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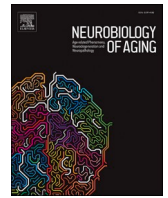
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Age-related differences in perception and coding of attractive odorants in mice

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ABSTRACT

Hedonic perception deeply changes with aging, significantly impacting health and quality of life in elderly. In young adult mice, an odor hedonic signature is represented along the antero-posterior axis of olfactory bulb, and transferred to the olfactory tubercle and ventral tegmental area, promoting approach behavior. Here, we show that while the perception of unattractive odorants was unchanged in older mice (22 months), the appreciation of some but not all attractive odorants declined. Neural activity in the olfactory bulb and tubercle of older mice was consistently altered when attraction to pleasant odorants was impaired while maintained when the odorants kept their attractiveness. Finally, in a self-stimulation paradigm, optogenetic stimulation of the olfactory bulb remained rewarding in older mice even without ventral tegmental area's response to the stimulation. Aging degrades behavioral and neural responses to some pleasant odorants but rewarding properties of olfactory bulb stimulation persisted, providing new insights into developing novel olfactory training strategies to elicit motivation even when the dopaminergic system is altered as observed in normal and/or neurodegenerative aging.

1. Introduction

Sensory information robustly elicits emotional responses which guide approach or avoidance to stimuli. Our unlearned emotional responses to stimuli, often referred to as their hedonics, possess major implications for aging and countless age-related neurological conditions wherein impairments in stimulus hedonics may impact quality of life as well as metabolic health. For example, deficit in hedonic evaluation of food odorants decreases food intake (Joussain et al. 2017) and may perpetuate bodily wasting disorders (Ali and Garcia, 2014; Frontera et al. 2000; Madsen et al. 1997) – which are common in aging and age-related dementias (Joussain et al. 2013, 2016) and represent a major public health problem. The lack of mechanistic understanding of age-related impairments in odor hedonics, especially at the neural circuit level, prevents us from developing remediation strategies.

Our team revealed the existence of a pathway in young adult mice that transmits the hedonic information of the odor from the olfactory system to the reward system to drive approach behavior. Specifically, we uncovered evidence that odor hedonics is represented along the antero-posterior axis of the olfactory bulb (OB) (Kermen, Midroit et al. 2016; Kermen, Mandairon, Chalençon, 2021) – the first cortical level for processing odor information. This neural signature of hedonic value present in the OB is transferred into down-stream brain structures. Among the main structures targeted by OB axons, we revealed recently that only the olfactory tubercle (OT, which is part of the ventral striatum) preferentially responds to attractive odorants in young adult mice, as does the ventral tegmental area (VTA) (Midroit, Chalençon et al. 2021). To further emphasize the functional connection between the OB and the reward system, we tested whether direct stimulation of OB neurons could be rewarding. Intracranial self-stimulation has been

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extensively used to determine brain areas belonging to the reward system and unravel the neural bases of motivation (Olds and Milner, 1954; Olds and Fobes, 1981; Carlezon and Chartoff, 2007). This technic consists in an operant conditioning in which the stimulation of a specific brain area serves as a reinforcer. We previously showed that self-stimulation of the OB is able to induce operant conditioning and is accompanied by an activation of the VTA in young adult mice (Midroit, Chalénçon et al. 2021). This accounted for how the olfactory system solicits the reward system to drive motivated behavior. Altogether these data showed that spontaneous attraction induced by pleasant odorants is dependent upon the solicitation of the reward circuit. However, whether this brain mechanism may be affected by aging remains unknown.

The biological substrate of the reward circuit includes dopamine (DA) release from VTA neurons in the ventral striatum. With aging and/or age-related neurological conditions, numbers of dopaminergic neurons and their receptors in the midbrain decline (Collier, Kanaan, and Kordower, 2017) as do forebrain DA levels (Araki et al. 1997; Irwin et al. 1994; Schuligoi et al. 1993; Barili et al. 1998; Watanabe, 1987; Norrara et al. 2018). Degeneration of this neuromodulatory pathway is involved in abnormal reward valuation in aging (Marschner et al. 2005; Sojitra et al. 2018) and thus may underlie odor hedonic dysfunction that is observed in aging (Wang et al. 2005; Markovic et al. 2007; Jousain et al. 2013; Vieillard et al. 2020). In this context, the first goal of this project was to assess how odor hedonics and its neural signature is modulated by aging. Since neural representation of odor hedonics has been found along the antero-posterior axis of the OB, we hypothesized changes in this neural representation linked to the change in behavioral performances. The second goal was to determine to what extent attractive odorants could still drive motivation and activation of the olfactory and reward circuits with aging as it was observed in young adult mice (Midroit, Chalénçon et al. 2021).

2. Materials and methods

2.1. Animals

133 adult male C57Bl6/J mice (Charles River Laboratories, L'Arbresle, France) were used for all experiments (86 mice in experiment 1, 47 mice in experiment 2). “younger” mice were aged of 2 months and “older” mice were aged 22 months at the beginning of the experiments. Experiments were done following procedures in accordance with the European Community Council Directive of 22nd September 2010 (2010/63/UE) and the National Ethics Committee (Agreement DR2013–48 (vM)). Mice were kept on a 12-hr light/dark cycle (22°C) with food and water *ad libitum*. Mice were housed by groups of 5, then individually after surgery (when applicable). All experiments started after at least 1 week of acclimatization to the animal facility.

2.2. Statistical analysis

All statistical analyses were conducted using Statistica. We verified normal distribution of data and homogeneity of variances for each group using Kolmogorov-Smirnov and Levene tests. If either normality or variance homogeneity was not met, non-parametric tests were applied (Friedman, Wilcoxon or Mann-Whitney tests). For data that met these two conditions, statistical analyses were performed using parametric tests (ANOVA or t-test). One-Tailed tests were performed when we had specific predictions about the direction of the difference based on the data of young adult mice. Bonferroni corrections for multiple comparisons were applied when needed. Heatmaps and permutation tests were performed using custom-made algorithms in MATLAB. No statistical methods were used to predetermine sample sizes, but sample sizes were similar to those reported in previous publications (Midroit, Chalénçon et al. 2021; Kermen, Midroit et al. 2016; Moreno et al. 2009; Mandairon et al. 2011a, 2011b; Sultan et al. 2011; Moreno et al. 2012).

2.3. Experiment 1. Olfactory performances and neuronal activity in response to odorants with different hedonic values

2.3.1. Odorants

Six monomolecular odorants were used: three attractive in young adult mice (camphor: CAM; citronellol: CITRO; +limonene: LIM) and three unattractive (pyridine: PYR; guaiacol: GUA; p-cresol: CRE). The behavioral attraction of the odorants was described previously (Kermen, Midroit et al. 2016) and replicated in two independent experiments (Midroit, Chalénçon et al. 2021; Athanassi, Breton et al. 2023). To ensure similar air concentration, the odorants were all diluted in mineral oil proportionally to their vapor pressure to reach a pressure of 1 Pascal (Pa) (Table S1) (Midroit, Chalénçon et al. 2021; Kermen, Midroit et al. 2016; Kermen et al. 2011).

2.3.2. Odorant detection

At 1 Pa, the odor concentration was shown to be supraliminal (above the detection threshold) and thus easily detected by older mice (Rey et al. 2012; Moreno et al. 2014). Here, we confirmed that older mice were able to detect odorants at 1 Pa by submitting three groups of mice to a preliminary detection test session. 28 mice aged of 22 months conducted the odorant detection test (n=10 CITRO, n=10 GUA, n=8 CAM). The session consisted of 4 successive trials (50-sec duration, 5 min inter-trial interval). During the first three trials, only mineral oil (no odor condition; NO) was presented in a tea ball hanging from the lid of the cage (habituation phase: NO1 to NO3). For the fourth trial, the tea ball was odorized with CITRO, CAM or GUA at 1 Pa (cross habituation). Time spent to investigate the tea ball was recorded (active sniffing 1 cm around the tea ball, using Volcan software (Hegoburu et al., 2009; Richard et al., 2010)). Detection was recognized as a significant change in the exploration time of the odorized tea ball when compared to the mean exploration time of the last habituation trial (NO3 versus odor trial; Two-Tailed Wilcoxon).

2.3.3. Assessment of odor attraction (or preference test)

10 younger and 25 older mice performed an olfactory preference test. Odor attraction was measured in mice using odorant investigation time (Kobayakawa et al. 2007; Mandairon, Poncelet, et al. 2009; Kermen, Midroit et al. 2016). All odorants were novel for the mice and tested once so that differences in investigation time could be ascribed to different levels of attraction and not novelty. As previously described (Kermen, Midroit et al. 2016), we used a computer-assisted one-hole-board apparatus fitted with sensors to automatically monitor the duration of nose poking into the central hole (Mandairon, Poncelet, et al. 2009; Mandairon, Sultan, et al. 2009). A polypropylene swab impregnated with 60 µL of odorant at 1 Pa was placed at the bottom of the hole, under a grid covered with clean bedding. The bedding was replaced after each trial. All mice were allowed to explore each of the six odorants and one trial was performed in a no odor control condition (mineral oil) (one odorant/day; 2 min/trial; Fig. 1A). Odorants were presented in random order and animals performed no more than two consecutive days of testing. Behavioral data from young adult mice (Figure 1B) replicated previous findings in young adult mice using an additional group (n=10 Fig. S2) (Athanassi, Breton et al. 2023; Midroit, Chalénçon et al. 2021; Kermen, Midroit et al. 2016). For each animal, investigation time of each odorant was expressed as the percentage of the no odor condition (used as a reference value, 100%) and differences from 100% was assessed by one sample t-tests.

2.3.4. Odorant stimulation and sacrifice

Over the 25 older mice that performed the olfactory preference test, 20 mice have been randomly selected to examine their odor-evoked neuronal activity. To do so, we mapped the expression of the immediate early gene c-Fos in several olfactory and reward areas. The protein c-Fos is a transcription factor whose expression reflects neural activity. Before sacrifice, mice were submitted to odorant stimulation for one

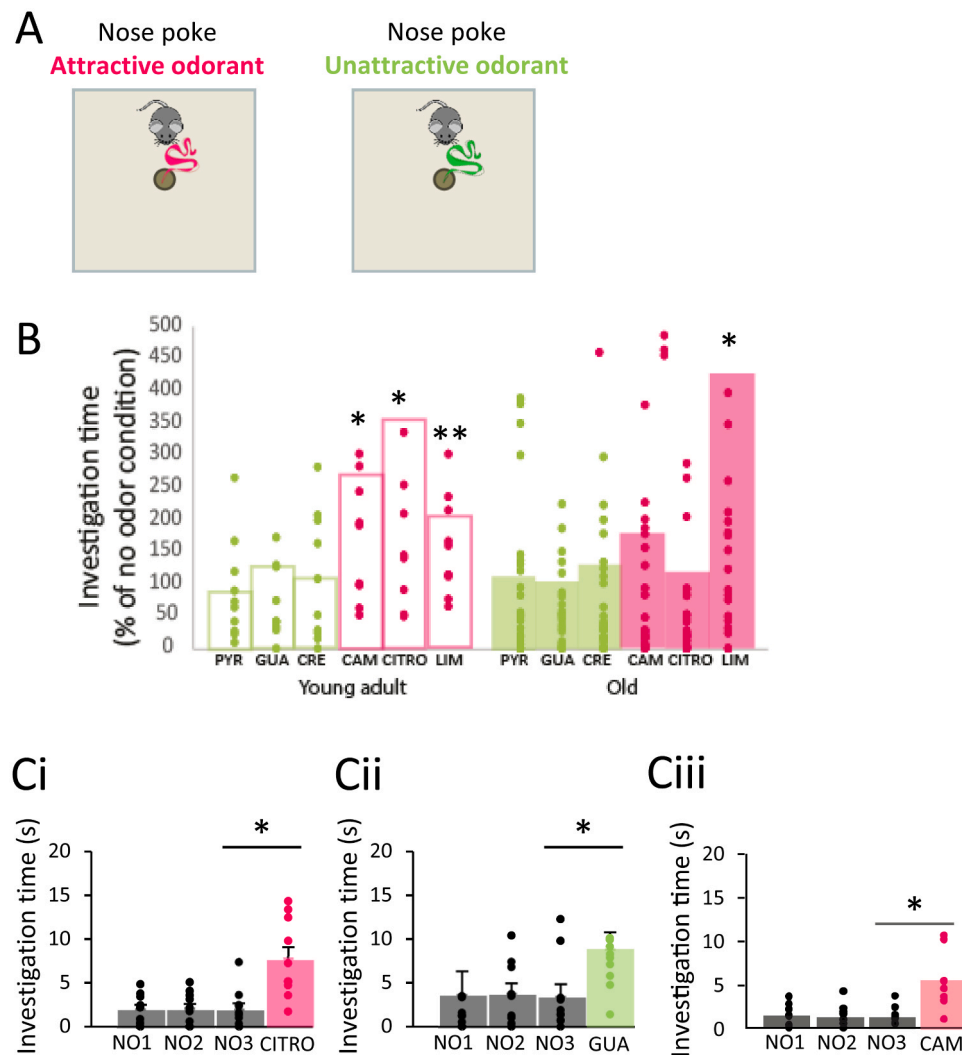


Fig. 1. Odor-driven attraction is altered during aging. **A.** Experimental setup. **B.** Investigation time measured in young adult ($n=10$) and older mice ($n=25$) in response to 3 odorants known to be attractive (pink: CAM, CITRO, LIM) and 3 unattractive in young adult mice (green: PYR, GUA, CRE). For each age condition, the mouse investigation time of each odorant was expressed as a percentage of the no odor condition (young mice, mean = 3.63 se.m. = 0.92 and aged mice, mean = 9.34 se.m. = 2.33) and analyzed by one sample t-test for difference from 100% (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). CAM, CITRO and LIM were explored more than no odor in young adult mice while in older mice CAM and CITRO were no longer explored more than no odor. LIM remained attractive and explored above no odor level. For clearer representation, 10 data points (out of 210) above 600% are not shown and are distributed as follows: in older animals, Lim: 1845.23, 1454.89, 2440.95, 1126.47; Citro: 1454.14; Cam: 1588.29, 733.33; Cre: 1522.66; Gua 681.8 and in young adult animals, Citro: 6442.94. **C.** To assess detection of odorants at 1 Pa, an habituation/cross habituation test was used. The significant difference of investigation time between NO3 and CITRO (Ci; ($n=10$)), NO3 and GUA (Cii, ($n=10$)) or NO3 and CAM (Ciii, ($n=8$)) indicated efficient odor detection by older mice. For all graphs, bars represent means of individual data points \pm sem. *: $p<0.05$; ***: $p<0.001$. PYR: pyridine, GUA: guaiacol, CRE: p-cresol, CAM: camphor, CITRO: citronellol, LIM: +limonene, NO: no odor. [Midroit et al., 2021](#).

hour and killed one hour after the end of the stimulation. This 1-hour delay has previously been shown to enable the expression of c-Fos in response to odorant stimulation ([Kermen, Midroit et al. 2016](#); [Dielenberg et al., 2001](#); [Kovács, 2008](#)). Briefly, mice were first placed in individual clean cages for 1 h, with an empty tea ball hanging from the top of the cage. A polypropylene swab impregnated with one odorant (60 μ L, 1 Pa) was then placed in the tea ball for an additional hour. Mice were sacrificed using dolethal (2 g/Kg) and intracardiac perfusion of 50 ml of fixative (paraformaldehyde 4%, pH = 7.4). The brains were removed, post-fixed overnight, cryoprotected in sucrose (20%), frozen rapidly, and then stored at -20°C before sectioning with a cryostat (14 μm thick; 210 μm intervals).

2.3.5. c-Fos immunohistochemistry and mapping in the ventral granule cell layer of the OB

For each animal, c-Fos immunohistochemistry was performed on OB sections ($n=5$ mice randomly chosen for each hedonic condition; 210

μm interval; coordinates from the bregma [antero-posterior (AP) +5.56 to +4 mm]). A rabbit anti-c-Fos primary antibody was used (1: 5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA; ref: Sc-189) and combined to biotinylated goat anti-rabbit secondary antibody (1: 200; Vector Laboratories, Burlingame, CA, USA; ref: 24541). Brain sections were then processed through an avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories). Following dehydration in graded ethanols, sections were defatted in xylene and coverslipped in DPX (Fluka, Sigma).

For each OB section analysed, the external contour of the right hemisphere was outlined and c-Fos⁺ cells were automatically counted in the ventral granule cell layer in line with our previous finding showing that the hedonic representation lies in this part of the OB. 14 sections were analysed per mouse using a mapping software (Mercator, Explora Nova, La Rochelle, France) coupled to a Zeiss microscope. All cell counts were conducted blind with regard to the experimental group. Then, the coordinates of each labelled cell (c-Fos⁺) and the contour point were

exported into a homemade software allowing high-accuracy matching of experimental brain sections with a reference brain atlas (Midroit et al. 2018). This method allows precise, automatic assignment of the labelled cells to a brain structure and permits between-group comparisons. In the atlas, we delineated the ventral granule cell layer (corresponding to the half-inferior part of the granule cell layer). The layer was represented along its antero-posterior axis with each bin corresponding to 120 μm of the layer along the antero-posterior axis (missing sections were interpolated; for more method explanations see Midroit et al. 2018). For each mouse, we first calculated the density of c-Fos-positive cells for each of the 14 bins. As the mean of the bins' density per animal could vary as a result of technical factors (i.e. difference in labeling intensity etc.). We computed z-scores on the c-Fos density of the 14 bins in order to standardize the data within each animal (mean density of the 14 bins of each animal = 0 and SD = ± 1). To compare spatial patterns of labelled cells between groups, we then averaged the standardized density maps across animals within each group and used Mann-Whitney tests to compare the c-Fos density of each bin between groups (mean density of a bin of "A" group compared to the respective bin of "UnA" or "PA" group). Non-parametric tests were used as the distribution of the density maps across animals was not normal. Importantly, z-scores and Mann-Whitney tests have been performed on different dataset (14 bins of one animal for the z-scores (1 z-score per animal), 5 animals of one group vs 5 animal of another group for the Mann-Whitney tests (1 Mann-Whitney per bin)). We compared maps of three groups of mice based on the behavioral results: unattractive (n=5), attractive in young but no more in older mice called previously attractive (n=5) and attractive (n=5) odorants. The statistical differences between groups were confirmed by odorant permutation (252 permutations) on each bin of the z-scored arrays followed by One-Tailed Mann-Whitney tests.

2.3.6. Double labelling analysis

To investigate the activity of medium spiny neurons of the OT (coordinates from the bregma [AP +2.09 to -0.11 mm]), we performed double immunostaining against c-Fos and DARPP-32 (a marker of medium spiny neuron of the striatum and the OT (Murata et al. 2015)). Briefly, rabbit anti-DARPP-32 (1: 500; AbCam, ref: 40801) and mouse anti-c-Fos (1: 500; ProteinTech, ref: 66590) primary antibodies were combined respectively with Alexa 488 goat anti-rabbit (1:250; Molecular Probes, ref: 11034) and Alexa 546 goat anti-mouse (1: 250; Molecular Probes, ref: 11030) antibodies. The percentage of double-labelled cells (c-Fos⁺ among DARPP-32⁺=DARPP-32⁺/c-Fos⁺) was assessed (5 sections/animal; ~ 220 cells/animal, 5 animals/group). One-Way ANOVA was used to test group differences followed by Bonferroni corrected *post hoc* t-tests for paired comparisons.

To investigate the activity of the VTA (coordinate from the bregma [AP -3.3 mm]), we performed double immunostaining against c-Fos and Tyrosine Hydroxylase (one section/animal; 5 animals/group). Briefly, chicken anti-TH (1: 2000; AbCam, ref: 76442) and rabbit anti-c-Fos (1: 2000; ProteinTech, ref: 26192) primary antibodies were combined respectively with Alexa 546 goat anti-chicken (1: 250; Molecular Probes, ref: 11040) and Alexa 488 goat anti-rabbit antibodies (1: 250; Vector Laboratories, Burlingame, CA, USA, ref: 32731). The percentage of double-labelled cells (c-Fos⁺ among TH⁺=TH⁺/c-Fos⁺) was calculated (1 section/animal; ~16 cells/animal, 5 animals/group) and Kruskal-Wallis ANOVA was used to test the group effect. The percentage of TH⁺/c-Fos⁺ cells was also calculated and One-Way ANOVA was used to assess the group effect.

After both immunohistochemistries, sections were cover-slipped in Vectashield medium combined with Dapi (Vector laboratories). All fluorescent counting was done blind with regards to the identity of the animal and using AxioVision (Zeiss) software coupled to a fluorescent pseudo-confocal Zeiss microscope.

2.4. Experiment 2. Optogenetic intracranial self-stimulation

The aim of the optogenetic experiment was to inhibit the inhibitory granule cells of the ventro-posterior OB using a NpHR expressing lentivirus that was shown to effectively inhibit granule cells and consequently disinhibit mitral cells using slice patch clamp recording (Kermen, Midroit et al. 2016).

2.4.1. Lentivirus injection and optical fiber implantation in the ventro-posterior OB

Prior to surgery, 47 mice aged of 22 months were anaesthetized with an intraperitoneal cocktail injection of 50 mg/kg ketamine and 7.5 mg/kg xylazine and secured in a stereotaxic instrument (Narishige Scientific Instruments, Tokyo, Japan). 150 nl of Lenti-hSyn-eNpHR3.0-EYFP lentivirus ($9,22 \times 10^6$ IU/ml, expressing halorhodopsin and the yellow fluorescent protein; NpHR mice, n=27) or 300 nl of control Lenti-hSyn-EYFP lentivirus ($1,1 \times 10^6$ IU/ml, expressing only EYFP; Ctrl mice, n=20) were injected bilaterally in the ventral granule cell layer of the ventro-posterior part of the OB (with respect to the bregma: AP, +4.3 mm; ml, ± 0.75 ; DV, -2 mm) at the rate of 150 nl/min. Following virus infusions, a dual optical fiber (200-nm core diameter, 0.22 N.A.; Doric Lenses) was implanted into the OB at the same coordinates as the virus infusion. Behavioral experiments started 8 weeks after surgery. 5 mice died immediately after surgery and 15 during the 8-week time window after surgery, resulting in 16 NpHR mice and 11 Ctrl mice available for the behavioral experiment. The rate of post-surgery death is higher than in our previous studies and could be attributed to the age of the mice (22 months) (Midroit, Chalçon et al. 2021; Forest et al. 2020; Forest, Moreno et al. 2019; Kermen, Midroit et al. 2016).

2.4.2. Optical self-stimulation in freely moving animals

8 weeks after surgery, NpHR and Ctrl mice were submitted to the conditioning phase during which they were allowed to move freely on the one-hole-board apparatus for a 2-min trial per day, during 5 days (Midroit, Chalçon et al. 2021). The bilateral continuous light stimulation (Crystal Laser, 561 nm, 10–15 mW) was automatically triggered by the entry of the mouse's nose within 1 cm (light-triggering zone) of the hole (VideoTrack, Viewpoint) and stopped automatically when the mouse's nose exited the zone (Light phase: Light1, Light2, Light3, Light4 and Light5). This phase was followed by an extinction phase (Extinc1, Extinc2, Extinc3, Extinc4 and Extinc5) in which the nose presence in the zone did not trigger light stimulation any longer and one last trial (Light1') in which light stimulation was available again (2 trials per day). Total duration of nose time spent in the light-triggering zone was recorded and Friedman tests were used to test trial effect and was followed by Two-Tailed Wilcoxon tests for specific trial comparisons.

2.4.3. Cellular analysis

All mice were sacrificed as described in Experiment 1 one hour after a behavioral session in which light stimulation was available. Behavioral performances of this session were not different from those of the last behavioral session (Light1') (Two-Tailed Wilcoxon for difference between session the day of sacrifice and Light1', NpHR post group: $p=0.782$; EYFP post group: $p=0.700$).

OB sections were double labelled after immunohistochemistries of EYFP and c-Fos proteins to control the effect of light stimulation on transfected granule cells. Briefly, rabbit anti-c-Fos (1: 5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA, ref: Sc-189) and chicken anti-EYFP (1: 1000; Anaspec TEBU, ref: 55423) primary antibodies were combined respectively with Alexa 546 goat anti-rabbit (1: 250; Vector Laboratories, Burlingame, CA, USA; ref: 11035) and Alexa 488 goat anti-chicken (1: 250; Molecular Probes; ref: A32931TR) antibodies. The total density of EYFP⁺ and the percentage of double-labelled cells (EYFP⁺/c-Fos⁺) were assessed in the ventral granule cell layer of the OB at the level of the injection site (ventro-posterior OB, 2 sections/animal; ~50 cells/animal; 27 animals) and at 0.96 mm anterior to the injection

(2 sections/animal; ~8 cells/animal) (ventro-anterior OB; coordinates from the bregma [AP +5.33 to +4.85 mm]; ventro-posterior OB; coordinates from the bregma [AP +4.49 to +3.89 mm]). Bonferroni corrected Two-Tailed Wilcoxon were used to compare EYFP⁺ density between the injection site (ventro-posterior OB) and ventro-anterior OB. Two-Tailed Mann-Whitney was used to compare the percentage of EYFP⁺/c-Fos⁺ between groups. Neural activity in the VTA and OT was assessed by calculating the percentage of c-Fos-TH and c-Fos-DARPP-32 double-labelled cells as described in Experiment 1 (Two-Tailed Mann-Whitney for between group comparisons).

3. Results

3.1. Perception of some but not all pleasant odorants becomes aberrant with aging

To test whether odor hedonics is altered during aging, we analyzed the duration of odor investigation as a reflection of attraction to odorant (Kobayakawa et al. 2007; Mandairon, Poncelet, et al. 2009; Kermen, Midroit et al. 2016; Midroit, Chalçon et al. 2021) (Figure 1A) in 22-month-old mice and in younger mice. The 6 odorants used (Supplementary Table 1) were novel for the mice and tested once. Replicating previous finding (Kermen, Midroit et al. 2016; Midroit, Chalçon et al. 2021), PYR, GUA, CRE, 3 odorants documented as unattractive in young adult mice, induced investigation times in young adults similar those of the no odor condition (one Sample t-test, PYR $p=0.1$; GUA $p=0.79$; CRE, $p=0.76$), thus showing low level of attractivity. This was also true for older mice (one Sample t-test, PYR $p=0.54$; GUA $p=0.86$; CRE, $p=0.62$; Fig. 1B). Odorants documented as attractive in young adult mice (CAM, CITRO and LIM) showed here also increased attractivity compared to the no odor control condition in young mice (one Sample t-test, CAM $p=0.01$; CITRO $p=0.03$; LIM, $p=0.005$). In older mice, CAM and CITRO were no longer attractive (one Sample t-test, CAM $p=0.22$; CITRO $p=0.77$; Fig. 1B) and only LIM remained as attractive as in young adult mice (One Sample t-test, LIM $p=0.01$; Fig. 1B).

To ensure that the changes in hedonic perception observed in aged mice was not due to a lack of odor detection in older mice, we performed a cross habituation test. In this task, a tea ball containing odorless mineral oil was presented 3 times before one presentation of a pleasant (CITRO or CAM, Figure Ci and Ciii) or unpleasant odorant (GUA, Figure Cii), at the same concentration than during the preference test (1 Pa). An increase of investigation time was observed in response to odorants compared to the last trial of mineral oil indicating that the three odorants were detected at 1 Pa (Friedman trial effect, CITRO $F_{(3,6)}=13.163$ $p=0.001$, GUA $F_{(3,6)}=9.000$ $p=0.029$, CAM $F_{(3,8)}=12.26$ $p=0.006$; Two-Tailed Wilcoxon for difference between NO3 and odorant, CITRO $p=0.013$, GUA $p=0.037$, CAM $p=0.012$; Fig. 1C).

Altogether, our data revealed an age-dependent decrease of odor-driven attraction specific to some attractive odorants that we will call now previously attractive odorants (PA).

3.2. Neural activity in the OB reflects odor attractiveness

To investigate the neural bases of the alteration of odorant attraction with aging, we compared neural activity in response to odorants that are unattractive (UnA; PYR GUA CRE), that were attractive in young adult mice but no longer in older mice (PA; CAM CITRO) and that were still attractive in aged mice (A; LIM). The expression of the immediate early gene c-Fos was used to evaluate neural activity in the OB, OT and VTA in response to the three types of odorants (Midroit et al. 2018; Midroit, Chalçon et al. 2021).

Regarding the OB, we first found no difference in global c-Fos density between the three experimental groups (One-Way ANOVA group effect, $F_{(2, 12)}=0.513$, $p=0.611$). We then focused our analysis on the antero-posterior axis of the ventral granule cell layer (Figure 2Ai-Aii) as the neural signature of odor hedonics was previously identified in this

region (Kermen, Midroit et al. 2016). Indeed, in this previous study performed in young adult mice, a higher activity was found in the ventro-anterior granule cell layer in response to attractive odorants and in the ventro-posterior granule cell layer in response to unattractive odorants (Kermen, Midroit et al. 2016). The pattern of activity in the ventral granule cell layer in older mice evoked by odorants is depicted in Figure 2Aiii and statistical comparisons between these patterns in Fig. 2B. C-Fos positive cell density was higher in the ventro-anterior OB in response to still attractive compared to unattractive odorants (A>UnA, Figure 2Aiii and 2B first line) and higher in the ventro-posterior OB in response to unattractive compared to attractive odorants (UnA>A, Figure 2Aiii and 2B first line). These results are in line with our previous study (Kermen, Midroit et al. 2016). Odorants that lost their attractive power with aging showed a pattern different from that of attractive odorants in the ventro-anterior OB (A>PA, Figure 2Aiii and 2B second line) and did not differ neither from the attractive nor from the unattractive patterns in the ventro-posterior OB (Fig. 2B, PA>A second line; UnA>PA and PA>UnA third line). The statistical significance of these results was confirmed by permutation tests (Fig. S1). Our current data revealed the persistence of a neural signature of odor hedonics within the granule cell layer similar to that of young mice, characterized by a representation of attractive odorants in the ventro-anterior OB and of unattractive odorants in the ventro-posterior OB in older mice. In addition, attractive odorant becoming unattractive with aging showed a pattern similar to that of initially unattractive odorant. The concomitant changes of odorant driven-attraction and OB odorant-evoked activity observed with aging support the functionality of this neural trace to represent hedonics, even in older mice.

3.3. Activity in the OT is enhanced only in response the odorant remaining attractive

Since the OB projects to the OT which is involved in olfactory perception and hedonic processing, we analyzed neural activity in the OT and more particularly in the relay cells (medium spiny neurons) of the OT using the medium spiny neurons-specific DARPP-32 marker. We found different levels of neural activity based on the odorant groups (One-Way ANOVA group effect, $F_{(2,3)}=13.146$ $p<0.001$; Fig. 2C). Indeed, we observed an increase of double labelled DARPP-32⁺/c-Fos⁺ cells in A group compared to UnA group (Bonferroni corrected *post hoc* t-test $p<0.001$) or compared to PA group (Bonferroni corrected *post hoc* t-test $p=0.019$). We found no difference between PA and UnA (Bonferroni corrected *post hoc* t-test, $p=0.325$). Together these data indicated an increase of medium spiny neuron activity, specifically in response to odorants that remain attractive in older mice.

To better understand the role of the reward system in hedonic processing in aged mice, we further investigated the neural activity of dopaminergic neurons (TH⁺ cells) of the VTA in the three odorant groups. Surprisingly, we found no difference in TH⁺/c-Fos⁺ cells between the three groups (Kruskal-Wallis ANOVA group effect, $H_{(2,3)}=1.078$ $p=0.583$; Fig. 2D) suggesting that attraction induced by odorants in older mice is not underlined by an increased activity in the VTA. No difference in the activity of non-dopaminergic (TH⁻) VTA neurons was neither observed between the groups (One-Way ANOVA attractiveness effect, $F_{(2,3)}=0.205$ $p=0.818$; Fig. 2E).

Altogether, these results revealed that when attraction to odorants was altered in older mice, the neural activity in the OT and VTA was impaired. Moreover, when the odorant retained its attractive power, an increased activity in the OT was observed compared to unattractive odorants, but not in VTA. This suggests that the increased in OT activity is sufficient to lead to LIM attraction. Whether behavioral attraction and its associated activity in the OT will vanish as aging progresses remains unclear.

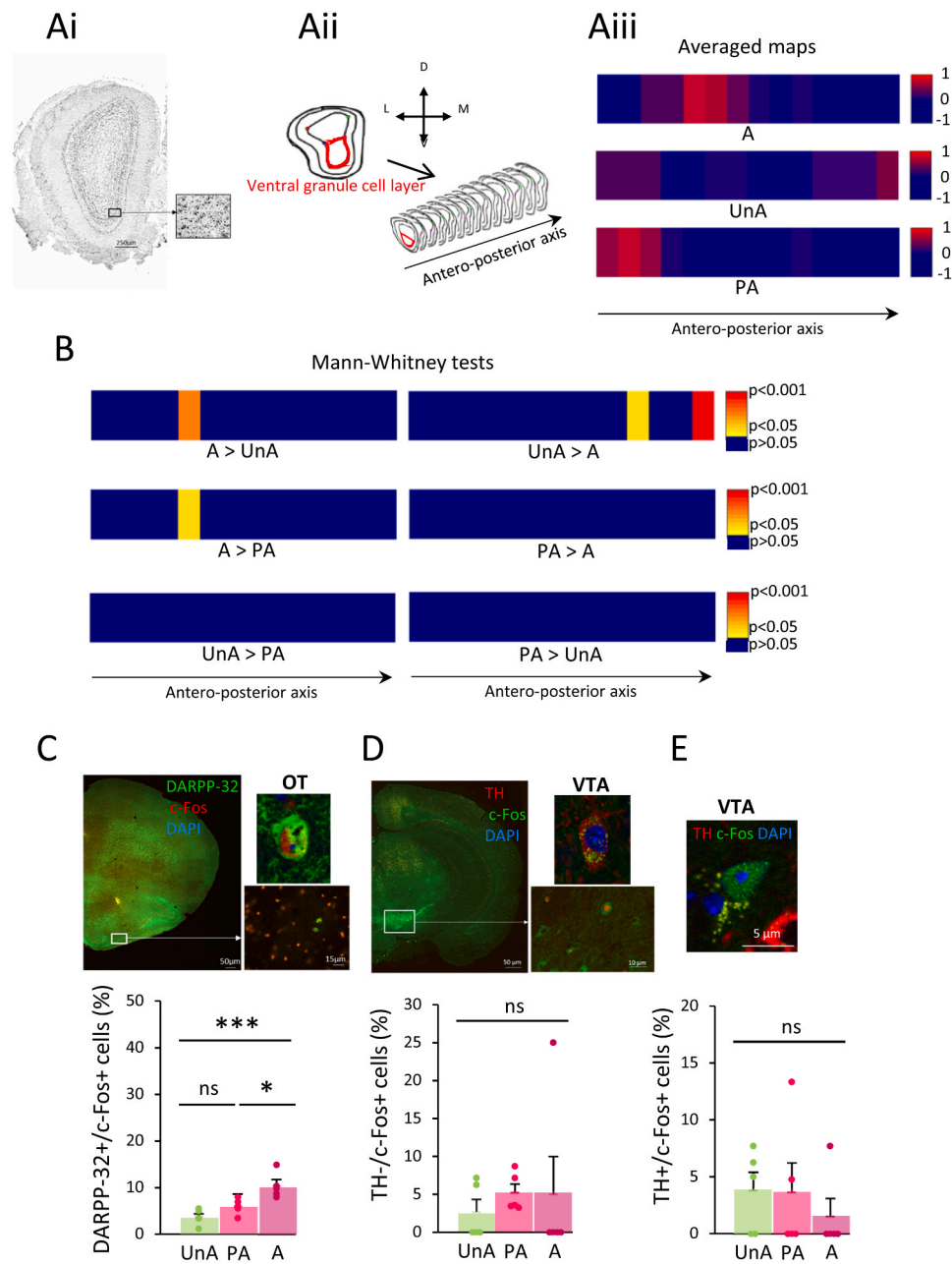


Fig. 2. Neural activity in the pOB, OT and VTA depends on the attractive power of odors. **A.** C-Fos positive-cell mapping. **(Ai).** Photo of c-Fos labelling in the OB. **(Aii).** Delineation of the ventral granule cell layer of in the OB. **(Aiii).** Averaged maps of z-scored c-Fos density (cells/mm²) in the OB ventral granule cell layer represented along the antero-posterior axis in response to attractive odors (A, n=5), unattractive (UnA, n=5) and previously attractive (PA, n=5). **B.** Mann-Whitney tests for between-group comparisons of the maps. Significance cutoff was set at $p < 0.05$. **C.** Up. Representative image of the OT on a section (left) and double labeled DARPP-32+/c-Fos+ cell in the OT (right). Down. The percentage of DARPP-32+/c-Fos+ cells was higher in response to A compared to PA and UnA odors. There was no difference between PA and UnA groups (n=5/group). **D.** Up. Representative image of the VTA on a section (left) and double labeled TH+/c-Fos+ cell in the VTA (right). Down. There was no difference in the percentage of TH+ cells expressing c-Fos in the VTA among experimental groups. **E.** Up. Representative image of TH- cell expressing c-Fos in the VTA (n=5/group). Down. There was no difference in the percentage of TH-/c-Fos+ cells in the VTA among experimental groups (n=5/group). For all graphs, bars represent means of individual data points \pm sem. * $p < 0.05$ *** $p < 0.001$.

3.4. The olfactory bulb remains a rewarding site for intracranial self-stimulation in older mice

We previously showed that the granule cell layer of the ventro-posterior OB is a site for intracranial self-stimulation in young adult mice (Midroit, Chalénçon et al. 2021), revealing the capacity of this sensory area to recruit the reward circuit to drive motivated behaviors. To evaluate the functional integrity of the connection between the OB and the reward system, we investigated here whether optogenetic stimulation in the ventro-posterior OB would retain the ability to

support operant conditioning in older mice. To this aim, a bilateral injection of Lenti-hSyn-eNpHR3.0-EYFP virus was performed in the ventro-posterior granule cell layer in 22-month old mice (NpHR group) in order to reduce granule interneuron activity and leading to a disinhibition of OB relay cells (mitral/tufted cells) (Kermen, Midroit et al. 2016). In other words, self-stimulation is applied to the ventro-posterior granule cell layer in order to inhibit the inhibitory granule cells that are preferentially activated in this area by unattractive odors (Kermen, Midroit et al. 2016). A control group was injected with a control Lenti-hSyn-EYFP virus at the same coordinates (Ctrl group). During

behavioral testing, bilateral light stimulation was automatically triggered by mice nose poking within 1 cm around the hole of a one-hole-board apparatus (light-triggering zone), and lasted as long as the nose poke (Fig. 3A). Total nose pokes duration of NpHR mice increased across days of training (Friedman trial effect $F_{(4,10)}=28.45$ $p<0.001$, Wilcoxon Light1 vs Light5 $p<0.001$; Fig. 3B), revealing that older mice learned to nose poke to receive optogenetic stimulation in the ventro-posterior OB. During the extinction phase, when the light was no longer available, nose pokes duration quickly decreased (Friedman trial effect $F_{(4,10)}=27.97$ $p<0.001$, Wilcoxon Light5 vs Extinc5 $p<0.001$). On the last trial, when the light was available again, nose pokes duration increased again (Wilcoxon Extinc5 vs Light1' $p<0.001$), revealing a reinstatement of self-stimulation. This learning/extinction/reinstatement sequence confirmed that self-stimulation behavior is timely dependent on optogenetic inhibition of granule cells and that optogenetic stimulation serves as reinforcer in the operant learning in older mice. In Ctrl mice, a modulation of nose pokes duration was observed during the initial sessions of light stimulation (Friedman trial effect $F_{(4,$

$10)=10.109$ $p=0.039$; Fig. 3B), but no significant change was observed between Light 1 and Light 5 (Wilcoxon Light1 vs Light5 $p=0.929$). The investigation time remained stable during extinction (Friedman trial effect $F_{(4,10)}=0.218$ $p=0.994$, Wilcoxon Light5 vs Extinc5 $p=0.155$) and reinstatement (Wilcoxon Extinc5 vs Light1' $p=0.131$) phases. Interestingly, during the first trial, nose pokes duration in NpHR mice was lower than in Ctrl mice suggesting a neophobic response induced by the optogenetic manipulation (Bonferroni corrected One-Tailed Mann-Whitney, Light1 $p=0.039$). It is worth noting that nose pokes duration differed between NpHR and Ctrl mice from the 4th trial of training (Bonferroni corrected One-Tailed Mann-Whitney, Light2 $p=0.812$, Light3 $p=0.081$, Light4 $p=0.010$, Light5 $p=0.008$, Fig. 3B). This time lag was no longer observed during the extinction phase (Bonferroni corrected One-Tailed Mann-Whitney; Extinc1 $p=1$, Extinc2 $p=1$, Extinc3 $p=1$, Extinc4 $p=1$, Extinc5 $p=0.315$) but reappears during re-instatement (Light1' $p=0.030$).

To verify the posterior location of viral injection and of the effect of light on neuronal activity, we quantified the density of EYFP⁺ cells and

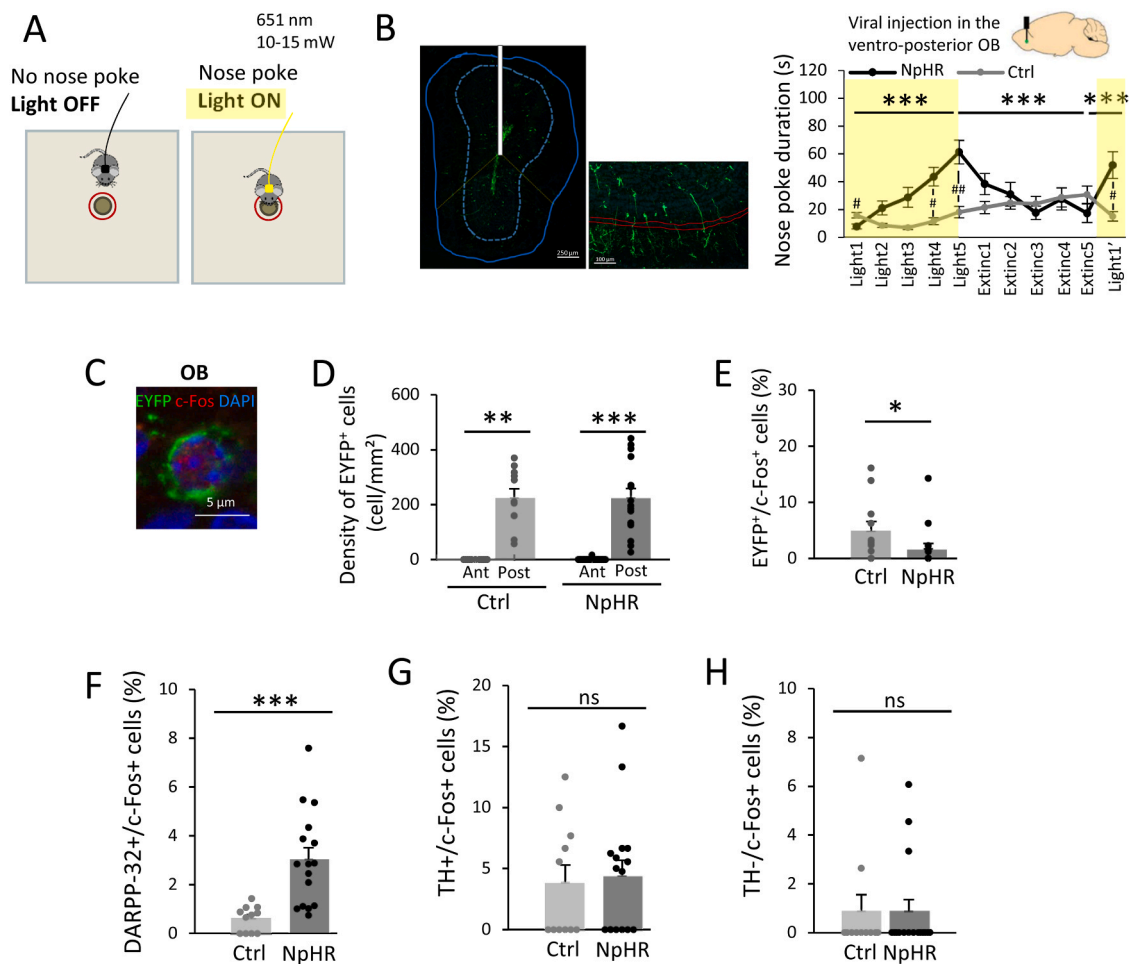


Fig. 3. The pOB remains a site for optogenetic self-stimulation in old mice. **A.** Experimental setup. **B. Left.** Image of EYFP expression in the OB. Blue line: OB boundary; dotted blue line: OB granule cell layer boundary, red line: mitral cell layer. **Right. Up.** Injection of NpHR-EYFP (NpHR, $n=16$) or control virus (Ctrl, $n=11$) in the ventro-posterior granule cell layer of the OB. **Down.** NpHR mice learned to nose poke to trigger light stimulation. This learning was subjected to extinction when light was no more available. On the last trial, light was available again and nose pokes started again. In Ctrl, nose pokes significantly decreased during the light stimulation phase, but no change was observed between Light1 and Light5. No difference was observed during extinction phase or when the light was available again. The difference between NpHR and Ctrl mice was observed from the 4th trial and was no longer observed during the extinction phase. Values represent means \pm sem. * stands for within-group differences and # for between-group differences. **C.** Representative image of double labelled EYFP⁺/c-Fos⁺ cell in the OB. **D.** Density of EYFP⁺ neurons is higher in ventro-posterior OB (the injection site) compared to ventro-anterior OB for both groups (NpHR, $n=16$; Ctrl, $n=11$). **E.** A lower percentage of EYFP⁺ cells expressing c-Fos was observed in ventro-posterior OB of NpHR compared to Ctrl mice NpHR, $n=16$; Ctrl, $n=11$. **F.** The percentage of DARPP-32⁺/c-Fos⁺ cells was higher in NpHR than in Ctrl mice NpHR, $n=16$; Ctrl, $n=11$. **G.** No difference in TH⁺/c-Fos⁺ cells was observed between NpHR and Ctrl groups NpHR, $n=16$; Ctrl, $n=11$. **H.** No difference in TH⁻/c-Fos⁺ cells was observed between NpHR and Ctrl mice. For D-H, bars represent means of individual data points \pm sem. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; #: $p<0.05$; ##: $p<0.01$.

analyzed the percentage of EYFP⁺ cells expressing c-Fos in the ventral granule cell layer of the OB. We found a higher density of EYFP⁺ cells in the ventro-posterior granule cell layer compared to the ventro-anterior part in both NpHR and Ctrl groups (Bonferroni corrected Two-Tailed Wilcoxon, NpHR $p < 0.001$ Ctrl $p = 0.007$; Fig. 3C-D) suggesting similar injection success. The percentage of EYFP⁺/c-Fos⁺ cells was lower in NpHR than in Ctrl mice (Two-Tailed Mann-Whitney, $p = 0.032$; Fig. 3E), indicating that the optogenetic manipulation efficiently induced an inhibition of ventro-posterior OB granule cells.

We then investigated the effect of self-stimulation behavior on neural activity in the OT and VTA. We found a higher percentage of activated relay cells double labelled by DARPP-32 and c-Fos in NpHR compared to Ctrl mice in the OT (Two-Tailed Mann-Whitney, $p < 0.001$; Fig. 3F). Remarkably, no difference between NpHR and Ctrl groups was observed for activated dopaminergic TH⁺/c-Fos⁺ cells (Two-Tailed Mann-Whitney, $p = 0.904$; Fig. 3G) and for the non-dopaminergic activated TH⁺/c-Fos⁺ cells (Two-Tailed Mann-Whitney, $p = 1$; Fig. 3H) in the VTA. These data indicated that while ventro-posterior OB self-stimulation is behaviorally rewarding, it led to no enhancement of the activity of the VTA in contrast to the OT suggesting less efficient recruitment of the reward system in older mice.

These results indicate that the optogenetic self-stimulation of the OB was reinforcing even without modulation of VTA activity.

4. Discussion

Age-related alteration of odor hedonics (Wang et al. 2005; Markovic et al. 2007; Joussain et al. 2013; Vieillard et al. 2020) is a major issue for the quality of life in human elderly (Joussain et al. 2017). While modifications of olfactory perception such as changes in odor detection (Bodyak and Slotnick, 1999; Kraemer and Apfelbach, 2004; Patel and Larson, 2009), fine but not gross discrimination (Moreno et al. 2014; Yoder et al. 2017; Rey et al. 2012) or learning and memory (Moreno et al. 2014; Dardou, Datiche, and Cattarelli, 2008; Kraemer and Apfelbach, 2004; LaSarge et al. 2007) have been already shown during aging in rodents, the present study revealed that as in humans (Joussain et al. 2013), an hedonic dysfunction specific to pleasant odorants is observed in older mice leading to a depreciation of these odorants. Indeed, we found that some odorants lost their attractive power while other remained attractive in aged mice. The behavioral changes we observed were ascribed to altered hedonic perception rather than to threshold or discrimination deficits. Indeed, we provided evidence that with the supraliminal odorant concentration we used, mice well detected the odorants. In addition, it appears that this age-dependent hedonic quality does not preclude discrimination between dissimilar odorants (Rey et al. 2012; Moreno et al. 2014).

Our results offer a good model to look for neural mechanisms underlying odor hedonic quality during aging and more specifically investigate the distinct pathways processing positive and negative odor hedonics.

The impact of aging at different levels of the olfactory circuit could explain changes in odor hedonics. A study in humans has shown that the spatial distribution of neural activity in the olfactory epithelium at least partly reflects olfactory pleasantness (Lapid et al. 2011). Alteration of olfactory epithelium integrity (Doty and Kamath, 2014; Naessen, 1971; Nakashima, Kimmelman, and Snow, 1984; Morrison and Costanzo, 1990; Paik et al. 1992), number of olfactory sensory neurons (Conley et al. 2003; Weiler and Farbman, 1997) and change in expression of some olfactory receptor genes have been observed with aging in humans (Cavallin et al. 2010; Khan, Vaes, and Mombaerts, 2013). In addition, some age-related modifications were observed also in the OB and secondary olfactory structures in term of volume in rodent model (Hinds and McNelly, 1981; Rey et al. 2012; Moreno et al. 2014) and human (Bhatnagar et al. 1987; Yousem et al. 1998; Sama-ul-Haq, Tahir, and Lone, 2008; Segura et al. 2013), synaptic and cellular organization (Richard, Taylor, and Greer, 2010; Gocel and Larson, 2013; Adjei et al.

2013) and odor-evoked activity (Wang et al. 2005; Suzuki et al. 2001). Interestingly, the age-related changes in hedonic appreciation has been correlated to EEG modification in beta-band oscillations in humans (Joussain et al. 2013). However, it remains unclear from these previous studies which neural alterations could be relevant to hedonic coding versus other dimension of olfactory perception such as thresholds of discrimination and importantly why the aged-related alteration in hedonics would be specific to some pleasant odorants. To shed light on this question, we analyzed odor-evoked activity in the OB, OT and VTA, a relevant pathway involved in odor-guided behavior (Midroit, Chalénçon et al. 2021).

Our previous study revealed that the ventral granule cell layer of the OB carries a neural signature of odor hedonics, with a higher activity in the ventro-anterior OB in response to pleasant odorants and in the ventro-posterior OB to unpleasant odorants (Kermen, Midroit et al. 2016). Moreover, optogenetic manipulation of these hot spots changed hedonic perception (Kermen, Midroit et al. 2016). Here, we confirmed the significance of these bulbar hot spots using aging as an ecological model of modifications of perception and of OB activity maps. Indeed, we confirmed in older mice an increase in neural activity in the ventro-anterior OB in response to an attractive odorant compared to unattractive odorants and in the ventro-posterior OB in response to unattractive compared to attractive odorant. Interestingly, when some odorants lose their attractive power, the increased neural activity in the ventro-anterior OB is no longer observed, further supporting the functional value of this pattern. The mitral cells of the OB, project onto the OT (Scott, McBride, and Schneider, 1980; Schwob and Price, 1984). Accordingly, in aged mice, we found an activation of the OT medium spiny neurons in response to attractive odorants, but not in response to odorants that have lost their attractive power or to unattractive ones. As a part of the ventral striatum receiving direct connections from the OB, the OT is considered as an open gate for olfactory information processing by the reward system (Wesson and Wilson, 2011). In addition, the OT is closely connected to the VTA (Ikemoto, 2007) and in young adult mice, pleasant odorants induced a higher activity of DAergic VTA neurons compared to unpleasant ones (Midroit, Chalénçon et al. 2021). Notably, this difference was not retrieved in older mice. In addition, since VTA glutamatergic and GABA neurons were involved in motivated behaviors (Wang et al., 2005; Morales and Margolis 2017), we also investigated the activity of non DAergic VTA neurons. No difference in activity depending on the hedonic value of the odorants was observed for non DAergic VTA neurons. These data suggest that the combined OB-OT activity is sufficient to determine the appreciation of pleasant odorants in older mice and that the lack of modulation of odor-evoked activity in the VTA may not reflect the lack of odorant positive appreciation.

Our data further raise the question of why certain odors maintain their attractiveness with aging while others do not. Our observation that LIM remained attractive in 22-month-old mice suggests that some odorants maintain their attractiveness and that neural circuits underlying these odorants are preserved. This could be due to higher ability of LIM to stimulate olfactory receptors or/and more remaining receptors able to bind LIM since individual patterns of aging related changes have been described for different receptors (no change, increase or decrease of expression; Khan, Vaes, and Mombaerts, 2013). We did not record overall higher activity in the OB in response to LIM compared to the previously attractive odorants to substantiate this hypothesis but more specific recording of individual receptor's activity would be necessary to conclude. In addition, increased activity in the OT is observed in response to attractive odorants but not unattractive or previously attractive ones. This suggests that some level of activation of the OT is required for perceived attraction to odorants. While the detailed central projection of individual mitral cells is currently unknown, it is possible that it differs depending on the receptors they activate which could contribute to this differential aging of odor hedonic perception. Furthermore, as the DAergic system is aging, an additional,

nonexclusive possibility is that the activation of the olfactory pathway by a given odorant becomes ineffective in recruiting the reward system (Collier, Kanaan, and Kordower, 2017). Still ongoing aging process that would have spared LIM perceptive properties cannot be fully excluded, even though 22-month-old is an age very close to the end point of laboratory mice life span.

Finally, from an ecological perspective, we could hypothesize that odor familiarity could play a role. The repeated exposition to an odor could strengthen its neuronal processing thanks to plasticity mechanisms but how this could apply to laboratory animals is unclear.

The results of the optogenetic experiment also support the theory that combined OB-OT activity is sufficient to drive motivated behavior in older mice, while VTA activity is impaired. Indeed, the OB has been recently described as a core component of the reward system, driving motivated behavior (Midroit, Chalénçon et al. 2021). Young adult mice are able to learn to self-stimulate their posterior OB. Here, we found that aged mice were also able to perform such OB self-stimulation. This conditioning induced an increase of OT activity compared to controls, indicating that the ventro-posterior OB was still able to recruit the reward system to drive motivated behavior. Remarkably, in aged mice the self-stimulation behavior is not accompanied by an increase of neural activity in the dopaminergic neurons of the VTA, revealing an only partial recruitment of the reward system. The lack of increased activation of the VTA in response to pleasant odorants or after optogenetic self-stimulation of the OB could reflect the overall degradation of the DAergic system that arises during aging. For instance, aging is accompanied by a reduction in the number of VTA neurons (Irwin et al. 1994; Barili et al. 1998; Norrara et al. 2018; Collier, Kanaan, and Kordower, 2017) or an impairment of DA synthesis (Araki et al. 1997; Irwin et al. 1994; Schuligoi et al. 1993; Barili et al. 1998; Watanabe, 1987; Norrara et al. 2018; Collier, Kanaan, and Kordower, 2017). Such alterations could lead to deficits in reward-related activity in the VTA. Nevertheless, self-stimulation remains effective in aged animals suggesting that the residual activity may be sufficient to drive attraction. In addition, we revealed that the OT showed increased activation during self-stimulation. As part of the ventral striatum, the OT is strongly inter-connected with the nucleus accumbens and sends projections to other structures of the reward system (e.g. orbitofrontal cortex et ventral pallidum) (Ikemoto, 2007; Wesson and Wilson, 2011; Xiong and Wesson, 2016). The activity of the OT could thus contribute to drive attraction as a gateway for soliciting the extended neural network that underlies motivation. In support of this view, the present study is the first to provide evidence that self-stimulation behavior can be achieved in aged animals despite the impairment of the VTA activation. It is unknown if this observation reflects a general pattern of the reward system activation in aging or if it is specific to OB self-stimulation. In any case, this observation and the finding that some odorants may keep their attractiveness suggest that the olfactory pathway could be used in aging as an entry to stimulate and maintain the reward system.

5. Conclusions

The present study highlights the key role of OB-OT network in olfactory perception and odor-motivated behaviors. Aged mice provide an ecological model showing that attraction to pleasant odorants is impaired when activity in these structures is altered. In addition, it revealed new functional properties of the aged brain in which the OB-OT pathway is activated for odorants that are perceived as attractive and despite no increased activity in the VTA. This opens new avenues on the use of odorant training in the elderly to elicit motivation even when the DAergic system is altered as it is observed in normal and/or neurodegenerative aging (Araki et al. 1997; Irwin et al. 1994; Schuligoi et al. 1993; Barili et al. 1998; Watanabe, 1987; Norrara et al. 2018; Collier, Kanaan, and Kordower, 2017).

CRediT authorship contribution statement

Didier Anne: Writing – review & editing, Writing – original draft, Conceptualization. **Mandaïron Nathalie:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Forest Jérémy:** Investigation. **Richard Marion:** Writing – original draft. **Thevenet Marc:** Methodology, Formal analysis. **Breton Marine:** Investigation. **Athanassi Anna:** Formal analysis, Data curation. **Chalénçon Laura:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Midroit Maëllie:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors have no actual or potential conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.neurobiolaging.2024.02.003](https://doi.org/10.1016/j.neurobiolaging.2024.02.003).

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