 20 μm.

(B) Number of caspase-3-activated new GCs of various ages before and 2 hr after the start of food supply (green bars). GCs were BrdU-labeled for 7 days and analyzed at the indicated periods. Total number of caspase-3-activated GCs is also shown (orange bars), to confirm its ~2-fold increase 2 hr after food supply in every analysis ($p < 0.01$).

(C) Percentage of the number of BrdU-labeled GCs of various ages among total number of caspase-3-activated GCs.

(D) Number of caspase-3-activated neonate-born GCs before and 2 hr after the start of food supply (left graph). BrdU was injected on postnatal days 4–5 and the mice were analyzed in adulthood. Total number of caspase-3-activated GCs is also shown (right graph).

(E) Number of caspase-3-activated newly generated GCs that are positive for either BrdU or DCX in the hippocampal DG before and 2 hr after the start of food supply. BrdU-labeled cells are 14–20 days of age.

In (B)–(E), 6 or 7 mice were analyzed for individual groups. Data represent the average ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., not significant (t test). See also Figure S2.

Adult neurogenesis also occurs in GCs of the hippocampal dentate gyrus (DG) (Lledo et al., 2006; Zhao et al., 2008). To examine whether apoptosis of adult-born GCs in the DG was also enhanced during feeding and postprandial period, caspase-3-activated GCs labeled with BrdU (14–20 days of age) were examined in the DG of the same mice used for OB analysis. At 2 hr after food supply, no significant increase was seen in the number of either caspase-3-activated GCs or caspase-3-activated GCs labeled with BrdU or DCX (Figures 2E and S2F–S2H). Thus, enhanced apoptosis during feeding and postprandial period occurs in the OB but not in the hippocampal DG.

Enhanced GC Apoptosis Occurs in Association with Postprandial Behaviors

We then addressed the question of why apoptosis of adult-born GCs is enhanced during the feeding and postprandial period. Although all mice examined were confirmed to have eaten food pellets during the feeding time, some showed no apparent increase in GC apoptosis (see Figure 1E). No significant correlation was seen between the amount of food consumed and number of caspase-3-activated GCs (data not shown). We therefore speculated that the enhancement of GC apoptosis was correlated with behavior other than eating.

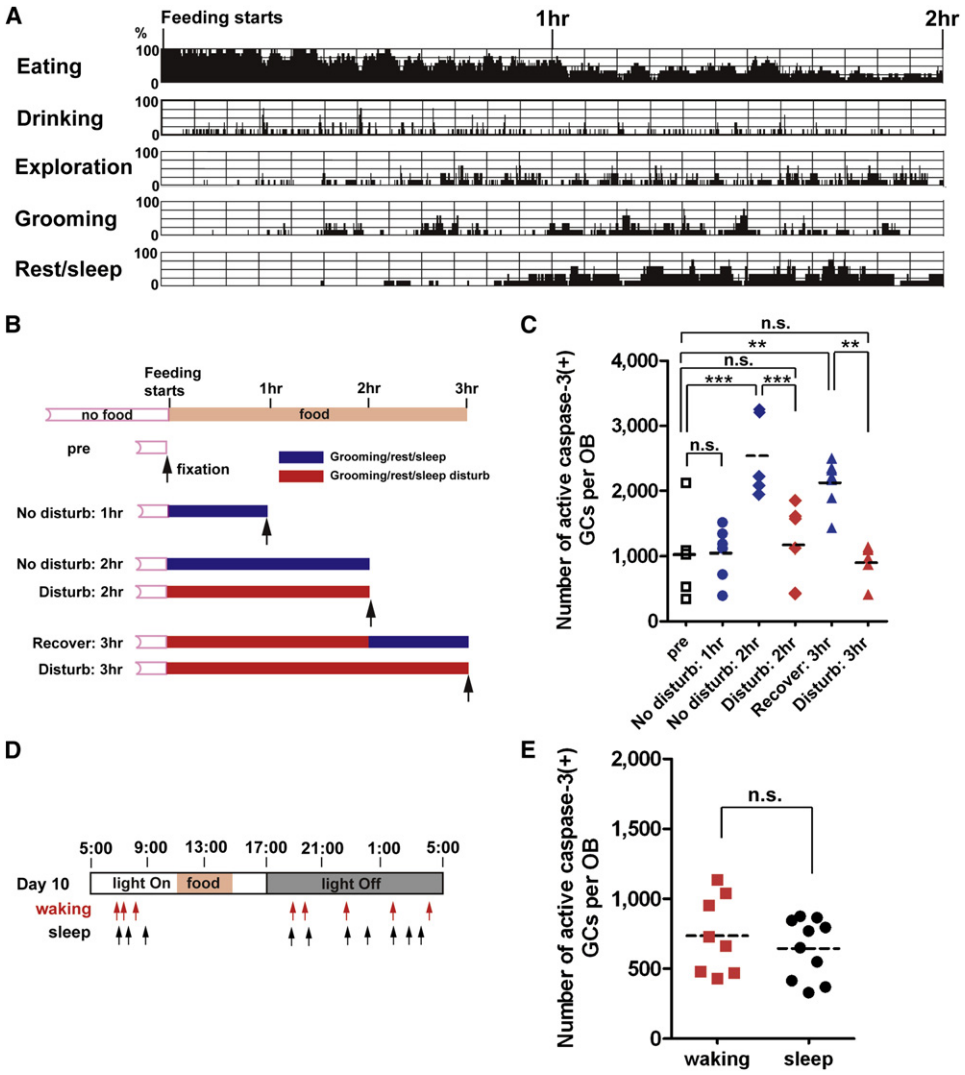

Figure 3. Enhanced GC Apoptosis Occurs in Association with Postprandial Behaviors

(A) Behaviors during the initial 2 hr after the start of food supply. Behaviors are categorized and the frequency of each behavior is shown in the histograms. Data represent the frequency averaged from 11 animals.

(B) Protocol used for the disturbance of postprandial behavior. The mice were classified into six groups and respectively allowed to behave freely (blue), and disturbed from resting, sleeping, and extended periods of grooming by gentle handling (red) during the indicated periods. Arrows indicate analysis times.

(C) Number of caspase-3-activated GCs in the six groups of mice shown in (B).

(D) Examination of GC apoptosis during the waking (red arrows) and sleeping period (black arrows) outside feeding time.

(E) Number of caspase-3-activated GCs in mice shown in (D).

In (C) and (E), each dot represents the number of caspase-3-activated GCs in one animal (average of left and right OBs). Bars indicate the average. $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not significant; one-way ANOVA with post hoc Bonferroni test (C) and unpaired t test (E). See also Figure S3 and Movie S1.

We therefore analyzed the behavior of mice during the initial 2 hr of feeding and postprandial period (Figure 3A and Movie S1). Before food presentation, mice showed extensive exploratory behavior. During the initial hour of supply, they were mostly occupied with eating and drinking, and also exhibited a small amount of exploratory and grooming behaviors. During the following hour, in contrast, various postprandial behaviors dominated over eating behavior, including grooming, resting, and sleeping.

Given the apparently distinct behaviors between the first and second hours, we examined the number of apoptotic GCs at 1 hr after the start of feeding (Figures 3B and 3C). The number did not significantly increase over this period, when the mice were mostly occupied with eating and drinking. In contrast, the number substantially increased during the following hour, when postprandial behaviors became conspicuous. To examine the contribution of postprandial behaviors to GC apoptosis, we suppressed postprandial behaviors, namely resting, sleeping, and

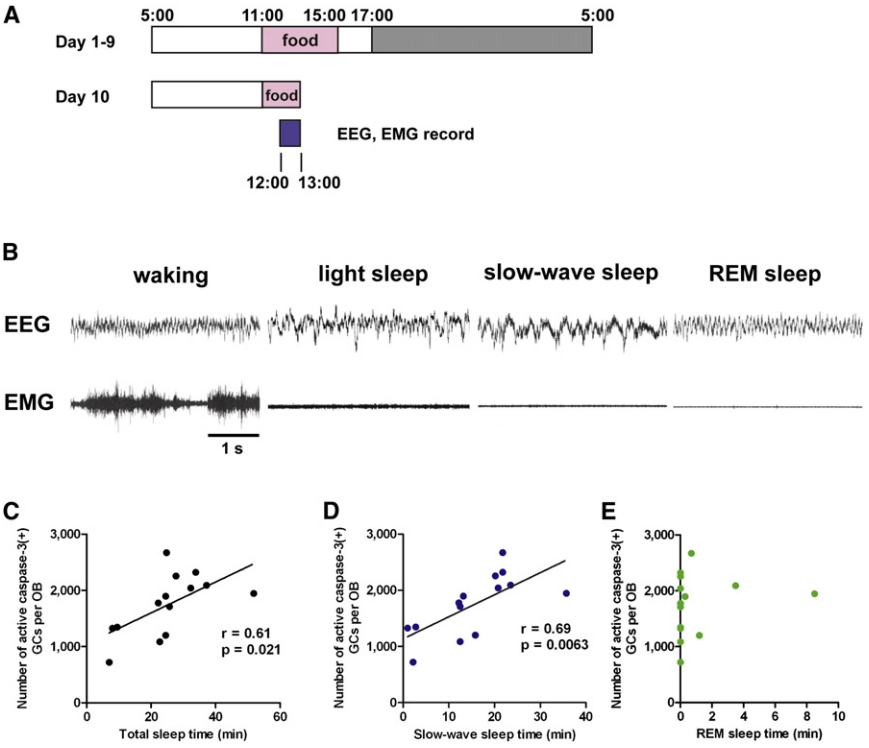

Figure 4. Length of Postprandial Sleep Correlates with the Extent of GC Elimination

(A) Protocol for evaluation of sleep behavior by EEG and EMG recording. On day 10, the EEG from the occipital cortex and EMG from the neck muscle were recorded during 1 hr of the postprandial period.

(B) Behavioral state was classified into waking, light sleep, slow-wave sleep, and REM sleep states according to EEG, EMG, and behavior.

(C–E) Correlation of the number of caspase-3-activated GCs with the length of total sleep (C), slow-wave sleep (D), and REM sleep (E). Each dot represents the data from one animal (n = 14). Regression line, Pearson's r and p value are indicated (C and D).

See also Table S1.

extended periods of grooming (longer than 5 s), by gently handling the mice during the feeding and postprandial period, without disturbing their eating, drinking and exploratory behaviors (see Supplemental Experimental Procedures; Mistlberger et al., 2003; Figures 3B and 3C). A group of control mice that were allowed to behave freely during the feeding and postprandial period showed a two-fold increase in apoptotic GCs (Figures 3B and 3C; No disturb: 2 hr). In contrast, this increase in GC apoptosis was significantly inhibited in a second group whose postprandial behaviors during the feeding and postprandial period were disrupted (Disturb: 2 hr). We confirmed that the gentle handling did not reduce the amount of food pellet consumed during the 2 hr (2.1 ± 0.2 g for control mice and 2.0 ± 0.1 g for handled mice, p = 0.22). When postprandial behaviors were disrupted for 2 hr and then allowed for the following 1 hr, GC apoptosis increased (Recover: 3 hr). This increase was in turn inhibited by continual gentle handling disruption (Disturb: 3 hr). These results indicate that enhanced GC apoptosis during feeding and postprandial period occurred in association with postprandial behaviors.

Length of Postprandial Sleep Correlates with the Extent of GC Apoptosis

Given that sleeping behavior was the most conspicuous postprandial behavior in the present analysis (Figure 3A) and that sleep plays a crucial role in brain plasticity (Buzsáki, 1989; Diekelmann and Born, 2010), we next examined the contribution of sleeping behavior during the postprandial period to GC apoptosis. Postprandial sleep was evaluated using EEG and EMG recording in freely behaving mice. After implantation of recording electrodes, mice were subjected to restricted feeding and analyzed on day 10 (Figure 4A). During the initial 1 hr of feeding, mice engaged in continuous eating without resting or sleeping. The EEG of the occipital cortex and EMG of the neck muscles were recorded during the following hour, and the mice were then perfusion fixed. Using video recordings of behavior, EEG, and EMG, the behavioral state was classified every 10 s into the waking, light sleep, slow-wave sleep, and REM sleep states (Radulovacki et al., 1984; Tsuno et al., 2008; Figure 4B). Waking-sleeping behaviors during postprandial period were fragmented into episodes of short duration. Thus the total time of each state during the 1 hr postprandial period was calculated and evaluated.

All mice examined showed various sleep states with various lengths (Table S1). The length of total sleep (sum of light, slow-wave, and REM sleep) positively correlated with apoptotic GC number (Figure 4C). By state, the length of slow-wave sleep correlated well with apoptotic GC number (Figure 4D). On the other hand, REM sleep was not necessarily observed during the postprandial period, and many mice without REM sleep showed an increase in GC apoptosis (Figure 4E). These results confirmed that most mice slept during the postprandial period and suggested that slow-wave sleep or total sleep promoted GC apoptosis. They also suggested that a brief period of sleep of 20–40 min exerted a potent effect in enhancing GC apoptosis (Figure 4C). We also confirmed in EEG- and EMG-recorded mice that the gentle handling efficiently inhibited sleep states during the postprandial period (data not shown), supporting the potent role of sleep in enhancing GC apoptosis.

The occurrence of sleep during the postprandial period is in accord with the notion that satiety induces sleep (Mieda and Yanagisawa, 2002). One question is whether sleep per se has a potent role in enhancing GC apoptosis, or whether this is due to a combination of feeding and sleep. Continuous behavioral analysis of food-restricted mice showed that they also slept outside the postprandial period (Figure S3), whereas

enhanced GC apoptosis was apparent only during the postprandial period (Figure 1). Outside the feeding time, the number of apoptotic GCs during the sleeping period was low and did not differ significantly from that during the waking period (Figures 3D and 3E). These results suggest that a sequence of feeding followed by sleep had a specific effect on the enhancement of GC apoptosis.

Sensory Deprivation Enhances GC Apoptosis Specifically during the Postprandial Period

During waking, mice receive various odor inputs from the external environment. Deprivation of olfactory sensory input greatly increases the number of apoptotic GCs (Corotto et al., 1994; Fiske and Brunjes, 2001; Petreanu and Alvarez-Buylla, 2002; Yamaguchi and Mori, 2005). To examine the influence of olfactory sensory input on GC elimination during the postprandial period, one nostril was occluded in mice prior to food restriction (Figure 5A). Sensory deprivation was confirmed by reduced expression of phosphorylated ERK in GCs (Figures S4A and S4B; Miwa and Storm, 2005). Results showed a 7.4-fold increase in the number of apoptotic GCs 2 hr after the start of food supply compared to that before supply in the sensory-deprived OB (Figures 5B and 5D), indicating that the extent of GC elimination during the feeding and postprandial period is regulated by olfactory sensory input. The number of apoptotic GCs increased 2.5-fold 2 hr after food supply in the normal side of the OB of nostril-occluded mice (Figure 5C). Importantly, the number of apoptotic GCs between the deprived and normal OBs did not differ outside the time window of the feeding and postprandial period ($p > 0.05$, t test), indicating that sensory input-dependent GC apoptosis specifically occurs during the feeding and postprandial period, and that deprivation of sensory input to the OB does not affect the time window of enhanced GC elimination.

Examination of caspase-3-activated GCs with the BrdU-labeling method and DCX-immunohistochemistry showed that more than half of caspase-3-activated GCs were either BrdU-positive (14–20 days of age) or DCX-positive newly generated GCs both before and at 2 hr after the start of food supply (52.0% ± 4.6% before feeding and 55.3% ± 3.5% at 2 hr after supply; Figures 5E and S4C). The results show also that apoptosis of newly generated GCs increased (5.3-fold) in the sensory-deprived OB during the feeding and postprandial period. Analysis of TUNEL-positive cells also confirmed the large increase in apoptotic GCs in the sensory-deprived OB during this period (Figure S4D).

To address the question of whether postprandial behaviors contribute to the enhanced GC apoptosis in the sensory-deprived OB, behaviors of nostril-occluded mice were examined. As in nostril-intact mice, extensive eating behavior during the initial hour and postprandial behaviors during the subsequent hour occurred in the nostril-occluded mice (Figure S4E). Intriguingly, apoptotic GC number in sensory-deprived OB increased as early as 1 hr after the start of food supply in many mice, without apparent resting and sleeping behavior (Figure 5F; No disturb: 1 hr; Figure S4F). However, this increase during the initial hour was suppressed when grooming behavior (longer than 5 s) was disturbed by gentle handling (Figure 5F; Disturb: 1 hr). The larger increase in apoptotic GCs at 2 hr was partially but significantly suppressed by disturbing grooming, resting and sleeping behavior during the 2 hr. Gentle handling in nostril-occluded mice did not reduce the amount of food pellet consumed (data not shown). These results indicate that enhanced GC apoptosis occurred in association with postprandial behaviors in sensory-deprived OB. Under unilateral sensory deprivation, enhanced GC apoptosis can occur in association with postprandial extended grooming even without apparent sleep. GC apoptosis in the open side of the OB of the nostril-occluded mice also showed an increase in GC apoptosis at 1 hr, and this increase was also suppressed by gentle handling (Figure 5G). The presence of olfactory sensory input to the open side of the OB and its absence to the closed side during feeding time was confirmed by examining the presence and absence of induced arc expression in GCs (Figure S4G; Guthrie et al., 2000).

Local Deprivation of Olfactory Sensory Input Causes Local Enhancement of GC Elimination during the Postprandial Period

The odor map of the OB shows domain and cluster organization (Mori et al., 2006). The survival rate of adult-born GCs is regulated in local OB areas by local activation with odor learning (Alonso et al., 2006). Does local sensory input regulate the extent of GC elimination during the postprandial period in local OB areas? To address this question, we utilized dorsal zone-depleted mice (ΔD mice), in which olfactory sensory neurons (OSNs) in the dorsal zone (D-zone) of the epithelium were selectively ablated (Kobayakawa et al., 2007). Glomerular structure was lacking in the D-domain of the ΔD mouse OB due to the depletion of OSNs targeting the D-domain (Figure S5A). Other layers were largely maintained, including the granule cell layer (GCL), the majority of cells in which were NeuN-expressing GCs (data not shown). As expected, the number of GCs expressing an immediate early gene c-fos with odor stimulation (Magavi et al., 2005) was drastically reduced in the D-domain (Figure S5B). The quantitative analyses in the paragraph below were conducted in coronal sections at the central portion in the rostrocaudal axis of ΔD and wild-type mouse OBs, which include a considerable volume of both the D-domain and ventral domain (V-domain) (Figure S5C).

ΔD mice and wild-type mice were subjected to food restriction and examined for caspase-3-activated GCs in the D- and V-domains (Figures 6A and S5D). In the ΔD mouse OB, the density of caspase-3-activated GCs in the D-domain increased 3.2-fold during the postprandial period compared to that before feeding, while that in the V-domain increased 2.2-fold (Figures 6A and 6B). The ratio of caspase-3-activated GC density in the D-domain to that in the V-domain was greater in the postprandial period (2.0 ± 0.2; average ± SEM) than before food (1.3 ± 0.1) (Figure 6D; $p = 0.009$). In wild-type mouse OB, the density of caspase-3-activated GCs increased during the postprandial period by 2.3-fold in the D-domain and 2.0-fold in the V-domain (Figures 6C and S5D). The ratio of caspase-3-activated GC density in the D-domain to that in the V-domain was 1.5 ± 0.1 before food and 1.8 ± 0.1 in the postprandial period (Figure 6D), showing no significant difference between the two time points ($p = 0.09$). These results indicate that the deprivation of sensory input in the local OB area in ΔD mice greatly enhanced GC

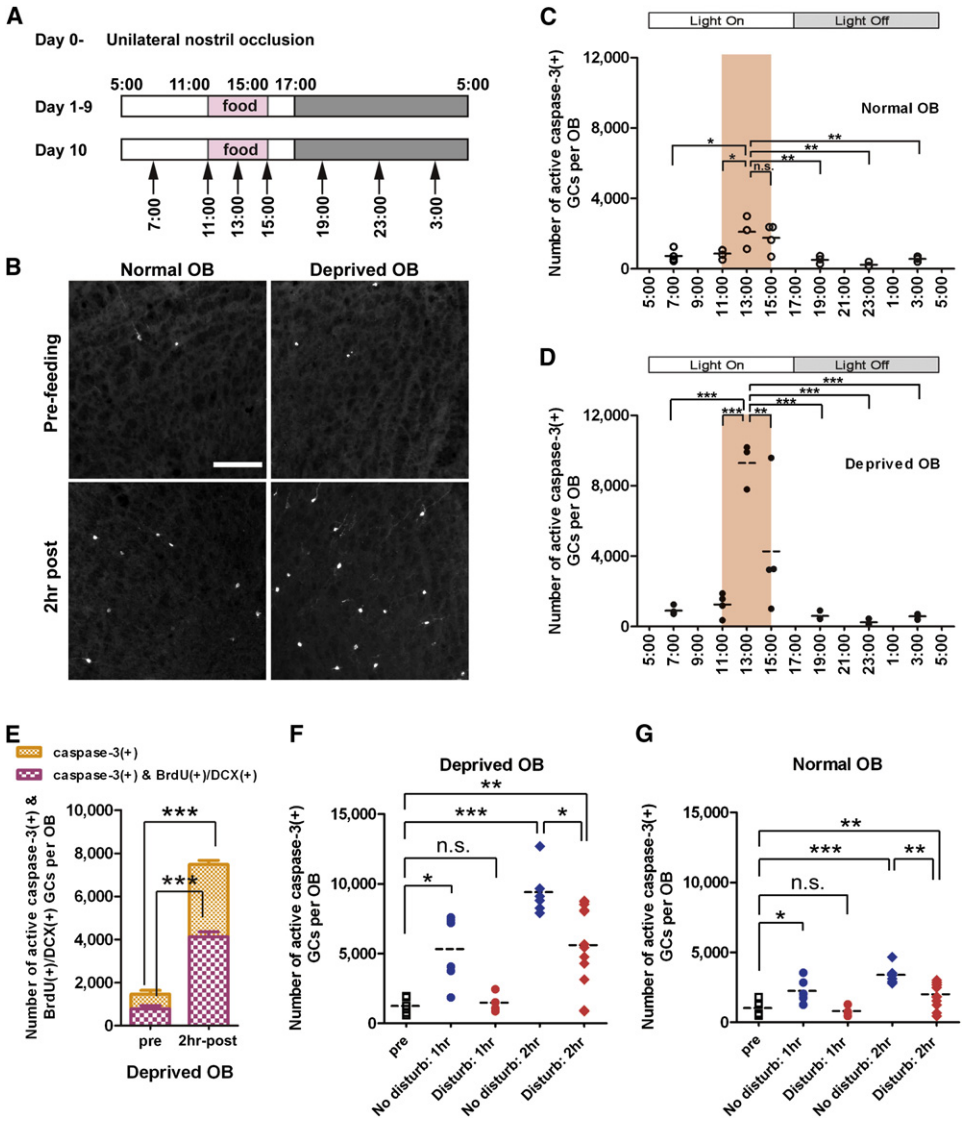

Figure 5. Sensory Deprivation Greatly Enhances GC Elimination during the Postprandial Period

(A) Protocol for olfactory sensory deprivation and restricted feeding. On day 0, one nostril was cauterized in each mouse. Food was supplied from 11:00 to 15:00.

(B) Caspase-3-activated GCs in deprived and normal OBs. Panels show normal (left) and deprived (right) OBs before feeding (upper) and 2 hr after the start of food supply (lower). Scale: 100 μm.

(C and D) Caspase-3-activated GCs in normal (C) and deprived OBs (D) at different time points. Number of mice analyzed was 4 (7:00), 4 (11:00), 3 (13:00), 4 (15:00), 3 (19:00), 3 (23:00), and 4 (3:00).

(E) Number of caspase-3-activated GCs in sensory-deprived OB that are positive for either BrdU (14–20 days of age) or DCX before and 2 hr after the start of food supply. n = 3 mice for each group. Data represent the average ± SEM.

(F and G) Effect of disturbance of postprandial behavior on enhanced GC apoptosis in deprived (F) and normal OBs (G). Mice were analyzed before food (pre), 1 hr after the start of food supply without (No disturb: 1 hr) or with postprandial behavior disturbance (Disturb: 1 hr), and 2 hr after the supply without (No disturb: 2 hr) or with postprandial behavior disturbance (Disturb: 2 hr). Each dot in graphs represents the number of caspase-3-activated GCs in one animal.

Bars represent the average. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., not significant; t test (E) and one-way ANOVA with post hoc Bonferroni test (other graphs). See also Figure S4.

elimination in that local area during the postprandial period. An increase in apoptotic GCs in the D-domain of ΔD mice during the postprandial period was also confirmed by an increase in TUNEL-positive cells (Figure S5F). Disturbance of postprandial behaviors of ΔD mice suppressed the enhanced GC apoptosis 2 hr after the start of food supply in both the D- and V-domains (Figures 6E, 6F, and S5E). Apoptotic GCs in ΔD mice showed no significant increase 1 hr after the start of food supply, as was also seen in wild-type mice with intact nostrils (Figures 6E and 6F).

In the D-domain of ΔD mice, more than half of caspase-3-activated GCs were either BrdU-positive (14–20 days of age)

or DCX-positive new GCs both before and 2 hr after the start of food supply (Figures 6G and S5G). To examine whether enhanced apoptosis of new GCs in locally sensory-deprived areas leads to a decrease in their long-term survival in these areas, adult-born GCs were BrdU-labeled and followed-up for 2 months (Figures 6H–6K and S5H). In the ΔD mice OB, the total number of BrdU-labeled cells per OB on days 9–13 was 72.1% of that in wild-type mice OB (Figure S5I), reflecting the small volume of the ΔD mouse OB. Interestingly, however, the density of labeled GCs in the D-domain of ΔD mice on days 9–13 was 1.7-fold larger than that in the V-domain of these mice (Figures 6H and 6J), which was also larger than that in the D- and V-domains of wild-type mice (Figures 6I and 6J). In this period, the density of BrdU and DCX double-positive GCs remarkably increased in the D-domain of ΔD mice (Figure S5J), indicating the enhanced recruitment of immature GCs in the area. Labeled cell density in the D-domain of ΔD mice decreased remarkably thereafter, becoming comparable to that in the V-domain on days 28–32 and 56–60 (ratio, ~1.0; Figures 6H and 6J). Survival rate of adult-born GCs (density ratio of BrdU-labeled cells, days 56–60/days 9–13) in the D-domain was 34.7%, which was significantly lower than that in the V-domain (62.3%; Figure 6K). In wild-type mouse OB, the density of labeled GCs in the D-domain was slightly higher than that in the V-domain, and the ratio (D-domain/V-domain) was constant across all time points examined (nearly 1.2; Figures 6I and 6J). Survival rates of adult-born GCs in the D- and V-domains of wild-type mice were comparable to that in the V-domain of ΔD mouse OB (Figure 6K). These results indicate the local regulation of (1) immature GC recruitment, (2) sensory input-dependent apoptosis of new GCs, and (3) long-term survival of new GCs, in the ΔD mouse OB.

Food Intake Is Not an Absolute Requirement for the Enhancement of GC Apoptosis

Postprandial period-specific enhancement of GC apoptosis in food-restricted mice may raise the general idea that food intake is an absolute requirement to triggering the enhanced GC apoptosis. To address this possibility, mice habituated to restricted feeding were left without food at the presumptive feeding time (Figure 7A; no food). In contrast to mice that ate food, those without food continued to show exploratory behavior, without resting, sleeping, or extended periods of grooming, during the initial 2 hr of the presumptive feeding time (data not shown). In this period, there was no increase in apoptotic GC number (Figure 7B; 2 hr—no food). In addition, mice with restricted feeding that were allowed to smell food odor but were prevented from eating (Figure 7A; food odor) also showed continual exploratory and sniffing behaviors during the presumptive feeding time, and also exhibited no enhancement of GC apoptosis (Figure 7B; 2 hr—food odor).

The observation period of the food-deprived mice was then prolonged beyond the presumptive feeding time (Figure 7C). After many hours, the mice showed various behaviors including grooming, resting, and sleeping. When examined after showing sleeping behavior (Figure 7C, arrows), some showed a several-fold increase in GC apoptosis (Figure 7D). This observation indicates that actual food intake is not an absolute requirement for enhanced GC apoptosis in food-restricted mice and also suggests that the postprandial period is a typical but not the only period in which GC apoptosis can be enhanced (see Discussion).

Enhanced GC Apoptosis during the Postprandial Period Occurs without Long-Term Entrainment to Food Restriction

The enhanced GC apoptosis observed so far might largely depend on the specific paradigm of food restriction. Alterations in body status such as hormonal levels and energy metabolism in long-term food-restricted mice (Gao and Horvath, 2007) may be important to the enhancement of GC apoptosis during the postprandial period. To examine whether GC apoptosis during the postprandial period is enhanced in mice without long-term food restriction, we designed a one-time food restriction paradigm. In this paradigm, food was abruptly removed for 4 hr and 20 min in ad libitum feeding mice and then made available again to efficiently induce feeding and postprandial behaviors (Figure 7E, middle bar). Food was removed during the early dark phase of the circadian cycle, because this was the period in which ad libitum feeding mice ate most extensively (data not shown; Zucker, 1971). Following food redelivery, the mice successfully showed feeding and subsequent postprandial behaviors, including grooming, resting, and sleeping.

Under this paradigm, GC apoptosis was enhanced in mice with feeding and postprandial behaviors compared to mice before food supply (Figure 7F). Because under this condition the time of eating and postprandial behaviors after food redelivery varied widely among mice, the redelivery period was limited to 1 hr only (Figure 7E, bottom bar), which efficiently induced postprandial behaviors and enhanced GC apoptosis within 2.5 hr after the start of food redelivery (Figure 7G). Disruption of postprandial behaviors during these hours inhibited the enhancement of GC apoptosis (Figure 7G). Further, in nostril-occluded mice subjected to this feeding paradigm, apoptotic GCs remarkably increased in the sensory-deprived OB after the postprandial behaviors, which was suppressed by gentle handling (Figure 7H). We confirmed that gentle handling did not reduce the amount of food pellet consumed (data not shown). These results indicated that sensory experience-dependent enhancement of GC apoptosis during the postprandial period did not depend on long-term food restriction. Another group of ad libitum feeding mice in which the period of food removal and re-delivery was set at a different circadian time (late dark period) also showed enhanced GC apoptosis during the postprandial period, indicating that the enhancement can occur at different circadian times in ad libitum feeding mice with one-time food restriction (Figures S6A and S6B).

Finally, we examined GC apoptosis during the postprandial period in ad libitum feeding mice without any short period of food removal (Figure 7I). Mice that showed sleeping behavior after eating in the early dark phase showed a larger number of caspase-3-activated GCs than mice whose postprandial behavior was disturbed (Figure 7J). Enhancement of GC apoptosis by postprandial behavior was thus also observed in ad libitum feeding mice without any food deprivation period.

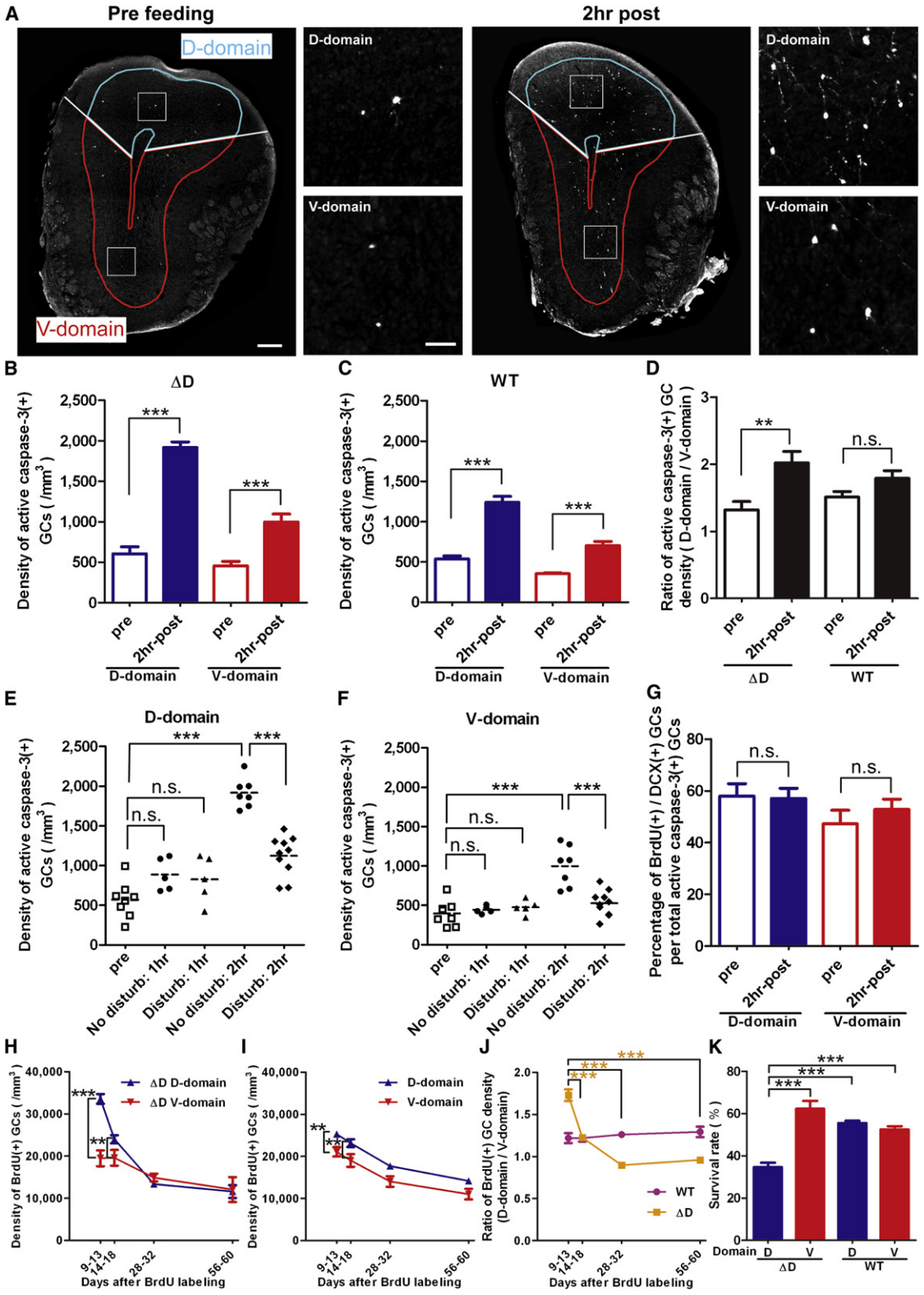

Figure 6. Local Sensory Deprivation Enhances Local GC Elimination during the Postprandial Period

(A) Caspase-3-activated GCs in ΔD mouse OB before (left) and 2 hr after food supply (right). Blue lines surround D-domain GCL and red lines surround V-domain GCL. Boundary between the D- and V-domain is indicated by white lines. Boxed areas are magnified and shown on the right. Scale: 200 μm (low power view) and 50 μm (magnified view).

DISCUSSION

Two-Stage Model for GC Elimination

These results in food-restricted and ad libitum-fed mice indicate that the elimination of GCs does not occur evenly across the day but is rather enhanced during the postprandial period. The results suggest that an active "reorganizing signal" occurs in the OB during the postprandial period, and that olfactory sensory inputs during waking periods regulate the extent of GC elimination during the subsequent postprandial period. The majority of apoptotic GCs were adult-born GCs. Based on these results, we propose the following two-stage model for the sensory experience-dependent elimination of a subset of adult-born GCs (Figure 8).

During the waking period, when mice show food-finding and eating behavior, a subset of newly generated adult-born GCs receives local olfactory sensory inputs (lower-left diagram in Figure 8) while the remaining subset does not (upper-left diagram). However, the putative "reorganizing signal" may be relatively small, if any, during waking periods. Rather, an active "reorganizing signal" enters the OB during the subsequent postprandial period (right diagrams) such that the sensory experienced subset of adult-born GCs is selected to survive (lower-right diagram), whereas other adult-born GCs without sensory experience are eliminated (upper-right diagram). Thus, the fate of individual adult-born GCs might be determined by the interplay between the "reorganizing signal" and the trace of sensory experience. This two-stage model of GC elimination resembles the two-stage model of memory formation in the hippocampus, which proposes memory-related structural changes occur by the combination of learning experience during waking and neuronal activities during subsequent sleep and rest periods (Buzsáki, 1989; Diekelmann and Born, 2010).

Enhanced GC elimination during the postprandial period resembles homeostatic synaptic downscaling during sleep (Tononi and Cirelli, 2006; Vyazovskiy et al., 2008). Because a large number of adult-born GCs are recruited in the OB every day, elimination of adult-born and preexisting GCs (Figure 2) is necessary to maintain the overall number of GCs in the entire OB within an appropriate range. Sensory experience-dependent elimination of adult-born and preexisting GCs during the postprandial period downscales the GC number and may increase the ratio of useful versus useless GCs. In fact, GC elimination optimizes such olfactory functions as odorant exploration and discrimination (Mouret et al., 2009).

Possible Candidates for the Reorganizing Signal that Occurs during the Postprandial Period

What neuronal mechanisms generate the putative reorganizing signal that leads to the enhanced elimination of adult-born GCs during the postprandial period? The OB receives a variety of behavioral state-dependent signals, including cholinergic and catecholaminergic neuromodulatory signals and hormonal signals (Adamantidis and de Lecea, 2008; Hasselmo, 1999; Figure 8). In addition, proximal dendrites of GCs in the OB receive massive centrifugal excitatory synaptic input from the olfactory cortex (Price and Powell, 1970), which shows behavioral state-dependent change in information processing mode (Murakami et al., 2005).

Given the correlation between apoptotic GC number and postprandial sleep length in wild-type mice (Figure 4), we consider that the reorganization signal occurs strongly during the postprandial sleep period. We recently found that neurons in anterior regions of the olfactory cortex repeatedly generate synchronized spike discharges during slow-wave sleep, but not during waking or REM sleep (Manabe et al., 2011). These synchronized spike discharges of numerous olfactory cortical neurons drive synchronized top-down centrifugal inputs to GCs in the OB during slow-wave sleep, raising the possibility that these inputs to GCs during postprandial sleep serve as the reorganizing signal to GCs. Excitatory synaptic inputs to the proximal dendrites of GCs, particularly those of new GCs, show high plasticity (Gao and Strowbridge, 2009; Nissant et al., 2009). The synchronized centrifugal inputs might induce not only synaptic plasticity but also regulate GC elimination. It is intriguing that very immature GCs (7–13 days of age) showed no significant increase in cell death during the postprandial period (Figure 2). This might be due to the scarcity of synapse formation on these GCs, which occurs extensively after this cellular age (Carleton et al., 2003; Kelsch et al., 2008; Whitman and Greer, 2007).

Our present study also suggests that the reorganizing signal can occur without apparent sleep during the postprandial period, as shown by the enhanced GC apoptosis in nostril-occluded mice at 1 hr after the start of food supply (Figure 5). In the hippocampus, synchronous discharges of neurons represented by sharp waves occur most frequently during slow-wave

(B and C) Density of caspase-3-activated GCs in the D- and V-domain of ΔD mouse (B) and wild-type mouse (C) OBs before and 2 hr after the start of food supply.
(D) Ratio of the density of caspase-3-activated GCs (D-domain/V-domain) in ΔD and wild-type mouse OBs before and 2 hr after the start of food supply. The ratio between domains is calculated in each OB and the data from different mice are averaged.
(E and F) Effect of disturbance of postprandial behavior on enhanced GC apoptosis in D-domain (E) and V-domain (F) of ΔD mouse OB. Each group of mice was analyzed at the indicated times with or without postprandial behavior disturbance.
(G) Percentage of either BrdU- (14–20 days of age) or DCX-positive GCs among caspase-3-activated GCs in the D- and V-domains of ΔD mouse OB before and 2 hr after the start of food supply.
(H–K) Decreased survival of new GCs in the D-domain of ΔD mouse OB. BrdU was injected for 5 days and labeled GCs were analyzed at the indicated periods. Labeled GC density in the D- and V-domain of ΔD mouse OBs (H), wild-type mouse OBs (I), and ratio of labeled GC density between domains (D-domain/V-domain) of ΔD and wild-type mouse OBs (J) are shown. Survival rates of BrdU-labeled GCs in the D- and V-domains of ΔD and wild-type mouse OBs are indicated (K). Survival rate was calculated by dividing the labeled GC density at days 56–60 by that at days 9–13.
In (E) and (F), each dot represents the number of caspase-3-activated GCs in one animal and bars represent the average. Data in (B)–(D) and (G)–(K) indicate average ± SEM. Numbers of mice analyzed were 6 (ΔD, pre), 7 (ΔD, 2 hr-post), 4 (wild-type, pre), and 4 (wild-type, 2 hr-post) in (B)–(D), 5 for individual groups in (G), and 4, 4, 3, and 4 ΔD and 4, 4, 3, and 4 wild-type mice at days 9–13, 14–18, 28–32, and 56–60, respectively, in (H)–(K). $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not significant; unpaired t test (B, C, D, and G) and one-way ANOVA with post hoc Bonferroni test (E, F, and H–K). See also Figure S5.

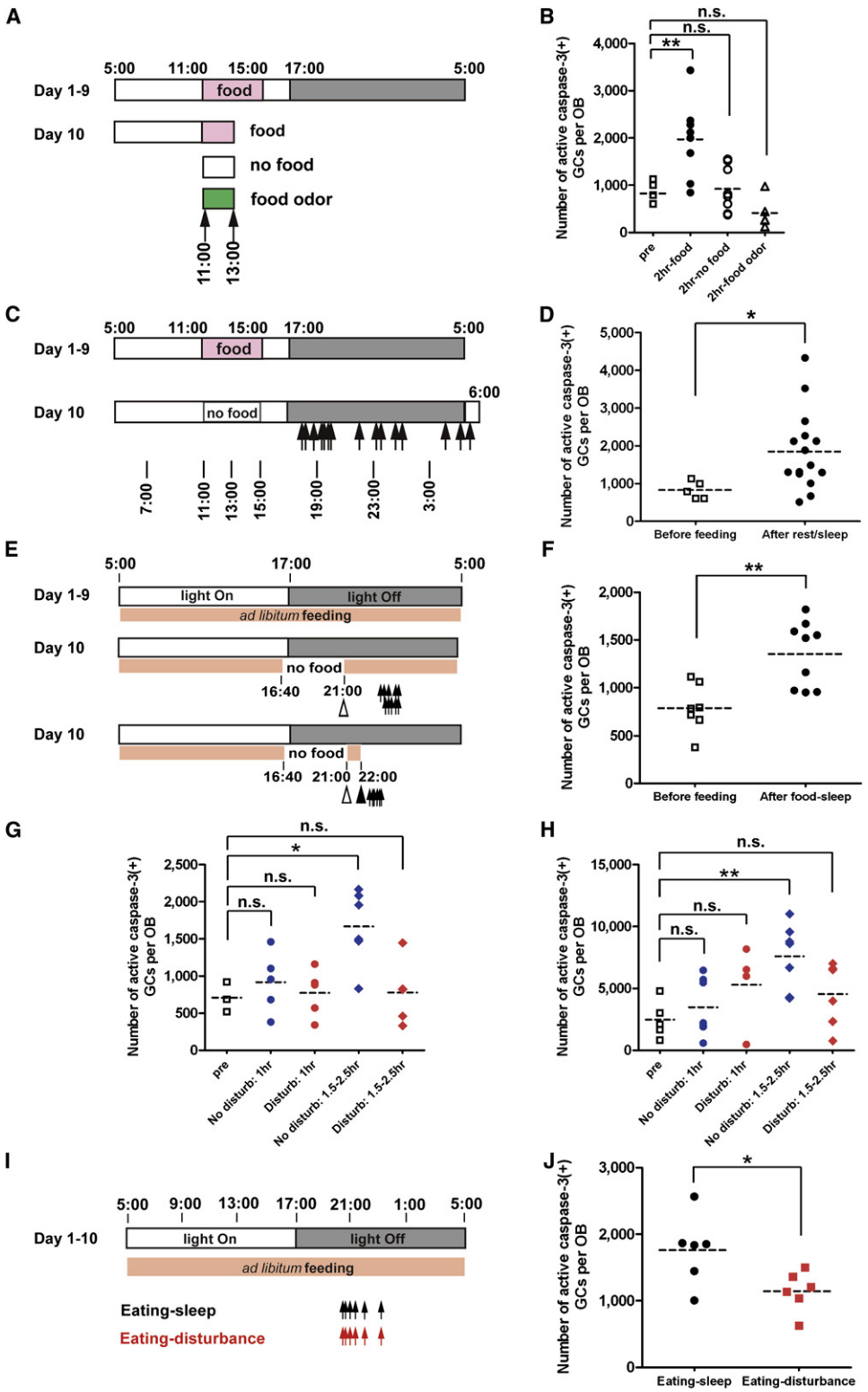

Figure 7. Enhanced GC Elimination Can Occur without Food Intake or without Entrainment to Food Restriction

Food intake is not an absolute requirement for enhanced GC apoptosis (A–D). Enhanced GC apoptosis during the postprandial period occurs without long-term entrainment to food restriction (E–J).

(A and B) No enhancement of GC apoptosis during the presumptive feeding time in mice without food intake. (A) On day 10 at the expected feeding time, mice were either given food (food), deprived of food (no food), or exposed to food odor only (food odor). (B), number of caspase-3-activated GCs in four groups

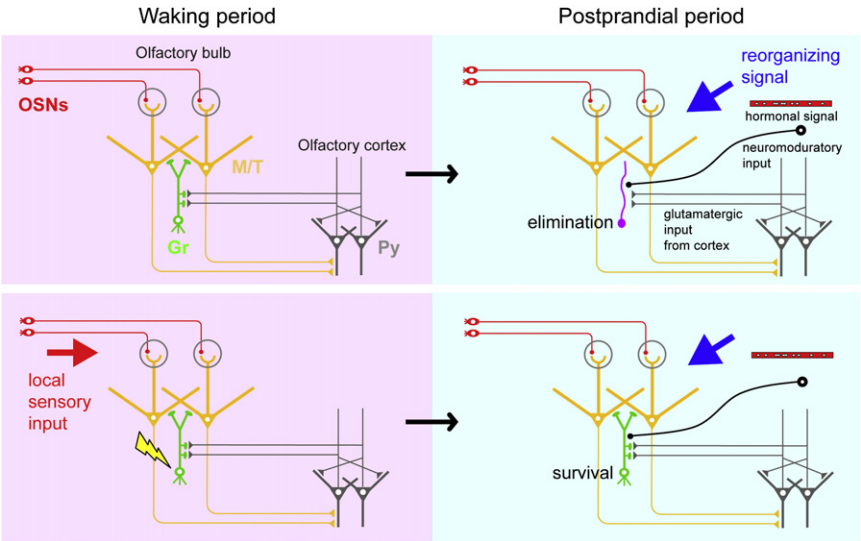

Figure 8. Schematic Diagram of the Two-Stage Model for GC Elimination

Adult-born GCs (green, Gr) make reciprocal synapses with mitral/tufted cells (yellow, M/T) and receive top-down synaptic input from pyramidal cells in the olfactory cortex (gray, Py). (Left panels) During waking, local sensory input from olfactory sensory neurons (red, OSNs) activates a subset of adult-born GCs (lower panel). The activated GCs might deposit "sensory experience-dependent tags." Other adult-born GCs lack activation by sensory experience (upper panel). (Right panels) During the following postprandial period, a reorganizing signal enters the OB. Adult-born GCs activated during the waking period survive (lower), while those without activation are eliminated (upper). Candidates for the reorganizing signal are glutamatergic input from the olfactory cortex, neuromodulatory input, and hormonal signals.

sleep, but also in other behavioral states such as awake immobility, grooming, and consuming behaviors (Buzsáki et al., 1983). It will be important to examine the top-down input from the olfactory cortex to the OB during various behavioral states of nostril-intact and -occluded mice. Overall, we regard the top-down synaptic input as a plausible candidate for the reorganizing signal, and are currently examining the causal link between the synchronized top-down signal and GC elimination. At the same time, we do not deny other possibilities, for example that alterations in neuromodulatory and hormonal signals during the postprandial period act as the reorganizing signal.

Role of Olfactory Activities during the Waking Period in the Regulation of GC Elimination during the Subsequent Postbehavioral Period

Our present observations in nostril-occluded mice and ΔD mice indicated that sensory deprivation did not affect the time window of enhanced GC elimination, but rather shifted the direction of GC response to the reorganizing signal during the postprandial period from survival to elimination. Olfactory sensory input is likely to drive glutamatergic synaptic inputs to adult-born GCs. Drawing from the general idea that experience puts "tags" on specific synapses which serve as substrates for the subsequent synapse-specific plastic modulation (Frey and Morris, 1997), olfactory sensory inputs are considered to put tags on glutamatergic synapses of particular adult-born GCs. We speculate that GCs with tagged synapses are prevented from elimination by the putative reorganizing signal during the postprandial period, while nontagged GCs are eliminated by the signal. The sensory deprivation models in the present study appear to be helpful in understanding this tagging mechanism.

The occurrence of enhanced GC elimination in mice without food intake (Figure 7) suggests that the postprandial period is a typical but not the only time window in which GC elimination

of mice. Mice were tested before food or 2 hr after the various feeding conditions in (A). During the 2 hr, mice without food showed no resting, sleeping or sustained grooming.

(C and D) Enhanced GC apoptosis in mice without food intake at many hours after the presumptive feeding time. (C) On day 10, mice were not given food and observed continually after the presumptive feeding time. Mice were analyzed 1 hr after showing initial sleep behavior. Arrows indicate the fixation times (n = 15 mice). (D) Number of caspase-3-activated GCs in mice shown in (C) (filled circles). Data of mice before feeding (11:00) are also indicated for comparison (open squares).

(E–H) Enhanced GC apoptosis during the postprandial period of ad libitum feeding mice under one-time food restriction. (E) One-time food restriction paradigm. Ad libitum feeding mice were deprived of food from 16:40 to 21:00 and then redelivered with food. Food was redelivered continually (middle bar) or only for 1 hr (bottom bar; 21:00–22:00). Mice were analyzed before food redelivery (open triangle) and 30–40 min after showing initial postprandial sleep behavior (arrows). Mice were also analyzed 1 hr after the food redelivery (closed triangle in the bottom bar). (F) Number of caspase-3-activated GCs in mice under one-time food restriction. One group of mice was analyzed before food redelivery (open triangle in the middle bar in E; open squares in F). The other group was analyzed after the continual food redelivery and postprandial sleep behavior (arrows in the middle bar in E; closed circles in F). (G and H) Effect of disturbance of postprandial behavior on enhanced GC apoptosis in mice with one-time food restriction followed by 1 hr of food redelivery (bottom bar in E). Number of caspase-3-activated GCs in nostril-intact mouse OB (G) and sensory-deprived OB of nostril-occluded mice (H) was analyzed at indicated times after the start of food redelivery with or without postprandial behavior disturbance.

(I and J) Enhanced GC apoptosis during the postprandial period of ad libitum feeding mice without any food deprivation period. (I) One group of mice was analyzed after showing eating and sleeping behaviors during the early dark phase (black arrows). They were analyzed 30–40 min after showing initial postprandial sleep behavior. In another group of mice, postprandial behavior during the period was disturbed. They were analyzed at the same time points (red arrows). (J) Number of caspase-3-activated GCs of the eating-sleep group (black circles) and the eating-disturbance group (red squares).

Each dot in graphs represents the number of caspase-3-activated GCs in one animal. Bars represent the average. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., not significant; one-way ANOVA with post hoc Bonferroni test (B, G, and H) and unpaired t test (D, F, and J). See also Figure S6.

is enhanced. We are currently examining the possibility that other behaviors, such as olfaction-mediated avoidance behavior and mating behavior (Kobayakawa et al., 2007; Mak et al., 2007), also lead to enhanced GC elimination during the postbehavioral period. These olfactory behaviors are accompanied by alterations in neuromodulatory and hormonal signals. For example, norepinephrine signals are stimulated by feeding and mating (Brennan et al., 1990; Wellman, 2000), and metabolic hormones and the dopaminergic system work together in controlling feeding (Hommel et al., 2006). We speculate that such waking behavior-related signals play crucial roles in the subsequent GC elimination. These signals may promote the generation of putative reorganizing signal during the postbehavioral period, or potentiate GC responsiveness to it. Such mechanisms may explain why the enhancement of GC elimination was restricted to the postprandial period in food-restricted mice, and similar mechanisms might be at work in the feeding and postprandial period of ad libitum feeding mice. The combinatory role of olfactory sensory experience and neuromodulatory/hormonal signals during waking behavior and signals during the postbehavioral period will likely be revealed as the key mechanisms of the experience-dependent reorganization of the bulbar circuit.

EXPERIMENTAL PROCEDURES

Animals and Housing

C57BL/6 male mice (8 weeks old) were used for most experiments. ΔD male mice and age-matched C57BL/6 male mice (11–12 weeks old) were also used. They were housed individually under a 12 hr light-dark cycle. All experiments were conducted in accord with the guidelines of the Physiological Society of Japan and were approved by the Experimental Animal Research Committee of the University of Tokyo.

Restricted Feeding

Food was supplied for only 4 hr per day (11:00–15:00). The mice were analyzed on day 10. To analyze ad libitum feeding mice, food was removed for about 4 hr on the day of analysis and then delivered again.

Behavioral Analysis and Disturbance of Postprandial Behavior

Animal behavior was video recorded and analyzed. Behavior was categorized as eating, drinking, grooming, exploratory, or resting/sleeping. Postprandial resting, sleeping, and extended grooming (more than 5 s) were disrupted by gentle handling (Mistlberger et al., 2003), in which mice were stimulated by stroking the body with a plastic ruler.

EEG and EMG Recording

Electrodes were implanted in the neck muscle for EMG and in the bone above the occipital cortex for EEG. The mice were subjected to food restriction. EMG and EEG during the postprandial period were captured and analyzed. The mice were perfusion-fixed immediately after data acquisition.

Nostril Cauterization

Olfactory sensory deprivation was conducted by nostril cauterization as described previously (Yamaguchi and Mori, 2005).

BrdU Labeling

Adult-born GCs were labeled by intraperitoneal BrdU injection for 7 days and analyzed at various periods. Neonate-born GCs were BrdU-labeled on postnatal days 4 and 5. Adult-born GCs in ΔD mice was examined by BrdU labeling for 5 or 7 days.

Immunohistochemistry

Mice were deeply anesthetized with pentobarbital and transcardially perfused with PFA. Coronal OB sections (20 μm thickness) were immunostained and examined. TUNEL assay was conducted as described in Supplemental Experimental Procedures.

Cell Counting

Coronal sections of the entire OB were selected at the rate of 1 in every 10 serial sections. The number of caspase-3-activated GCs in the GCL was counted, summed, and multiplied by 10 to obtain the total number per OB. Comparative analysis of ΔD and wild-type mouse OBs was done using coronal sections at the central portion in the rostro-caudal axis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, one movie, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.05.046.

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