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Research Article

Western Diet Accelerates the Impairment of Odor-Related Learning and Olfactory Memory in the Mouse

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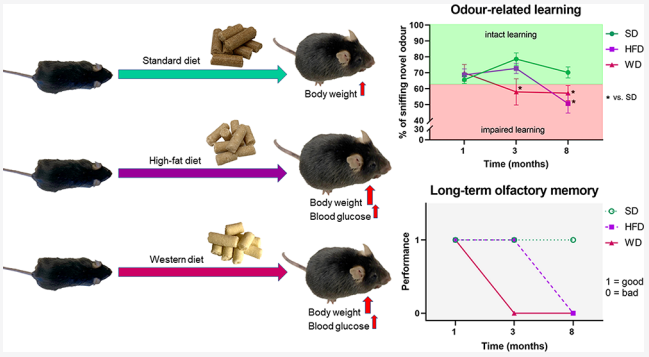
ABSTRACT: Olfactory dysfunction could be an early indicator of cognitive decline in type 2 diabetes (T2D). However, whether obesity affects olfaction in people with T2D is unclear. This question needs to be addressed, because most people with T2D are obese. Importantly, whether different contributing factors leading to obesity (e.g., different components of diet or gain in weight) affect specific olfactory functions and underlying mechanisms is unknown. We examined whether two T2D-inducing obesogenic diets, one containing a high proportion of fat (HFD) and one with moderate fat and high sugar (Western diet, WD), affect odor detection/discrimination, odor-related learning, and olfactory memory in the mouse. We also investigated whether the diets impair adult neurogenesis, GABAergic interneurons, and neuroblasts in the olfactory system. Here, we further assessed olfactory cortex volume and cFos expression-based neuronal activity. The WD-fed mice showed declined odor-related learning and olfactory memory already after 3 months of diet intake ($p = 0.046$), although both diets induced similar hyperglycemia and weight gain compared to those of standard diet-fed mice ($p = 0.0001$ and $p < 0.0001$, respectively) at this time point. Eight months of HFD and WD diminished odor detection ($p = 0.016$ and $p = 0.045$, respectively), odor-related learning ($p = 0.015$ and $p = 0.049$, respectively), and olfactory memory. We observed no changes in the investigated cellular mechanisms. We show that the early deterioration of olfactory parameters related to learning and memory is associated with a high content of sugar in the diet rather than with hyperglycemia or weight gain. This finding could be exploited for understanding, and potentially preventing, cognitive decline/dementia in people with T2D. The mechanisms behind this finding remain to be elucidated.



KEYWORDS: *Diabetes, high fat diet, obesity, olfaction, olfactory memory, Western diet*

INTRODUCTION

Olfaction, by influencing vital activities such as food intake, social behavior, and reproduction, plays a crucial role in animal behavior. This behavior is partially driven by olfactory networks that are involved in detection and processing of changes in the surrounding chemical environment.[1,2] Olfactory impairment is a predictor of several neurodegenerative diseases.[3] The olfactory system is also tightly linked with the endocrine system,[4] and not surprisingly, impaired olfaction has been observed in people with diabetes[5] who show reduced odor detection (ability to detect odors) and odor discrimination (ability to discriminate odors), loss of olfactory memory (ability to recall previously learned odors), and increased risk for anosmia (loss of the sense of smell).[6−9] Furthermore, olfactory dysfunctions in type 2 diabetes (T2D) are strongly associated with cognitive impairment,[10] and T2D can induce early alterations in the olfactory system preceding cognitive decline.[11] Overall, these data suggest that olfactory deficits in T2D could represent an early marker of cognitive decline.

Obesity is the main risk factor for T2D, but the interplay between obesity and olfaction is unclear. In fact, obesity has been associated with olfactory dysfunctions in humans.[12−15] However, increased olfactory sensitivity has also been reported in individuals with obesity.[16] Whether or not obesity affects olfaction within the T2D population is unknown. However, it is crucial to address this, because 85% of T2D individuals are obese. In fact, some clinical studies showing olfactory impairment in diabetics did not provide body mass index (BMI) data for the T2D group,[17] or they showed no significant difference in this parameter between T2D and controls.[18,19] Results of other studies have been conflicting. Some showed no correlation between BMI and olfactory scores,[6,7,18] while

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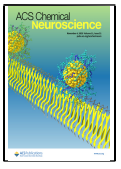

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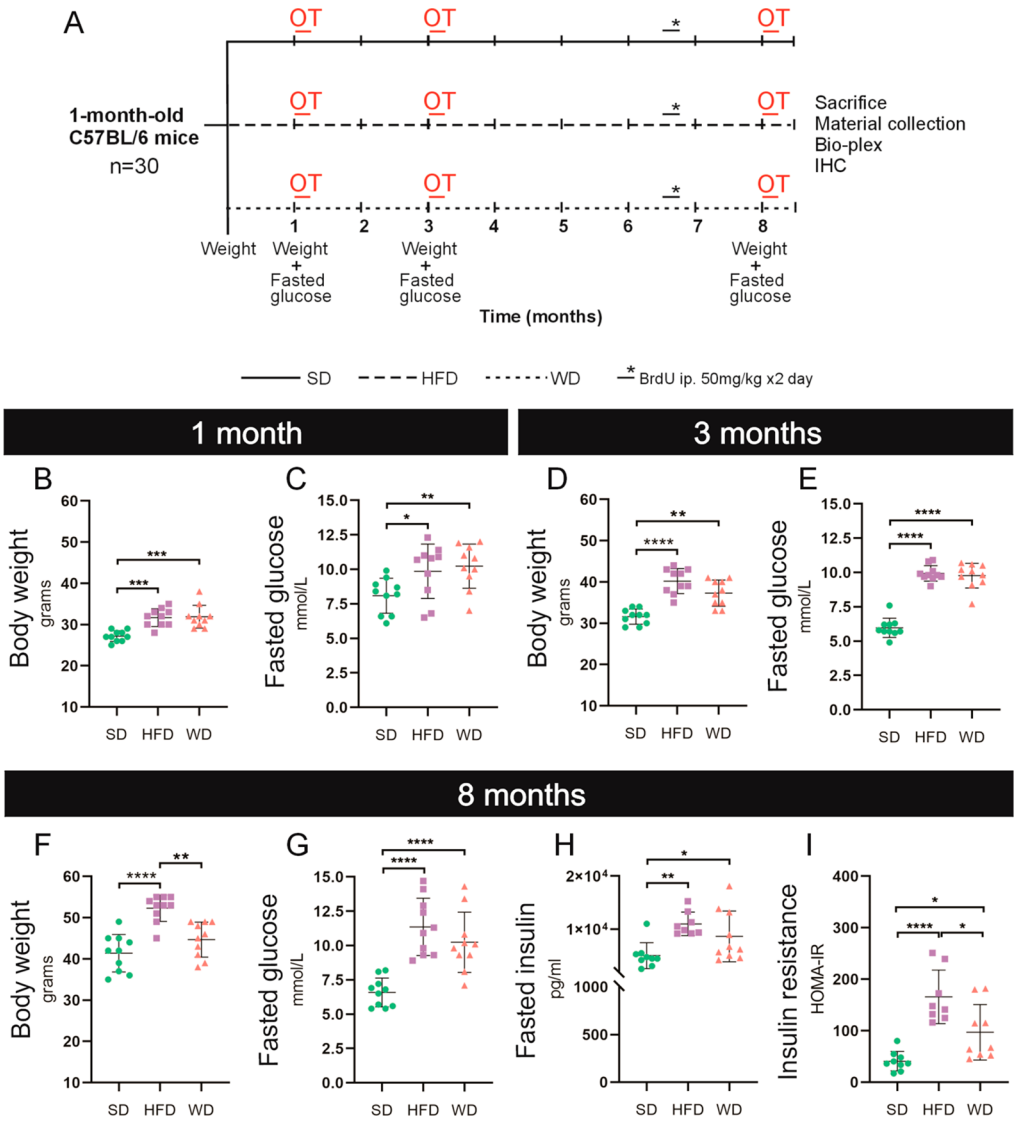

Figure 1. High fat diet and Western diet induce obesity and fasted hyperglycemia, as well as increase insulin levels and insulin resistance. Study design: 30 1-month-old C57BL/6 male mice were randomly assigned to three experimental groups (n = 10 each) (A). Obesity/T2D was induced either by continuous feeding with the high fat diet (HFD) or Western diet (WD) for 8 months. The control group were mice fed with the standard diet (SD). Olfactory tests (OTs) were performed after 1, 3, and 8 months of diet intake. To quantify the number of proliferating cells, we used 5-bromo-2′-deoxyuridine (BrdU) which is incorporated into the DNA during its synthesis. The mice received BrdU for 6 days, 8 weeks before they were sacrificed. Body weight (B, D, F) and fasted blood glucose concentration (C, E, G) after 1, 3, and 8 months of diet intake, respectively. Fasted plasma insulin concentration (H) and HOMA-IR (I) after 8 months. Welch's ANOVA test followed by two-stage step-up method of Benjamini, Krieger, and Yekutieli. Histograms show means ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

others reported a negative correlation;[8,15] one study even indicated a positive correlation between olfactory sensitivity and BMI[20] in individuals with diabetes. Therefore, these discrepancies warrant further studies. It is also worth emphasizing that the tested olfactory parameters mainly addressed odor identification/discrimination.[21] Thus, the effects of T2D on olfaction-related cognitive functions (e.g., olfactory memory) remain undetermined. Indeed, these functions are key to identifying changes in the olfactory system that precede cognitive decline in T2D.[11]

The complex interplay between the olfactory and endocrine systems is also reflected in animal studies where both olfactory deficits[22−27] and increased olfactory sensitivity[28,29] have been reported in animal models of obesity/diabetes. However, in most of these studies, olfactory assessments were performed only at one time point; thus, the temporal dynamics of the effects in relation to T2D development are not addressed. Furthermore, it remains unknown whether or not (or how) specific factors leading to obesity, such as different compositions of obesogenic diets with various fat and sugar contents, can affect olfaction. Indeed, nutrients play a key role in modulating olfactory sensitivity.[4,30]

Only a few studies have addressed the potential mechanisms at the basis of the diet-induced changes in olfaction. Results of these studies showed that reduced olfactory functions induced by moderate high fat and high fructose diets in the mouse were associated with olfactory sensory neuron alterations.[26,27] Other studies reported molecular changes in the olfactory system of obese rats such as a decrease in tyrosine-phosphorylated proteins in the piriform cortex (PC)[31] or a decrease of insulin binding in the main olfactory bulb (MOB).[32] However, how

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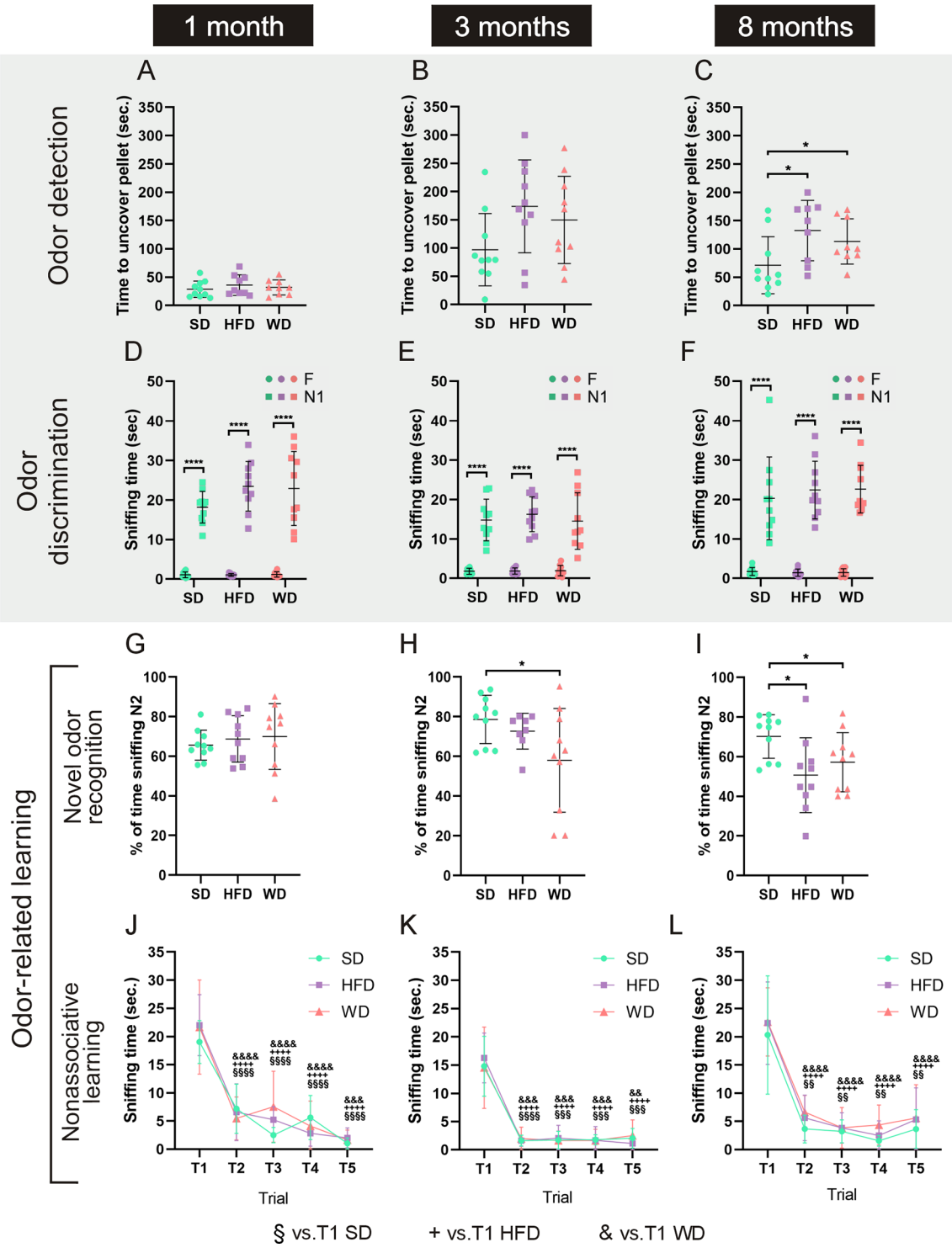

Figure 2. Obesogenic diets induce odor detection impairment and novel odor recognition deficit. The latter is already impaired after 3 months in the WD group. (A–C) Mean time to find pellet in the buried pellet test performed after 1, 3, and 8 months of diet intake in the three experimental groups. Kruskal–Wallis test followed by two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. (D–F) Mean sniffing time of wooden blocks covered with a scent of the tested mouse ("F") and unknown mouse ("N1") in the block test performed after 1, 3, and 8 months of diet intake. Two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. (G–I) Percent of sniffing time of unknown social scent ("N2") when presented along with familiar social scent ("N1") in the block test performed after 1, 3, and 8 months of diet intake. Welch's ANOVA test followed by two-stage step-up procedure of Benjamini, Krieger, and Yekutieli. (J–L) Mean sniffing time of a new social scent during 5 trials (T1–T5) of the habituation test performed after 1, 3, and 8 months of diet intake. Repeated measures of two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. Histograms show means ± SD, $^{*}p < 0.05$, $^{\&\&,\S\S}p < 0.01$, $^{\&\&\&,\S\S\S}p < 0.001$, $^{****,\&\&\&\&,++++,\S\S\S\S}p < 0.0001$.

these changes could relate to olfactory deficits is unknown, and consequently, additional studies are needed.

Adult neurogenesis in the MOB is limited in humans, but it is an important cellular process at the basis of olfaction-related

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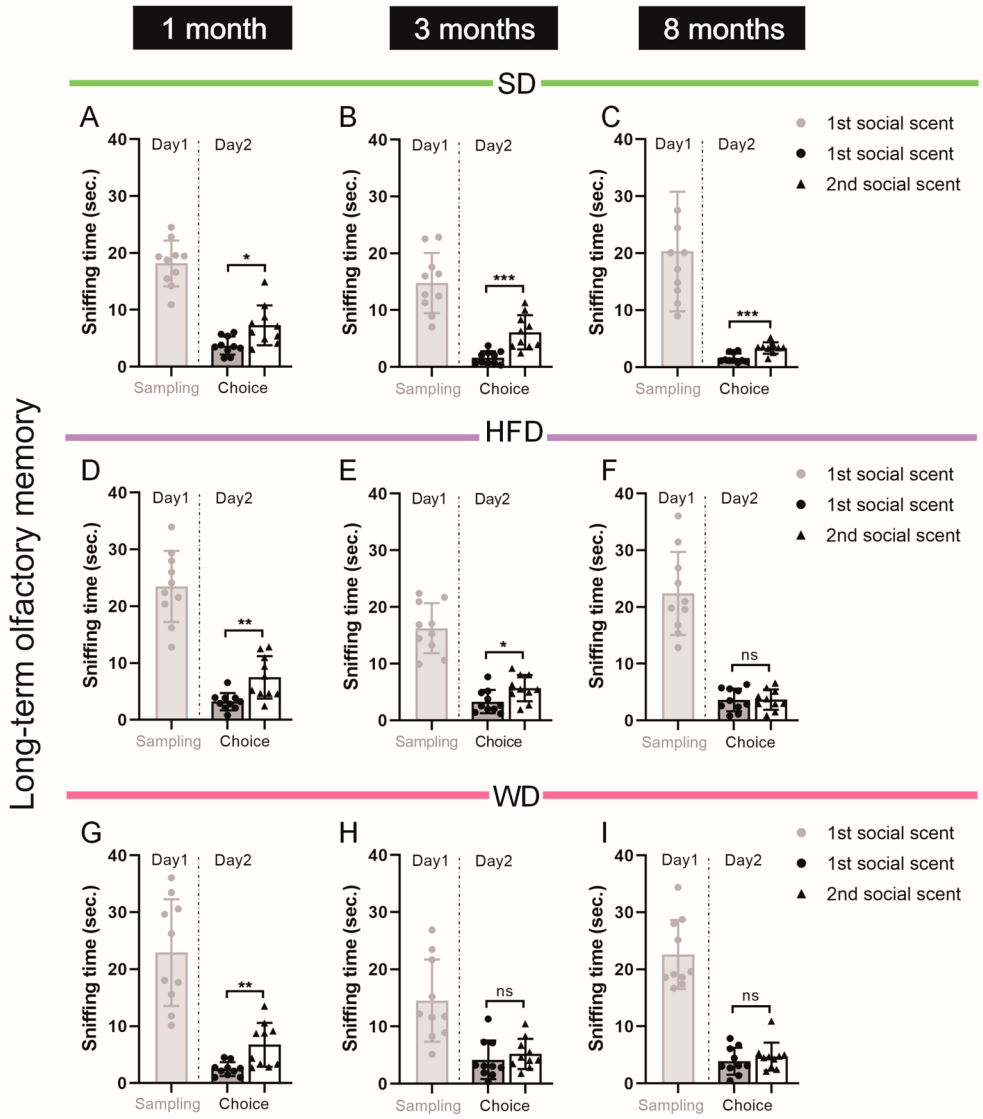

Figure 3. Obesogenic diets induce long-term olfactory memory dysfunction. This impairment is already present after 3 months in WD-fed mice, while in HFD-fed mice, it is present after 8 months of diet intake. Sniffing time of social scent with which the mice were familiarized the day before (1st social scent) and new social scent that mice had no previous contact with (2nd social scent) on day 2 in SD, HFD, and WD mice after 1 (A, D, and G), 3 (B, E, and H), and 8 months (C, F, and I) of the diet, respectively. Mann−Whitney test. Histograms show means ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

neuroplasticity in rodents.[33] Previous studies have reported diabetes-induced impairment of early neurogenesis in the rodent subventricular zone (SVZ)[34,35] and *in vitro*.[36,37] Nevertheless, so far only two studies have reported the impairment of adult neurogenesis by diabetes in the MOB: in a type 1 diabetic model[38] and in a genetic, lean model of T2D.[22] Therefore, it remains undetermined whether neurogenesis in the MOB is affected by obesity-induced T2D.

Important players involved in the olfactory neuroplasticity are GABAergic inhibitory interneurons.[39] Interestingly, one study showed their vulnerability in patients with Alzheimer's disease,[40] and our group has reported alterations in calbindin+ GABAergic interneurons in the PC in a lean T2D model.[22] However, the potential relationship between olfactory dysfunctions and alterations to GABAergic interneurons in obesity-induced T2D is undetermined.

Another less explored form of neuroplasticity in the olfactory system is represented by doublecortin (DCX)+ neurons in the PC. In contrast to DCX+ cells in the MOB, these cells are nonproliferative immature neurons of embryonic origin.[41] The pool of these cells decreases during aging due to continuous neuronal differentiation following new olfactory demands,[42] and we showed that this process is impaired in lean T2D rats.[22] Whether or not obesity-induced T2D affects these cells in the PC is unknown.

In this study, we compared the effects of two diets leading to obesity and T2D-like features: a high fat and moderate sugar diet (hereafter referred as "high fat diet" (HFD)) and a high fat and high sugar diet (hereafter referred as "Western diet" (WD)) on different olfactory functions. The hypothesis was that different nutrients in these diets can affect specific olfactory functions. This head-to-head comparison encompassed 8 months and specifically addressed odor detection/discrimination, odor-related learning, and long-term olfactory memory. We have also investigated whether or not these diets induce morphometric changes and impaired neuroplasticity

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mechanisms in the olfactory system, i.e., adult neurogenesis in the MOB, GABAergic interneurons, and doublecortin (DCX)+ immature neurons in the PC. Finally, we have explored whether HFD and WD induce changes in the basal neuronal activation of PC neurons by measuring cFos expression in these cells.

RESULTS

HFD and WD Increase the Body Weight, Glycemic Levels, and Insulin Resistance. Both HFD and WD feeding significantly increased the body weight in comparison to SD; they already started increasing the weight after 1 month (p = 0.0002) (Figure 1B). This effect was maintained in both groups after 3 months of diet intake (Figure 1D). Interestingly, we did not observe a significant difference in body weight between the SD and the WD groups after 8 months (Figure 1F). However, the results showed that HFD-fed mice had increased body weights compared to those of both SD- and WD-fed mice (p < 0.0001 and p = 0.002, respectively), at this time point (Figure 1F). Fasted glycemia was increased by HFD and WD, in comparison with SD, already after 1 month (p = 0.011 and p = 0.006, respectively) (Figure 1C). This effect was maintained after 3 and 8 months of diet intake (Figure 1E,G). After 8 months, both HFD- and WD-fed mice also had increased fasted plasma insulin levels (p = 0.002 and p = 0.046, respectively) compared to those of SD-fed mice (Figure 1H). Additionally, a trend toward an increase in insulin concentration, albeit not statistically significant (p = 0.1), was observed in the HFD-fed mice compared to that of the WD-fed mice (Figure 1H). The homeostatic model assessment (HOMA) index, being an indicator of insulin resistance, was increased in both the HFD and WD groups compared to that of the SD-fed mice (p < 0.0001 and p = 0.0136, respectively), at this time point (Figure 1I). Furthermore, we recorded a significant increase in homeostatic model assessment for insulin resistance (HOMA-IR) in HFD-fed mice compared to that of WD-fed mice (p = 0.0175) (Figure 1I).

HFD and WD Impairs Odor Detection But Not Odor Discrimination. No difference in odor detection between the experimental groups was observed, after 1 month of HFD/WD intake (Figure 2A). After 3 months of diet intake, a trend toward an increase in the time needed to find the hidden treat was recorded in both HFD- and WD-fed mice compared to that of the SD group (p = 0.159 and p = 0.191, respectively) (Figure 2B). After 8 months of obesogenic diet exposure, both HFD- and WD-fed mice required significantly more time to find the hidden treat compared to that of nondiabetic SD-fed controls (132.4 ± 17.80 s for HFD and 113.2 ± 13.33 s for WD), when compared with that of the SD control (71.30 ± 15.95 s), (p = 0.016 and p = 0.045, respectively) (Figure 2C), thus signaling diminished odor detection. We report no difference in odor discrimination among the groups, because at all time points, mice sniffed the unknown scent significantly longer compared to the familiar one (Figure 2D−F). We conclude that odor detection, but not odor discrimination, is progressively affected by both HFD and WD in the mouse.

HFD and WD Have a Differential Effect on Odor-Related Learning Functions. We observed no difference in sniffing time of novel social scent between obese and nonobese mice, after 1 month of HFD/WD intake (Figure 2G). After 3 months, mice fed with WD sniffed the novel social scent for a significantly shorter time compared to that of the control group (58.01 ± 8.25 vs 78.64 ± 3.84, respectively, p = 0.046; Figure 2H). No difference in sniffing duration was observed between SD and HFD mice, at this time point. After 8 months, we observed a significant decrease in the novel scent sniffing duration in both HFD- and WD-fed mice compared to that of the SD-fed mice (50.70 ± 5.97 s and 57.24 ± 4.72 s vs 70.23 ± 3.47 s, p = 0.015 and p = 0.049, respectively) (Figure 2I).

At all three time points, mice from the experimental groups presented a decrease in sniffing duration of the same social scent during the consecutive trials (T2−T5) compared to that of the time it was introduced (T1) (Figure 2J−L), which is evidence for successful habituation, i.e., no impairment of nonassociative learning.

We conclude that both diets progressively impair novel odor recognition (but not nonassociative learning), and this deficit appears sooner in WD-fed mice.

HFD and WD Progressively Impair Long-Term Olfactory Memory, and This Effect Is More Pronounced After WD Feeding. At all time points, SD-fed mice sniffed the new scent (2nd social scent) significantly longer compared to the time spent sniffing the familiar one (1st social scent) (p = 0.012, p = 0.001, and p = 0.0007 after 1, 3, and 8 months of diet intake, respectively) (Figure 3A−C), indicating no impairment of long-term olfactory memory.

HFD-fed mice sniffed the second scent longer after 1 and 3 months of diet intake (p = 0.006 and p = 0.027, respectively) (Figure 3D,E). After 8 months of diet, however, HFD-fed mice spent a comparable amount of time sniffing each scent (p = 0.929) (Figure 3F), indicating long-term olfactory memory impairment. After 1 month of diet, WD-fed mice also sniffed the second scent longer compared to the time spent sniffing first one (p = 0.007; Figure 3G). However, starting from the 3 month time point, the results showed no difference in sniffing time between the first and second scents in WD-fed mice (p = 0.454 and p = 0.464 after 3 and 8 months of diet intake, respectively), which implied that these mice did not remember the first scent and treated both scents as novel, i.e., showed long-term olfactory memory impairment.

In summary, these results indicate that the obesity-induced T2D progressively impairs olfactory memory. This olfactory dysfunction is induced earlier by WD than by HFD.

HFD and WD Have No Effect on Adult Neurogenesis in the Main Olfactory Bulb. To assess successful migration of neural stem cells from the SVZ of the lateral ventricle (where these cells were generated) to MOB, we quantified the density of BrdU+ cells in the MOB. To investigate whether HFD and/or WD affected adult neurogenesis in the MOB, we calculated the percentage of NeuN/BrdU+ neurons in the studied groups.

Neither the density of BrdU+ cells (Figure 4A,B) nor the percentage of NeuN/BrdU+ cells (Figure 4C,D) were affected by either HFD or WD in the MOB. This indicates that adult neurogenesis, at least after 8 months of HFD/WD feeding, is not affected by the obesogenic diets.

HFD and WD Have No Effect on the Volume of Piriform Cortex and Density of Calbindin+ and Somatostatin+ Interneurons in This Brain Area. Results of our previous study showed smaller cortical volume in the middle-aged diabetic rats (genetic, lean model of T2D) compared to that of age-matched Wistars.[43] To evaluate whether HFD and/or WD induced macroscopic changes in the PC, an olfactory area responsible for odor coding and transferring olfactory information to other brain regions, the morphometry of the PC was performed. We observed no

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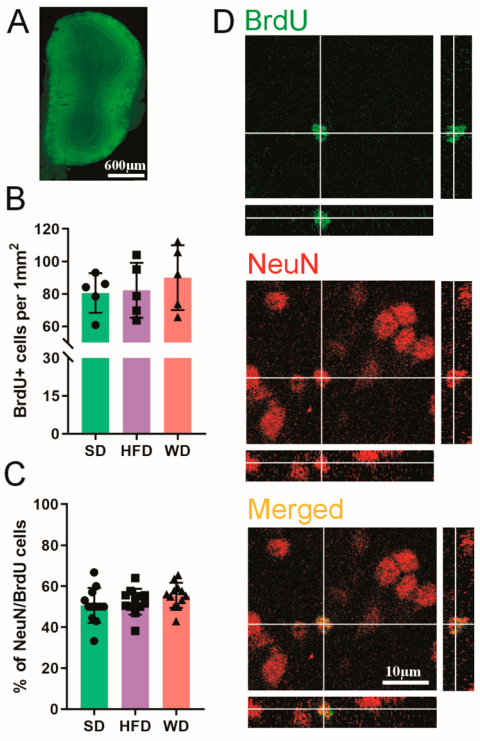

Figure 4. Obesogenic diets have no effect on neurogenesis in the main olfactory bulb. Representative microphotograph of BrdU staining (A) and density of BrdU+ cells in the main olfactory bulb of SD-, HFD-, and WD-fed mice (B). Percent of double stained NeuN/BrdU+ cells (C) and representative confocal images (D). Welch's ANOVA test followed by two-stage step-up method of Benjamini, Krieger, and Yekutieli. Histograms show means ± SD.

significant difference in PC volume in the obese HFD- and WD-fed mice compared to that of the SD-fed controls (Figure 5A,B).

In our previous study, we showed the vulnerability of GABAergic calbindin+ interneurons in the PC during aging in a genetic, lean model of T2D (GK rat).[44] In the present study, we investigated whether similar effects have also occurred in obesity-induced T2D. Thus, we assessed calbindin+ and somatostatin+ GABAergic interneurons, which mediate important effects in the PC.[45,46] The results do not show a difference in density of calbindin+ interneurons (Figure 5C,D) nor in density of somatostatin+ interneurons (Figure 5E,F) between those of the obesogenic diet-fed and those of the SD-fed mice.

Overall, these results indicate that, at least after 8 months of HFD/WD feeding, the PC is not altered morphologically, and the density of both calbindin+ and somatostatin+ interneurons in this brain area is unchanged.

HFD and WD Have No Effect on the Neuronal Differentiation of Doublecortin+ Cells in the Piriform Cortex. Results of our previous study have shown impairment of the differentiation rate of DCX+ immature neurons in the PC, in a genetic rat model of T2D.[22] These cells have been proposed to play an important role in neuroplasticity within the olfactory system (see the Introduction). In this study, we evaluated the potential effect of HFD and WD on this pool of cells. The results show no significant difference in the total number of DCX+ immature neurons in PC of HFD- and WD-fed mice *versus* that of the SD-fed control group (Figure 5G,H), at least after 8 months of HFD/WD.

HFD and WD Have No Effect on Neuronal Activation Based on Increased cFos Expression in the Piriform Cortex. The immediate early gene *c-fos* is an accepted molecular marker of neural activity.[47] To investigate potential differences in neuronal activity in the PC between the SD and HFD or WD groups, we quantified cFos/NeuN+ neurons in this brain area. The results showed no significant changes among the groups after 8 months of HFD/WD feeding (Figure 5I,J).

DISCUSSION

The results of this study show that both HFD and WD impair odor detection, odor-related learning, and long-term olfactory memory after 8 months of diet intake. Interestingly, odor-related learning and long-term olfactory memory were impaired earlier in the WD group, after only 3 months. At this time point, both body weight and hyperglycemia were similar in HFD- and WD-fed mice suggesting that the sugar content in WD, rather than the weight gain *per se* or differences in blood glucose, is associated with deficits in odor-related learning and olfactory memory. We observed no effect of either HFD or WD on PC volume, neuronal activation, or on any of the studied neuroplasticity markers, at least after 8 months of HFD/WD intake.

Clinical data from studies investigating whether obesity affects olfactory functions in T2D are conflicting (see the Introduction). Explanations of these discrepancies could be numerous, ranging from the diverse study design with different inclusion criteria (e.g., not a defined type of diabetes or participants with T2D and T1D included in the same study group) to, in some cases, the low number of participants underlining the need in the field for large prospective studies. Moreover, no universal, validated olfactory test has been used, but various tests with different sensitivities, specificities, and replicabilities have been used (revised in ref 21). Finally, most of the T2D participants in these studies were under diabetic treatments, and there is a paucity of data in rodents;[22,44] also, there is only one recent clinical report[8] regarding the positive effect of these therapies in the olfactory system. Therefore, experimental studies modeling the effects of different obesogenic diets on the olfactory functions, as well as investigating the underling mechanisms, are needed.

In the present study, we tested different olfactory functions related not only to odor detection and discrimination but also to odor-related learning and memory, during the progression of obesity-induced T2D after sustained HFD and WD feeding in the mouse. While the detrimental effects of HFD on the olfactory system have been more extensively characterized, studies on WD may better represent eating patterns in developed nations;[48] also, a recent report showed that this type of obesogenic diet is harmful to the brain.[49] We hypothesized that a high amount of sugar in the WD could further exacerbate the negative effect of a high content of the saturated fatty acids, present in the HFD, on olfactory functions. Our hypothesis was based on the fact that WD has been strongly associated with T2D, and a high sugar content in the diet has been shown to exacerbate glucose intolerance and insulin resistance.[50] Indeed, a head-to-head comparison of the effects of these two diets on olfaction over time has not been previously reported.

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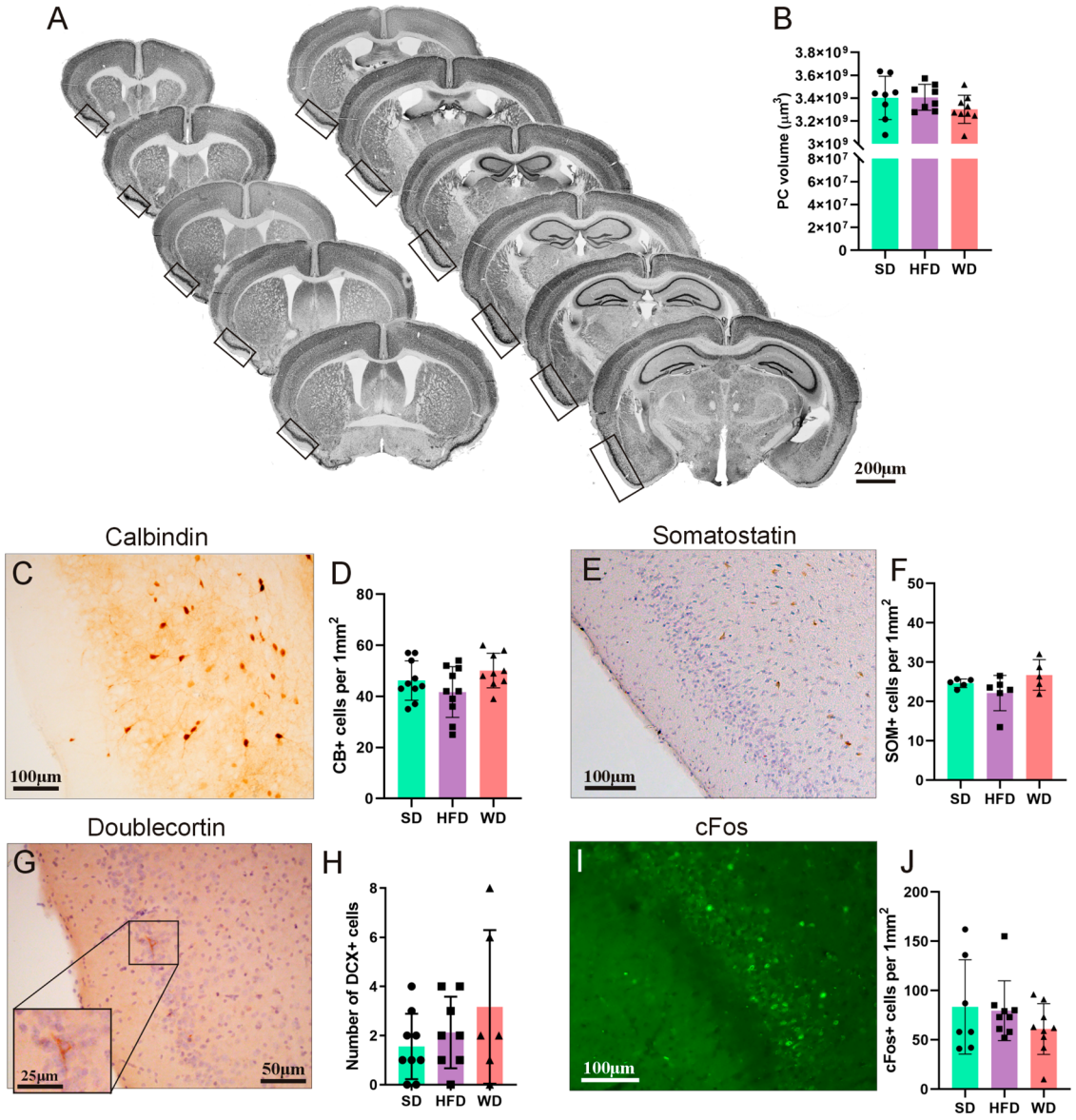

Figure 5. Obesogenic diets did not affect the total area or the number of DCX+ neuroblasts or neuronal activation in the piriform cortex. Coronal sections with marked piriform cortex (A) and the piriform cortex total area in the mouse brain (B). Representative microphotograph of calbindin (CB) staining (C) and density of CB+ interneurons in the piriform cortex of SD-, HFD-, and WD-fed mice (D). Representative microphotograph of somatostatin (SOM) staining (counterstained with hematoxylin) (E) and density of SOM+ interneurons (F) in the piriform cortex of the three experimental groups. Representative microphotograph of doublecortin (DCX+) staining (counterstained with hematoxylin) (G) and number of DCX+ immature neurons (H) in the piriform cortex of middle-aged SD-, HFD-, and WD-fed mice. Representative microphotograph of cFos staining (I) and density of cFos+ cells (J) in the piriform cortex of the three experimental groups. Welch's ANOVA test followed by two-stage step-up method of Benjamini, Krieger, and Yekutieli. Histograms show means ± SD.

HFD and WD Similarly Impair Odor Detection. Herein, we show that long-term intake of HFD and WD similarly impairs odor detection (but not odor discrimination) after 8 months but not at earlier time points. Other interesting animal studies employing obesogenic diets have shown odor detection impairment.[23,25−27] It must be underlined, however, that, in most of these studies, olfactory testing was performed only at one time point, thus providing limited information about the potential progression of olfactory impairment. For instance, Lacroix and colleagues reported impaired odor perception and decreased odor threshold in the obesity-prone rats after 8 months of WD intake.[23] Rivière and colleagues showed decreased odor detection and impaired odor discrimination in early diabetic mice fed with a diet based on high fructose for 4 weeks.[27] Moreover, results of an interesting study by Tucker and colleagues showed impairment in the detection of fatty scents, but not sweet scents, induced by 6.5 months of moderately HFD intake.[25] Authors interpreted these data as resulting from negative alliesthesia (sensation change from pleasure to displeasure), not odor detection impairment. In our study, we applied the same olfactory assay as that of Tucker and colleagues (buried pellet test) and used "sweet" treat, and mice receiving both HFD and WD showed a comparable odor detection impairment compared to that of aged-matched SD controls after 8 months of diet intake. A possible explanation for the discrepancy between the two studies could be related to differences in the diet content, because Tucker and colleagues used moderately HFD (32% energy from fat). Meanwhile, we

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used diets with higher fat intakes (54% and 42% energy from fat in the cases of HFD and WD, respectively). The same study also showed no obesity-induced changes in odor discrimination.[25] Another study of the same research group, however, reported reduced olfactory discrimination in the specific obesity-prone mouse strain that we employed, after 6 months of HFD.[26] We did not observe impairment of this olfactory parameter in our study. This could result from the fact that Thiebaud and colleagues used an olfactometer, which is a very sensitive instrument dedicated for olfactory testing.

Most of the clinical studies investigating olfactory functions related to odor sensitivity (such as detection, discrimination, and threshold) in T2D did not provide detailed information regarding the specific factors (e.g., the individual components of the diet) that could potentially influence the studied parameters. Stevenson and colleagues, however, applied a food frequency questionnaire and reported a poorer odor identification ability, but no difference in odor discrimination, in individuals consuming a diet rich in saturated fat and sugar (WD).[51]

In summary, in controlled experimental conditions, we observed a similar decrease in odor detection induced by the long-term intake of both HFD and WD diets after 8 months, suggesting that different components of the two employed obesogenic diets do not affect the deterioration speed of this process. The gain in weight was different between the two diets at 8 months also suggesting that this parameter *per se* does not play a major role. Because both groups showed significant hyperglycemia at this time point, our results suggest that this factor is involved in diminished odor detection. Taking into account that, in diabetics, a measurable decrease in olfactory sensitivity can occur before cognitive decline (see the Introduction), our data support the use of olfactory testing in obese people with T2D for the early identification of individuals at a higher risk for dementia.

WD Accelerates the Decrease of Odor-Related Learning and Long-Term Olfactory Memory Induced by HFD. Other olfactory parameters that are related to cognitive functions are odor-related learning[23,24,26] and long-term olfactory memory.[52] Previous studies have shown impairment in learning of olfactory-driven behaviors both in WD- and HFD-fed rodents.[23,26] In line with these studies, we observed impairment of novel odor recognition induced by both obesogenic diets. However, unlike Takase and colleagues,[24] we have not recorded nonassociative learning impairment either in the HFD or the WD groups. This discrepancy might be a result of methodological differences between the studies.

Olfactory memory may be an important parameter to investigate in people with obesity-induced diabetes, because its impairment in nondiabetics has been associated with aging and dementia.[53] Importantly, one study showed an association between this parameter and specific memory impairment in a population with prediabetes and diabetes.[54] A few animal studies addressing olfactory memory in diet-induced diabetes also showed impairment of short-term olfactory memory by HFD[24] and impairment of long-term olfactory memory after 8 months of WD.[23]

In the present study, we confirm that both HFD and WD impairs odor-related learning and long-term olfactory memory after 8 months. Remarkably, the WD group already presented both impairments after 3 months. This suggests an acceleration of the exacerbation of these processes by WD that likely involves the high content of sugar present in this diet. In fact, both body weight and fasted blood glucose levels were similar in HFD and WD-fed mice at this time point (Figure 1D,E). We did not measure peripheral insulin levels after 3 months of diets, and thus, HOMA (an insulin resistance indicator) was also not assessed at this time point. Likely, differences in peripheral insulin signaling could be responsible for the early differences in the olfactory parameters between HFD- and WD-fed mice, and therefore, future studies employing accurate evaluation of peripheral insulin levels, insulin resistance, and glucose intolerance (using insulin/glucose tolerance tests) in both groups are needed. We also cannot ignore that the early behavioral differences after 3 months could be a consequence of differences in central insulin resistance, and studies addressing this question are warranted.

The implications of our findings in relation to odor-related learning and olfactory memory could be relevant for understanding the role of different nutrients in the development of cognitive decline. Because WD-induced decline in odor-related learning and long-term olfactory memory appear before the decrease in odor detection, testing of these parameters could be included, and possibly even prioritized, in the screening of T2D individuals with obesity as part of preventive strategies against neurodegenerative diseases.

Mechanisms at the Basis of Olfactory Deficits Induced by the Obesogenic Diets. Studies investigating the mechanisms at the basis of obesogenic diet-induced olfactory dysfunctions have primarily focused on the main olfactory epithelium (MOE) and the MOB. Thiebau et al. observed apoptosis of olfactory sensory neurons (OSNs) in the MOE of HFD-fed mice.[26] OSNs in mice fed with a high fructose diet showed a decreased response to odorant stimulation and reduced excitability.[27] Riera et al. reported that obesity-induced loss of smell perception normalized body mass and improved insulin resistance, and loss of IGF1 receptors in OSNs improved olfactory functions in mice.[55] However, others have suggested that olfactory dysfunction is also present in HFD-fed, nonobese mice.[24] Moreover, HFD can induce the functional impairment of mitral cells[56] and also reduce the activation of specific populations of neurons (juxtaglomerular/cFos+ cells) in the MOB.[57]

In this study, we investigated whether chronic HFD and WD intake affected important neuronal processes in the MOB and PC. Our hypotheses were refuted, at least in the case of8 months of HFD/WD intake. Neither HFD nor WD affected neurogenesis in the MOB at this time point, despite the fact that previous animal studies have shown impaired neurogenesis in this area in streptozotocin-induced T1D.[38] We have also hypothesized that impairment of neuroplasticity mechanisms in the olfactory system could lay at the basis of deficits in olfactory parameters observed in obese/T2D mice. Disturbed maturation of DCX+ neuroblasts in PC has been shown in a lean model of T2D,[22] and it could be related to impaired glutamatergic differentiation of these neuronal progenitors during adult life.[58] However, we did not observe any diet-induced effects on these cells suggesting that the maturation of these specific neurons is not affected. It is also known that obesity/T2D affects GABAergic interneurons in the CNS in terms of the expression of different calcium-binding proteins and somatostatin. For instance, previous reports showed that calbindin+ interneurons in the PC were affected by T2D in a genetic rat model.[22,44] Chiazza et al. reported that HFD-induced T2D negatively affected somatostatin+ interneurons

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during poststroke recovery.[59] Considering the important modulatory function of GABAergic interneurons in the olfactory system, we investigated the potential effect of HFD/WD on these cells. We did not find any diet-induced effects either on the density of calbindin and somatostatin interneurons nor on the total PC volume or on the neuronal basal activation in this olfactory brain area. Application of semi/quantitative methods, other than IHC, could have allowed a more accurate expression assessment of the studied neuroplasticity markers in the olfactory system, and the lack of such data represents a weakness of the study. It must also be emphasized that the involvement of the investigated cellular mechanisms in olfactory decline in both PC and MOB cannot be excluded at earlier time points, and the fact that our experimental design did not include additional mice after 1 and 3 months of diet intake represents another weakness of this study.

In addition to the potential involvement of neuronal mechanisms in inducing the olfactory dysfunction, a growing body of evidence suggests that neuroglia can also be activated by obesogenic diets. Activation of microglia was observed in the MOE of HFD-fed mice,[26] and TNFα secreted by microglia can induce apoptosis in OSNs (reported in mice with olfactory deficits).[60] A recently published study has also shown an association between olfactory disfunction and IL-β-mediated inflammation and *miR-146a* overexpression in the MOB in the streptozotocin-induced T2D model.[61] Nonetheless, the detailed role of neuroglia in dysregulation of olfactory processes in obesity/T2D is still not fully understood and warrants further investigation.

Finally, future research will also have to investigate, more extensively, the interplay between the olfactory and endocrine systems in obesity/diabetes. In fact, mounting evidence suggests a direct action of metabolic peptides and nutrients on the olfactory networks (reviewed in ref 30). High mRNA levels for receptors of hormones such as leptin, insulin, and ghrelin were found in the olfactory system.[55] These hormones and their receptors take part in the modulation of olfaction depending on the nutritional status. Orexigenic (appetite stimulating) peptides such as ghrelin and orexin increase olfactory sensitivity, while anorexigenic (causing loss of appetite) hormones, such as insulin and leptin, decrease it. Although it has been known that levels of these hormones change in obesity/diabetes, the relationship between these alterations and olfactory dysfunction is not known and remains to be further studied.

CONCLUSIONS

The results of this study broaden our knowledge on the specific effect of obesogenic diets on olfactory functions in T2D. Apart from supporting the detrimental impact of HFD and WD on olfactory functions during the progression of T2D, we have also identified a specific effect induced only by WD that is an acceleration of odor-related learning and long-term olfactory memory deficits. This suggests that a high content of sugar in the diet, in addition to a substantial content of fat, may accelerate the decline of olfaction-related cognitive functions. The earlier appearance of odor-related learning and long-term olfactory memory deficits after employing the Western style diet, if confirmed in humans, could be considered when developing protocols used for screening for olfactory deficits in people with obesity and T2D. In the long-term perspective, this could allow for the application of preventive strategies against cognitive decline and dementia in these individuals.

MATERIALS AND METHODS

Ethical Approval. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed in this study. All experimental procedures were in accordance with the ethical standards of the Karolinska Institutet, where the studies were conducted (ethical approval no. S7−13).

Animal Model and Experimental Design. Thirty, male C57/BL6j mice (Charles River Laboratories, Germany) were housed in controlled conditions, in a 12 h light/dark cycle with free access to food and water. "Obesity-induced T2D" was achieved by sustained feeding with two different obesogenic diets for 8 months leading to obesity and fasted hyperglycemia (see the Results). Specifically, the mice were randomly assigned to 3 experimental groups. Starting at 5 weeks of age, they received either balanced standard chow (SD = control group; 58% energy from carbohydrates (mainly starch), 18% from fat, and 24% energy from proteins; ENVIGO 2018, Italy) (n = 10), HFD (54% energy from fat, 29% from carbohydrates (16.9% sugar), and 17% from proteins; ssniff E15126-34, Germany) (n = 10), or WD (42% energy from fat, 43% from carbohydrates (34.3% sugar), and 15% from proteins; ssniff E15721−34, Germany) (n = 10) for the next 8 months. Body weight and glycaemic levels were monitored during the study duration. Olfactory tests were performed after 1, 3, and 8 months of diet intake. Two months before they were sacrificed, mice received intraperitoneal (i.p.) injections of bromodeoxyuridine (BrdU) in doses of 50 mg/kg twice per day for 6 days. Mice were sacrificed, and their brains were collected for analyses from 9.5-month-old mice. The experimental design is presented in Figure 1A.

Body Weight, Fasted Glucose, and Insulin Levels. Body weight and blood glucose were measured in all mice 2 days before olfactory tests were performed (Figure 1A). Glucose concentration was measured using a ContourXT glucometer (Bayer, Sweden), after 10 h of fasting. Blood was collected by puncturing the tip of the mouse tail. To determine plasma insulin level, we applied Bio-Plex Pro Mouse Diabetes 8-plex assay (Bio-Rad), according to the manufacturer's instructions. Protein concentration was determined based on immunofluorescence measurement using Luminex xMAP (multianalyte profiling) technology on a MAGPIX multiplex reader (Bio-Rad, USA).

Insulin Resistance Index. HOMA-IR values were calculated from fasting insulin concentrations, using the formula HOMA-IR = [FPI (μU/mL) × FBG (mM)]/22.5, where FPI = fasting plasma insulin and FBG = fasting blood glucose.

Olfaction and Olfactory Memory Testing. To investigate potential changes induced by the obesogenic diets in olfactory functions, the following parameters were tested after 1, 3, and 8 months of diet intake:

Odor Detection. To assess odor detection ability in mice, we employed the buried pellet test, as previously described.[62] A detailed description of the test is provided in the Supporting Information. Briefly, the time for each mouse to uncover a treat hidden under the bedding was measured. If obesogenic diets significantly increased the time to find the scented treat compared to that of the SD-fed groups at the specific time point, this was interpreted as impaired odor detection ability.

Odor Discrimination. In order to test odor discrimination, we used the block test.[62] A detailed description of the test is provided in the Supporting Information. Briefly, the time the mouse sniffed each of the two wooden blocks covered with social scents, familiar (F) and unknown (N1), present in the cage was measured. Because mice should pay more attention to unknown odors, if the sniffing time of familiar and unknown scents were not significantly different, this was interpreted as odor discrimination impairment.

Odor-Related Learning. We investigated odor-related learning by testing novel odor recognition and nonassociative learning.

To test novel odor recognition, we applied the block test.[62] When presented with two wooden blocks covered with scents, familiar (F)

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and unknown (N2), the time each mouse sniffed a wooden block covered with novel, unknown scent (N2) was measured. Results were presented as the percent of total sniffing time for both scents at each time point. If the percent of the total time that the mouse spent sniffing N2 was decreased compared with that of the SD-fed control group, this was interpreted as novel odor recognition impairment.

For assessment of nonassociative learning, we performed a modified version of the Habituation Test[62] (see the Supporting Information). Briefly, the mouse was presented with a new social scent five times during one experimental day (i.e., 5 trials = T1−T5) in 5 min intervals. Because mice should spend less time sniffing the same scent after repeated presentations as they habituate to it, lack of significant difference in sniffing time between the first (T1) and any other trial (T2−T5) was interpreted as impaired nonassociative learning.

Long-Term Olfactory Memory. To test long-term olfactory memory, we employed the block test[62] (see the Supporting Information). Briefly, on the first day, the mice were familiarized with a novel social scent (1st social scent). The next day, sniffing time of the first social scent and an additional novel social scent (2nd social scent) placed together in the cage were measured for each mouse. Because mice should remember the scent presented the day before (1st) and pay more attention to the unknown scent (2nd), lack of significant difference between sniffing time of first and second scents was interpreted as long-term olfactory memory impairment.

To exclude possible differences between the experimental groups related to the motivation or activity, we also measured the time it took to approach the wooden block and movement activity. No difference in any of these parameters was detected between SD and HFD/WD mice (Figure 1S, Supporting Information).

Immunohistochemistry (IHC). Mice were administered with a lethal dose of sodium pentobarbital and perfused transcardially with saline followed by ice-cold 4% paraformaldehyde (PFA). Brains were extracted from skulls and postfixed overnight in 4% PFA and then placed in 20% sucrose solution for 3 days. Afterward, brains were cut into 30 μm thick coronal sections using sliding microtome (Leica, Germany). The sections were stained as free-floating, following antigen retrieval protocol: incubation either with 10 mM sodium citrate buffer with pH 6.0 at gradually increasing temperature from 70 to 95 °C for 25 min (anti-DCX and anti-cFos staining) or with 1 mM EDTA with pH 8.0 in 70 °C for 35 min (antisomatostatin and anticalbindin staining).

In the study, we used the following primary antibodies: rat monoclonal anti-5-bromo-2′-deoxyuridine (BrdU; Abcam, UK; Cat no. ab6326, Lot no. GR3173537-5, RRID = AB_305426, dilution 1:100), rabbit polyclonal anti-NeuN (Abcam; Cat no. ab104225, Lot no. GR247525-1, RRID = AB_10711153, 1:500), mouse monoclonal antidoublecortin (DCX; Santa Cruz Biotechnology, USA; Cat no. sc-271390, Lot no. D0517, RRID = AB_10610966, 1:200), mouse monoclonal anti-cFos (Abcam; Cat no. ab208942, Lot no. GR3264447-1, RRID = AB_2747772, 1:800), rat monoclonal antisomatostatin (Santa Cruz Biotechnology, Cat no. sc-47706, Lot no. B2806, RRID = AB_628268, 1:2000), and mouse monoclonal anticalbindin (Abcam, Cat no. ab82812, Lot no. GR137881-4, RRID = AB_1658451, 1:1500). Sections were incubated with primary antibodies for 24 or 48 h at 4 °C in PBS containing either 5% natural horse (NHS, Millipore) or goat serum (NGS, Gibco) and 0.25% Triton-X100 (BDH Laboratory Supplies, UK). Primary antibodies were detected either by biotin-conjugated (1:200, Vector Laboratories, Sweden) or by fluorescent dye-conjugated (Alexa Fluor488, and Alexa Fluor594; 1:200, Life Technologies) secondary antibodies. Sections were incubated with secondary antibodies for 2 h at RT in PBS containing 5% NHS or NGS and 0.25% Triton-X100. For chromogenic visualization of DCX and somatostatin- and calbindin-positive cells, the ABC kit (Vector Laboratories, USA) and 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, USA) was used. To counterstain the cell nuclei, we performed Mayer's hematoxylin staining according to the manufacturer's instructions (Histolab, Sweden). After we processed them, sections were mounted on microscope slides and covered with mounting medium: Pertex (Histolab, Sweden) or Vectashield (Vector Laboratories).

Quantitative Microscopy. Cells were visualized/quantified using an Olympus BX51 epifluorescent/light microscope (Olympus, Japan) and the stereology NewCAST software (Visiopharm, Denmark). However, the stereology option was not applied due to the low number of cells. Instead, microscope real-time images were displayed on the screen, and the total number of cells per section was quantified and adjusted on the area (1 mm^2). In MOB, BrdU- (density per 1 mm^2) and BrdU/NeuN-positive (%) cells were quantified on three coronal sections (from 4.28 to 3.92 mm in the distance from Bregma). PC volume measurement and quantification of total number of DCX-positive cells in PC was done on 11 coronal sections per animal (from 1.70 to −2.70 mm in the distance from Bregma). For other markers (calbindin, somatostatin, and NeuN/cFos), cell density per 1 mm^2 in PC was calculated on six coronal sections (1.10, 0.14, −0.82, −1.22, −1.82, and −2.46 mm distance from Bregma). Quantifications were performed by persons blinded to experimental groups and outcome assessment.

Statistical Analysis. Power analysis was performed to decide the adequate number of animals per group. To analyze potential differences induced by obesogenic diets in body weight, fasted glucose, fasted insulin, HOMA-IR, total cell number and cell density, and in odor detection and novel odor recognition, Welch's ANOVA followed by the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli were applied. Two values (one in WD group and one in HFD group) were outliers and have been excluded from the analyses.

To denote significant differences between the groups, we used the false-discovery-rate (FDR)-adjusted p value. To analyze potential differences induced by obesogenic diets in odor discrimination, two-way ANOVA followed by the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli were applied. To analyze differences in nonassociative learning, we applied two-way ANOVA RM. To analyze potential differences in long-term olfactory memory, the Mann−Whitney test was performed.

All data were analyzed using Graphpad Prism 8 (USA) and are presented as scatter plots or bar graphs showing means ± SD. Differences between the groups were considered significant when p values were less than 0.05 ($^*p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.0c00466.

Detailed description of olfactory tests and figure illustrating time to approach/movement activity during BT (PDF)

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Author Contributions

G.L. conceived and designed the study; performed behavioral assays, immunohistochemistry studies, and stereology analyses; acquired and processed images and figures; contributed to discussion; and wrote/edited the manuscript. T.N. provided expertise and resources, contributed to discussion, and edited the manuscript. Z.W. contributed to the conception of the study and edited the manuscript. V.D. performed part of the glycemic tests, helped with the statistical analysis, contributed to discussion, and edited the manuscript. C.P. conceived, designed, and coordinated the research plan; contributed to discussion; and wrote the manuscript.

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