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Investigation of olfactory function and its plasticity

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Contents

List of Abbreviations

OB	Olfactory Bulb
OFC	Orbitofrontal cortex
PET	Positron emission topography
fMRI	Functional magnetic resonance imaging
EEG	Electroencephalography
UPSIT	University of Pennsylvania Smell Identification test
CCCRC	Chemosensory Clinical Research Center test
OERP	Olfactory event related potentials
BOLD	Bold oxygenated level dependent signal
TDI	Threshold discrimination identification
CA	Congenital anosmia
IA	Idiopathic anosmia
NC	Normosmic controls
OW	Olfactory associated words
CW	Control words
EPI	Echo planar imaging
MPRAGE	magnetization prepared gradient rapid acquisition gradient echo
SPM12	Statistical Parametric Mapping
SD	Standard deviation
TR	Repetition time
TE	Echo time
PMC	Planimetric manual contouring
URTI	Upper respiratory tract
BF	Box-frame method
GC	Gum chewers
N'GC	Non gum chewers
α	Cronbach's alpha
ACC	Anterior cingulate cortex

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Introduction

Olfactory system

Humans use multiple senses to navigate around in life, among which the sense of smell is probably the most underrated and least explored one. Humans could perceive millions of odors, which are typically bimodal in nature, activating the olfactory and trigeminal systems.

The sense of smell or olfaction is important and humans rely on it in many ways. In general, there are three main functions of olfactory system: control of foods, avoidance of environmental hazards and social communication (Croy et al., 2012).

The sense of smell is important for eating and drinking. More than 50% of people with olfactory loss report loss of appetite (Mullol et al., 2020). Olfaction is important for food flavoring, which is a combined sensation of retronasal olfaction, orthonasal olfaction, taste and somatosensation. During the mastication process, odors from food we eat is released in the oral cavity and reaches the olfactory receptor neurons through the retronasal passage, the pharynx. Therefore, studies report effects of olfactory dysfunction on eating behavior, appetite regulation, and effect on weight as well. Olfactory dysfunction leads to a change in dietary habits, as patients tend to compensate their problem and make the food more palatable by adding sweeteners, salt or spices to improve their food taste by gustatory or trigeminal information. Foraging behavior in many mammals to detect their food has been shown to depend strongly on the olfactory system (Yeomans, 2006). Humans rarely use olfaction to detect distant food however, we have the capacity to follow scent trails and it has been shown to improve with practice (J. Porter et al., 2007).

Odor contribute to hazard avoidance. In the environment there exists a range of volatile chemicals signaling presence of pathogens, predators or kin (Ajmani et al., 2016). Environmental hazards entail microbial and non-microbial sources. People with smell loss report more household accidents (White and Cunningham 2017), gas leaks and smoke (Miwa et al., 2001). People with olfactory dysfunctions significantly report detection of non-food hazards.

Lastly, olfaction mediates social communication. Odors do have an important role in kin recognition (R. H. Porter, 1998). Human body odors are important for chemical communication and have a major role in developing mother child relationship or for newborns to identify nipples of mothers for feeding. Body odors also help in mediating attractiveness perception (Herz and Inzlicht 2002), developing romantic relationships and in selection of mating partners (White and Cunningham 2017). Research shows that body odors convey information about sickness, emotions as well as traits such as gender and individuality (Groot et al. 2017). Sick humans frequently emit odors that are different and often unpleasant from those emitted by healthy

individuals (Olsson et al., 2014). Another study showed that humans could detect fear- related cues. Results from this study suggest that female participants were able to identify sweat samples from fearful donors, suggesting detection and evaluation of stimuli under some circumstances (Ackerl et al., 2002). Most social interactions also involve act of eating or drinking where impaired sense of smell would influence eating behavior, limiting one's ability to detect rotten or spoiled food (Ackerl et al., 2002; Santos et al., 2004).

Odors are said to influence mood, evoke long forgotten memories, evoke powerful experiences of pleasure or displeasure, produce alertness or relaxation (Kontaris et al., 2020). Research suggests a role of odors in human sexual behavior, maintaining hygiene, familiarity along with a source of comfort (Hierl et al., 2021). Odors are directly linked to positive or negative emotions (Walliczek-Dworschak & Hummel, 2017). It is very well known that odors even when received unconsciously can modulate mood and emotion (De Luca & Botelho, 2021). An unpleasant odor induces negative mood, whereas presence of pleasant odors has been associated to reduce anxiety, release stress and improve sleep (Villemure et al., 2006). Odors can also evoke relaxation or anxiety depending on past associations (Krusemark et al., 2013). For example, odors can evoke panic or fear in patients with post-traumatic stress disorder, while maternal odors reduce crying in infants (Sullivan & Toubas, 1998). Therefore, we can say that odors have a direct link in modulating mood, emotions, cognition, and behavior.

The trigeminal system is an additional chemosensory system, apart from olfaction and gustation. The olfactory and trigeminal systems share a close relationship. Most odors also stimulate the trigeminal nerve (Doty, 1975; Doty et al., 1978). Trigeminal chemosensory system mediates the perception of sensations such as cooling, freshness, pain, stinging, warm and burning (Frasnelli et al., 2011; Laska et al., 1997; Wysocki et al., 2003). CO_2 is an example of a pure trigeminal odor whereas odors such as menthol and mustard oil are bimodal in nature leading to the combined activation of the olfactory and trigeminal systems. Odors when present in higher concentration can also produce trigeminal sensations (Frasnelli et al., 2011).

Olfactory dysfunction

Approximately 20-25% of general population is affected by olfactory deficits, with higher prevalence in older people (Landis, 2004; Murphy et al., 2002). Apart from aging major causes of olfactory loss include head injuries, sinonasal diseases, upper respiratory infection and neurodegeneration (Damm et al., 2004; Temmel et al., 2002). Olfactory loss is gaining attention with it being an early and clinical biomarker of people with neurodegenerative diseases (Fullard et al., 2017). For some individuals olfactory loss has a direct effect on our quality of life and mental health whereas for others it remains unnoticed. Depression and olfactory dysfunction affect individuals and these can have a major impact on patient's social skills, relationships, well-being and overall quality of life (Doty, 2009; Sivertsen et al., 2015).

Approximately one in 10,000 individual is born without an olfactory sense known as congenital anosmia (Alotaibi et al., 2022). These people are born with lifelong absence of olfactory perception and aplasia or hypoplasia of olfactory bulb; the first cerebral region of the olfactory pathway (Abolmaali et al., 2002). In contrast, people with specific anosmia only have the inability to perceive a specific odor. It is a non-pathological phenomenon (Croy et al., 2015). Hyposmia (13-18 %) explains the reduced ability to perceive certain odorants and anosmia explains the lack of olfactory function. Coronavirus, responsible for COVID-19 pandemic is one recent cause of smell and taste loss in a large fraction of patients. Chemosensory deficits being the earliest and sometimes the only sign in this viral infection (Butowt & von Bartheld, 2021). In addition to the obvious olfactory symptoms, loss of olfactory function also reduces trigeminal functions (Frasnelli et al., 2010, 2011; V. Gudziol et al., 2001).

Anatomy of olfactory system

Consciously or unconsciously, we can perceive millions of odors. The nasal cavity harbours the olfactory mucous membrane. Odorant molecules first encounter receptors that are present on the cilia of olfactory sensory neurons (OSNs). Each neuron expresses a single type of receptor protein on the dendrites. However, individual odorants can bind to different receptor proteins. The axons of individual OSNs combine to form neurovascular bundles. These bundles of axons form the olfactory nerves (T. D. Smith & Bhatnagar, 2019). Axonal projections of these olfactory nerves synapse with the dendrites of mitral and tufted cells in glomeruli which are found in the OB. The OB is the first central processing region of the olfactory system (representation in coronal brain scan in figure 1), involved in a list of olfactory tasks including odor discrimination (Wilson & Sullivan, 2011). Incoming olfactory information further transfers to the primary olfactory regions including the anterior olfactory nucleus, amygdala, anterior and posterior piriform cortex and entorhinal cortex (Cleland & Linster, 2019). The piriform cortex is the largest recipient of bulbar projections. It lies along the olfactory tract at the junction of temporal and frontal lobes and continues onto the dorsomedial aspect of the temporal lobe (Anderson et al., 2003; Gottfried, 2010a; Poo & Isaacson, 2011; Wilson & Sullivan, 2011). Activity in the piriform cortex is modulated by odor dimensions such as odor identification, pleasantness, intensity, quality (Li et al., 2020) as well as cognitive processing (Bensafi et al., 2007). Information further projects to the secondary olfactory regions including orbitofrontal cortex (OFC), insular cortex, thalamus and hypothalamus (Haberly, 1998) (representation of human olfactory system presented in figure 2).

Odors regulate mood and emotions. Both regions of olfactory cortex such as olfactory tubercle and regions of secondary olfactory pathways such as OFC, insular cortex, hippocampus are involved in emotional and memory regulation. Amygdala is another region involved in emotional processing. Research suggests enhanced functional connectivity between amygdala and olfactory cortex (Krusemark et al., 2013). The connection between mood and

olfaction is neuro-functionally sustained by an overlap between olfaction and emotional areas such as the amygdala, insula, OFC, and cingulate cortex (Gottfried, Deichmann, et al., 2002; Gottfried, O'Doherty, et al., 2002). When perceiving odors the insular cortex, part of the secondary olfactory cortex, was found to be associated with improved emotional awareness or assessment of emotional status (Paulus & Stein, 2006; Soudry et al., 2011). Another region of the secondary olfactory cortex, the OFC, also activates when odors influence cognitive, social and emotional processing (Hooker et al., 2006). Activation of the OFC is also associated with the pleasantness of stimuli. Part of it is involved in the processing of reward value and affective aspects of olfactory stimuli (O'Doherty et al., 2000).

Perception of selective olfactory stimuli lead to activation in the medial OFC, amygdala, parahippocampal gyrus and cerebellum. Whereas a pure trigeminal stimulus, activates brain stem, reward processing thalamus, caudate nucleus, OFC, cingulate gyrus, post central gyrus, medial frontal gyrus, superior temporal gyrus and frontal operculum. Functional overlap between olfactory and trigeminal regions has been observed in the piriform cortex, insular regions, medial OFC and in the secondary somatosensory cortex (Albrecht et al., 2010; Boyle et al., 2007; Hummel et al., 2009). In recent years, brain-imaging techniques such as positron emission topography (PET), functional magnetic resonance imaging (fMRI) has given insights into the processing of sensory information. Investigations of the trigeminal system rely predominantly on psychophysical or electrophysiological methods. Overlap in the brain structures mediating functional processing of olfactory and trigeminal systems has been found (Boyle et al., 2007; Hummel & Nordin, 2005) .

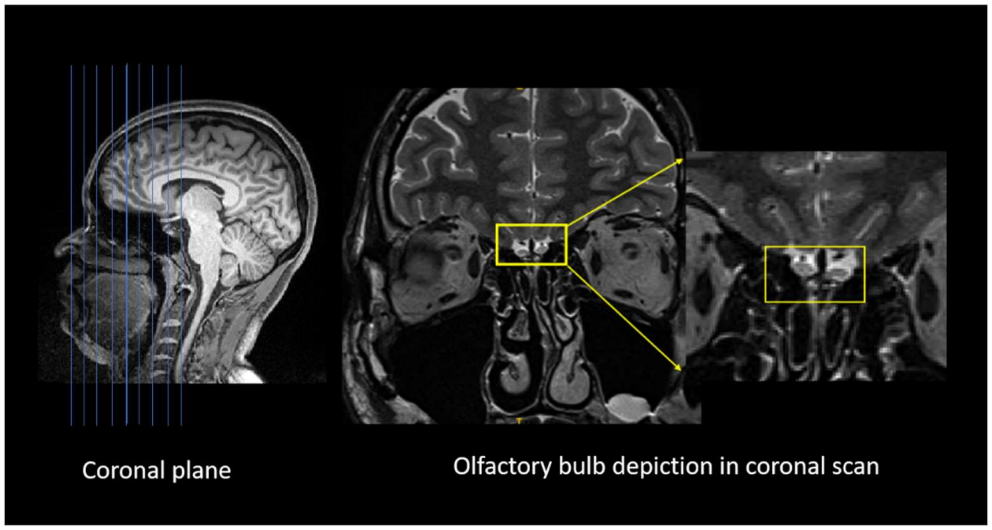

Figure 1- Olfactory bulb labelling. Coronal plane acquired perpendicular to sagittal plane to visualize olfactory bulb. Yellow boxed represent position of right and left OBs.

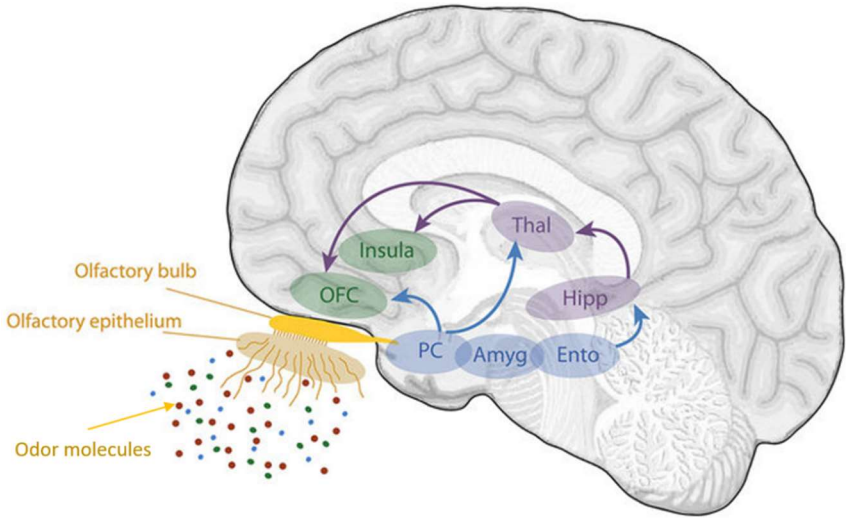

Figure 2- Schematic representation of human olfactory system. Areas in blue present primary olfactory regions; green regions represent secondary regions; purple represent tertiary regions, the dots represent binding of odors to olfactory receptors. Figure adapted from - (Saive et al., 2014, figure 1)

Chemosensory assessment

Psychophysical olfactory tests

When investigating olfactory dysfunction, the most important part is a detailed medical history. Duration of dysfunction, nature of impairments, medications should all be considered. ENT examination should also include nasal endoscopy. In special cases of idiopathic olfactory dysfunction, the patient is referred to a neurologist. When the dysfunction is suspected to be congenital, MRI is recommended. Two commonly used olfactory assessments worldwide are the 40- item University of Pennsylvania Smell Identification test (UPSIT) and the Sniffin' Sticks test. UPSIT (Doty et al., 1984) is a forced choice test kit used widely in North America with screening time of 10- 15 minutes. UPSIT comprises four booklets, each containing 10 stimuli to smell. It can be self- administered which uses microencapsulated odorants released by scratching. For identification of each stimulus, an individual chooses one of the four options provided. In addition to odor identification test, to get a clearer scenario, Cain and Rabin (Cain & Rabin, 1989) developed the Chemosensory Clinical Research Center test (CCCRC) that paired odor threshold component with odor identification to better evaluate olfactory function. Threshold testing was done by squeezing and sniffing the bottles using the method of ascending limits (Doty et al., 1996). UPSIT and CCCRC are used widely in the USA. Sniffin' Sticks is another extensive screening test used widely in Europe. It is a test battery utilizing felt-tip pens filled with odorants (Hummel et al., 2011). It is a forced choice screening consisting of three sub-sets: identification, discrimination and threshold testing. Testing starts with the threshold which is a staircase test procedure of 16 triplet pen sets. Test is prepared by diluting

the gradient odor to distinguish one pen that differs from the two odorless pens (blank). Score of threshold testing determines the lowest detectable odor concentration. Threshold test is available for n-butanol (cheese-like, harsh, alcoholic and sweet odor) or 2-phenylethanol (rose-like, flowery odor). Followed by it is another triple forced choice test of 16 sets known as discrimination test. Subject must identify one odor that is different from the other two. It is followed by 16-item identification test where the subject is asked to choose one of the four options displayed on a flash card. The total score allows to classify subjects as normosmic, hyposmic and anosmic (Oleszkiewicz et al., 2019a). In all our publications, Sniffin' Sticks kit was included to assess subject's olfactory sensitivity. If scoring is ≤ 16 points, subjects are declared anosmic; if scoring is between 16.25 and 30.5 points, subjects are declared hyposmic; and if scoring is between 30.75 and 48, subjects are declared normosmic. While testing the subject it is necessary that tests are conducted in a well-ventilated room. Examiners should wear gloves to avoid contamination as well as patients should not eat or drink 15 minutes prior to the experiment. Time gap of 3 minutes should be maintained between the sub-tests.

Psychophysical Trigeminal tests

Most odorants stimulate the olfactory and the trigeminal nerve (Doty et al. 1978; Wysocki et al. 2003). Lateralization task is one of the most used trigeminal tests. Trigeminal function is quantified by individual's ability to localize stimuli presented to either left or right nostril. Even patients with olfactory loss have persistent but reduced trigeminally mediated sensitivity. However, anosmic subjects show significantly reduced trigeminal sensitivity when compared to healthy controls (Hummel et al., 2003; Kendal-Reed et al., 2001; Walker et al., 2001). Neat 99% eucalyptol is used for the odor lateralization paradigm (Frasnelli, La Buissonnière Ariza, et al., 2010; Hummel et al., 2003). The odor is presented to one of the nostrils in a pseudo-randomized order in squeeze bottles (volume capacity of 250 ml). 30 ml of odorant is filled in one of the bottles whereas 30 ml odorless propylene glycol filled in another. Using a hand-held squeezing device a puff of approximately 15 ml air is delivered to each of the subject's nostrils. The subject localizes the trigeminal effect or cooling sensation perceived in left or right nostrils. A total of 40 stimuli are presented to blindfolded subjects with an interstimulus interval of 40 seconds. Stimuli are provided either to the left or right nostril in a pseudorandomized order. After each stimulus subjects identify the nostril where trigeminal odorant is presented. To save time, a shorter test consisting of only 20 stimuli with a similar pseudorandomized design is also used at times, maintaining a interstimuli interval of 40 seconds (Frasnelli & Hummel, 2007). Lateralization testing was used in third publication to test trigeminal effect of odors perceived.

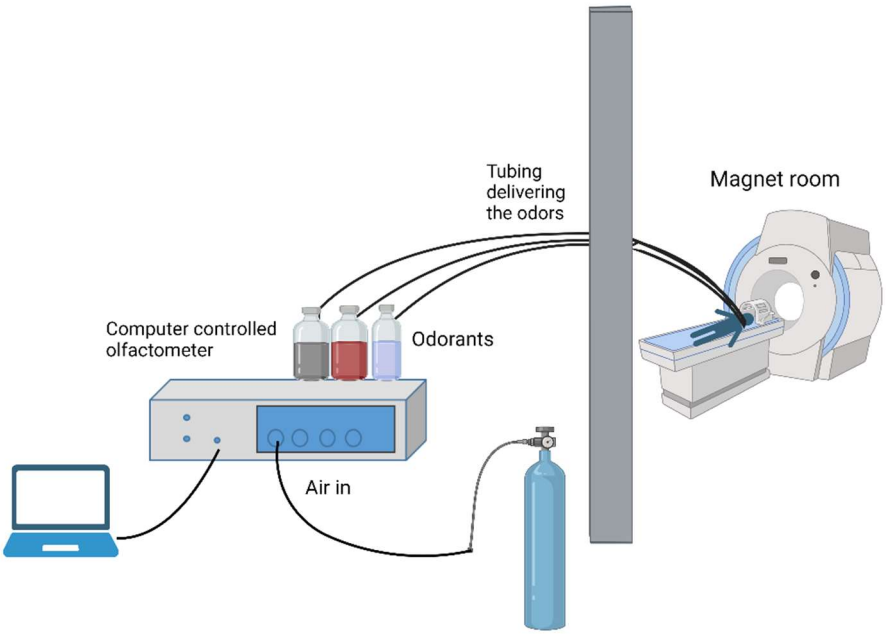

Figure 3- fMRI set up with olfactometer delivering the odors to the subject lying in the scanner (created in BioRender.com)

Electrophysiological tests - olfactory event related potentials (OERP)

Olfactory event-related potentials are a valid electrophysiological technique to study olfactory system. It is a result of sequential activation of different brain areas beginning from OB, tracts to higher order brain regions like orbitofrontal cortex, insular cortex along with the regions of temporal lobe. It is one of the objective ways to measure olfactory function and is independent of patient's response bias. Presence of OERP is a strong indicator of olfactory function whereas absence of it will indicate olfactory loss. OERP signals are characterized by three main parameters: latency, scalp topography and amplitude. We record waveforms from different areas of the scalp. This method is non-invasive and low cost. As compared to MRI, it has a much higher temporal resolution but a poor spatial resolution.

Functional magnetic resonance imaging (fMRI)

It is another non-invasive medical imaging technique used to image anatomical and physiological processes of body (Lin & Monica Way, 2014). MRI provides much greater contrast between different tissues as compared to computed tomography. It has a very high spatial resolution. Being non- invasive and without radiation exposure, it is used in human studies. The human body comprises of 3/4th water. When in presence of magnetic field, MRI takes advantage of high prevalence of water in the body that acts as dipole and causes the hydrogen atom to align in the direction of the magnetic field. When magnet switches off the proton gradually return to their original state in a process known as precession. fMRI detects

changes in the blood oxygen level- dependent (BOLD) level which is the indirect measure of neural activity. For example, when an unpleasant odor is presented in contrast to odorless air, our brain responds to the stimulus leading to deoxyhemoglobin by localized changes in the brain blood flow and influx of oxygenated blood coupled by underlying neuronal activity. Using MRI, several studies have investigated changes in odor related brain responses in patients with olfactory loss. Patients demonstrated widespread decrease of odor induced brain activation in the olfactory related regions which include OFC, piriform cortex, amygdala, insula (Han et al., 2018; Levy et al., 1999; Moon et al., 2018; Pellegrino et al., 2016). Altered odor induced brain responses were seen in patients with neurodegenerative disorders such as Parkinson's disease (Hummel et al., 2010; Takeda et al., 2010). With the help of functional imaging we found that olfactory perception is modulated by emotional, memory and cognitive processing, known as top-down modulation (Rolls, 2011a).

For olfactory based fMRI studies, variations in brain activity critically depends on factors such as odor selection and stimulation paradigm. For instance, active sniffing or passive odor exposure has a major impact on the BOLD signal in the primary olfactory areas (Wang et al., 2014). MRI has been useful to provide evidence about the functioning and the plasticity of the olfactory system. While designing an fMRI study, numerous things should be considered: choosing block design or event related design, duration of odor exposure (Han, Zang, et al., 2020; Pellegrino, Sinding, et al., 2017; Zang et al., 2020) to avoid habituation and mode of odor delivery for efficient exposure and to avoid contamination of the administered airstream with background odor.

Publication 1: Assessment of structural plasticity by measuring OB volume

Little is known about the plastic nature of the OB in humans. Its regenerative property in humans is still a topic of debate. A study by Bergmann et al., focusing on the age of OB neurons in humans concluded that age of the OB neurons equals the age of an individual and that less than 1% of OB neurons are replaced in one's entire lifetime (Bergmann et al., 2012). However, other groups reported indications for major regenerative activity in the OB (Curtis et al. 2007)(Lötsch et al., 2014).

Humans have varied OB volumes, which had been hypothesized to depend on synaptic input from olfactory receptor neurons (Hinds & McNelly, 1981; Patterson et al., 2015) In healthy subjects, OB volume was found to positively correlate with measured olfactory function, and decrease with aging (Buschhüter et al., 2008; Hummel et al., 2013; Mazal et al., 2016). OB volume varies in subjects with different olfactory pathologies. Hence, the OB volume is of clinical importance to gauge olfactory function (Buschhüter et al., 2008). As reported, change in its volume correlates well with change in odor threshold and odor identification (Haehner et al., 2008). Because assessment of OB volume requires manual delineation, it is time-

consuming and needs specific training of observers, its measurements are typically not used in routine examinations of patients with olfactory loss. This might change with the availability of tools allowing reliable but less investigator-biased and faster OB volume measurement. Hence, in publication 1 we aimed at introducing a novel method to calculate OB volumes, examining 1) test- retest reliability 2) validity comparing the generalized manual segmentation approach to the newly developed box- frame approach.

Publication 2: Assessing functional plasticity using bottom- up and top- down olfactory processing.

Stimulation with either odor molecules or olfactory associated non-chemical cues (e.g., pictures, words, metaphors) can activate the central olfactory system, representing the bottom-up and the top-down pathways for olfactory processing. For bottom-up processes, odor molecules bind to olfactory receptors before olfactory signals are transmitted via OB and are further processed in multiple olfactory related brain regions (e.g. piriform cortex, amygdala, OFC, insula, hippocampus, anterior cingulate cortex) (Djordjevic et al., 2005a; Seubert et al., 2013; Zhou et al., 2019). On the other hand, during top-down processing, absence of the physical olfactory stimuli leads to activation in the olfactory networks with the retrieval of cognitive information related to an odor (Rolls, 2011b). These top-down activations involve olfactory-related as well as other higher-order brain regions (Arshamian et al., 2013a; Djordjevic et al., 2004; González, Barros-Loscertales, Pulvermüller, Meseguer, Sanjuán, Belloch, & Ávila, 2006; Pomp et al., 2018b).

One's ability to imagine auditory and visual stimuli has been widely studied. However, it is very difficult to imagine odors. Still previous research has shown activation in the primary olfactory cortex by merely imagining the odors. Piriform cortex was activated by top- down modulating factors such as cognitive or neurophysiological processes, influenced by attention or expectation or by cross- modal associative learning (Gottfried et al., 2004; Gottfried & Dolan, 2004). In publication 2, we aimed at investigating the top-down olfactory processing in patients with smell loss and healthy controls. Using odor-related words versus control words, we hypothesized more activations in the olfactory processing areas in healthy controls and subjects with idiopathic olfactory loss as compared to individuals with congenital anosmia.

Publication 3: Assessing plasticity of chemosensory system

Adaptation is a major characteristic of the perception of odors (Pellegrino, Sinding, et al., 2017). Odor habituation is produced by repeated or continuous odor exposure leading to decreased peripheral and central responses. Odor habituation is also influenced by top-down modulators such as attention (Fallon et al., 2018). In mouse models, decreased electrical and BOLD signals were seen in the piriform cortex. It was previously reported that odor adaptation

leads to a decrease in piriform activity and increased activity in OFC (Pellegrino, Sinding, et al., 2017; Poellinger et al., 2001). Neural adaptation is also persistent to many other levels of the olfactory system including OSNs, OB, and piriform cortex (Chaudhury et al., 2010).

Repeated consumption of certain food items such as capsaicin or mint chewing gums, may lead to changes in the way we perceive them. In our publication 3, we aimed to investigate changes in chemosensory systems in response to prolonged exposure to the mixed olfactory/trigeminal stimuli (peppermint and spearmint) in frequent and non-frequent gum chewers. We hypothesized less habituation in frequent gum chewers when exposed to gum related trigeminal odors such as peppermint or spearmint.

Methods

Method 1:

Publication 1- A novel technique for olfactory bulb measurements

Subjects-

To calculate OB volumes, 52 subjects underwent magnetic resonance imaging (MRI) of the brain. All participating subjects visited the Smell and Taste Clinic at the Department of Otorhinolaryngology, University Hospital Carl Gustav Carus (Dresden, Germany) and were clinically diagnosed with smell loss. The local Ethics Committee approved the study. All subjects provided written informed consent and were tested for their orthonasal olfactory functioning using the “Sniffin’ Sticks” test battery (Hummel et al., 1997) which comprises three olfactory tests: olfactory threshold for phenyl ethyl alcohol (a rose-like odor), odor discrimination and odor identification. These tests were used to categorize olfactory loss patients as being either functionally anosmic, hyposmic or normosmic (Oleszkiewicz et al., 2019a).

MRI acquisition-

MRI data were acquired on a 3 Tesla scanner (model Prisma; Siemens, Erlangen, Germany). For the T2 weighted sequence a 32-channel head coil was used. The scanning parameters were: repetition time (TR) = 1500 ms; echo time (TE) = 78 ms; flip angle = 150˚; slice thickness = 1mm; field of view matrix = 256 x 320.

Measurement of OB volume-

OB volumes (shown in Fig 1B, publication 3) were calculated using two methods.

Manual segmentation method (MS). AMIRA 3D visualization and modeling system (Visage Imaging, Carlsbad, USA) was used to calculate the volume of right and left OB using

The plan metric manual contouring (PMC) technique (surface in mm2) (Fig 1A and 1C, publication 3). The OB sequence included acquisition of 1 mm thick T2- weighted fast spin images, in the coronal plane that covers middle and anterior portions of the skull base. A standardized PMC protocol was applied to all scans (Rombaux et al., 2012). Firstly, number of slices with clear visibility of the OB were selected. On each successive slice of brain, contours on left and right side of OB were manually drawn. The proximal end of the OB was defined by the abrupt change in the diameter at the beginning of the olfactory tract (Mueller et al., 2005; Rombaux et al., 2012).Two trained observers blind to the diagnosis and clinical characteristics of the subjects, calculated the volumes (in mm^3).

Box- frame method (BF). ITK-SNAP (version 3.8.0, University of Pennsylvania & University of Utah, www.itksnap.org) (Yushkevich et al., 2006) was used for the alternative calculations of OB volumes.

Firstly, the number of slices with distinct visibility of the OB was noted down. Further, the slice having the most visible voxels for both right and left side was chosen as the standard slice (in most cases it was the central slice). As the OB shape varies between individuals, we framed a box on it as shown in Fig 1A and 1D, publication 3. Annotations were drawn on the standard slice using Image annotation tool. With the help of this tool, we calculated the width (w) and height (h) by physically drawing a line between two extreme points of OB. For calculation of box volume, the length (l) was calculated by selecting the total number of slices which showed clear and distinct OB, multiplied by the slice thickness (1mm) ($V = l*w*h$, in mm^3). Two expert observers (AJ, XY), blind to the subject's condition calculated the volumes of right and left OB's. When the difference exceeded 10%, a third expert observer calculated the volumes again. After input of the third observer, two closest volumes with less than 10% difference were selected.

The idea for proposing the BF approach was also its usability by non-experts in neuroimaging. Accordingly, we checked its performance by non- expert observers who belonged to a different background with no imaging experience. They were well explained how the technique works and were asked to do the measurements in all of the subject population. Following the same rules, when the difference exceeded 10%, a third non-expert observer calculated the volumes again.

Out of the total 52 subjects, five subjects were excluded due to unclear OBs and lack of subject's information and finally, volumes of 47 subjects were analyzed and compared for left and right OB volumes. Out of them, 36 subjects had reduced olfactory functioning due to an infection in the upper respiratory tract (URTI), eight were diagnosed with idiopathic olfactory loss (ID) and three had congenital anosmia.

Statistics-

The Statistical Package for Social Sciences version 25.0 (IBM SPSS 25.0, Chicago, IL, USA) was used for statistical analysis. Table 1, publication 3 shows the characteristic information for all subjects (means ± SD). A paired t-test was done to compare volumes of right and left OB as calculated by observers 1 and 2 using both methods. Furthermore, using Pearson correlation, inter-observer reliability was investigated for the volumes calculated by MS (AMIRA) and BF (ITK-SNAP) method. The level of significance was set at 0.05.

Method 2

Publication 2- Neural processing of olfactory-related words in subjects with congenital and acquired olfactory dysfunction

Participants-

Participants were recruited from the residents of Dresden area (control participants) and the Smell and Taste Clinic, Department of Otorhinolaryngology, University Hospital Carl Gustav Carus, Dresden (patients). All participants received the “Sniffin’ Sticks” olfactory test (Hummel et al., 1997). A composite odor threshold, odor discrimination and odor identification score (TDI score) was used to classify normal olfaction (TDI > 30.5) and anosmia (TDI < 16.5). In order to ascertain anosmia in congenital anosmia (CA) patients, olfactory event related potentials recordings were done, and in none of the patient’s olfactory event related potentials were detected (Frasnelli et al., 2007). CA subjects were diagnosed with lack or hypoplasia of olfactory bulb and a life-long olfactory dysfunction without other known etiology. Patients with idiopathic anosmia (IA) were those patients with no cause for their olfactory dysfunction found after detailed clinical investigations (including medial history questionnaires, psychophysical olfactory testing, olfactory pathways morphology assessment) (Rombaux et al., 2006). In addition, participants completed the German version of Beck Inventory [ranging from normal state (1–10) to extreme depression (over 40)] (Beck et al., 1996) and the Montreal Cognitive assessment (ranging from 0 to 30) (T. Smith et al., 2007) for assessing the level of depression and executive functions, respectively.

Forty participants took part in the study. Of those, eighteen were control participants with normal olfaction (NC) (mean age 49.2 years; SD 12.2; 10 females), 14 were patients with congenital anosmia (CA, mean age 37.4 years, SD 18.9; 7 females) and eight patients with idiopathic anosmia (IA, mean age 56.4 years; SD 10.8; 4 females, disease duration ranging between 9 and 108 months). The study was approved by the Ethics committee at the medical faculty of the Technical University of Dresden. The experiment was conducted according to the Helsinki declaration. All participants provided written informed consent.

Study design-

For our experimental design, 36 words with strong olfactory association (OW) and 36 words with little or no olfactory association i.e. control words (CW) were presented to the participants lying in the scanner. Apart from the 24 new words as displayed in Table 1 (publication 2) for convenience of later analysis some words were randomly repeated to have a block time of 20 s each.

We chose the words with higher olfactory association as reported by (Han, Croy, et al., 2020). Briefly, 50 words with olfactory association and 50 words with little or no olfactory association were screened and rated by experts. Through a pilot study, 18 normosmics were asked to rate the randomly presented OW and CW words for the degree of olfactory association using a numerical scale ranging from 0 to 5. Combining the ratings from expert selection, CW had a mean score of 0.4 (SD 0.3) whereas OW had a mean rating score of 3.2 [(SD 0.9); t (17) = 13.5, $p < 0.001$](Han, Croy, et al., 2020).

The participants were instructed to covertly read the instructions and words. Cueing prior to word blocks was adopted to guide participants to (1) focus on the olfactory aspects of the displayed words (2) induce an expectation for the following words; and (3) to clearly separate the OW from the CW blocks. Olfactory related semantic differences were chosen as a criterion to differentiate between conditional activation. However, no control on the word frequency was marked on. The word length (e.g. number of characters in each word) was taken into consideration during selection; however, no statistical analysis was performed on this. Specifically, the experimental run contained 24 blocks in total with 12 blocks each of OW and CW; displayed in an alternating pattern. For each block, the expectation was induced with a slide showing for 2.5 s with the term 'Words with smell' (German: 'Wörter mit Duft') or "Words with no smell" (German: 'Wörter ohne Duft') followed by a 1-s fixation cross, making the expectation task for 3.5 s. The reading phase included three OW or CW presented for 2.5 s each, with 1-s intervals between words, making the reading task for 10.5 s. During inter-block intervals, a fixed cross was shown for 6 s. Each block was of 20 s. The order of the words within each block was randomized among participants. In the complete experiment, we had 36 OW + 36 CW words in total scan time of 480 s = 8 min ((12 + 12) × 20 s/block). A simplified diagram of the fMRI design is depicted in Fig. 1 (publication 1).

Imaging data acquisition and preprocessing-

Functional and structural brain images were acquired on a 3-T MRI scanner (Siemens Prisma, Erlangen, Germany) equipped with an 8-channel head coil. A total of 220 functional images were collected per individual using a T2 single-shot echo-planar imaging (EPI) sequence: TR = 2000 ms, TE = 40 ms, 90° flip angle13, voxel size 3 × 3 × 3.75 mm, no interslice gap, 192 × 192 mm field of view. A high-resolution structural T1 image was acquired using a 3D

magnetization prepared gradient rapid acquisition gradient echo (MPRAGE) sequence (TR = 2530 ms, TE = 2.34 ms, 256 × 256 mm field of view, voxel size 1 × 1 × 1 mm).

SPM12 (statistical parametric mapping) was used to analyze the functional MRI data, which is a MATLAB (The Mathworks Inc., Natick, MA, USA) based software from Welcome Trust Centre for Neuroimaging, London, UK. Default settings were used for pre-processing of data including-realignment, unwarping, co-registration, segmentation, smoothing and normalization. For all the subjects, head movement artifacts were further removed using ArtRepair software (version 4, Stanford University) (Pomp et al., 2018a), after which neuroimaging data of one control subject was discarded due to excessive movement. In the end, data set included functional images of 14 CA, 8 IA and 16 NC participants.

fMRI analysis-

On the single-subject level, the conditions for OW expect and OW read were calculated as follows: OW expect = (OW − NW) expect, and OW read = (OW − NW) read. Further, on the group level the contrast images from everyone were subjected to a random effect analysis to test specific research questions: (1) one-way ANOVA analysis was used to test between-group differences regarding OW expect; (2) the one-way ANOVA analysis was used to test between-group differences regarding OW read. Age, sex, and BDI scores were controlled in the models in the SPM 2nd level model. Significant brain activation was searched on the whole-brain level. To control for multiple statistical testing within the entire brain, we maintained a cluster-level false-positive detection rate at $p < 0.05$ using an initial voxel-level threshold of $p < 0.001$ with a cluster extent (k) empirically determined by Monte Carlo simulations (n = 1,000 iterations), by means of AlphaSim procedure (Forman et al., 1995). This was done using the REST toolbox (https://www.restfmri.net/forum/REST_V1.7) (Song et al., 2011). A minimum cluster size (number of contiguous voxels) was determined for each specific contrast to achieve a cluster-level Family-Wise Error corrected $p < 0.05$, and were reported as part of the results. Significant brain regions were labelled and reported with the AAL toolbox (Tzourio-Mazoyer et al., 2002) . The activation levels (contrast estimates) in significant clusters were plotted for each group (NC, IA, CA) using the plot function in SPM.

Statistical analyses for behavioral data-

Behavioral and socio-demographic measurements (“Sniffin’ Sticks” test score; BDI score; MCAT score) were analyzed using IBM SPSS version 2.4 (SPSS Inc., USA, Chicago) using one-way ANOVA, including age and sex as co-variables of no interest. The significance level for all the statistical tests was set at $p < 0.05$ unless specified. Results are represented as means ± standard deviation (SD).

Method 3:

Publication 3- Habitual Exposure to Trigeminal Stimuli and Its Effects on the processing of Chemosensory Stimuli

Participants-

Forty healthy subjects (m = 22, f = 18) with a mean age 25 ± 3 years (age range 18–40 years) were recruited in the study. Subjects received a structured medical history (Welge-Luessen et al., 2013), which, among others, included questions on demographics, smoking and drinking habits, medications, current disorders, family history of any neurodegenerative disease and general nasal health status. GC and N'GC subjects were identified based on a questionnaire asking about their mint consumption patterns. Subjects in the GC group (n = 20) chewed gums at least twice a day; used mint toothpaste, consumed peppermint tea or related foods frequently. N'GC (n = 20) consumed little or no chewing gums, mint toothpaste or any other mint related food items.

All subjects reported a normal sense of smell, which was ascertained using an odor identification test (with maximum score of 16) from the ''Sniffin' Sticks" olfactory test battery (Oleszkiewicz et al., 2019b). This test is performed within a forced choice paradigm where subjects have to identify 16 odors at supra- threshold concentrations using flash cards with four descriptors each (Cain & Rabin, 1989; Kobal et al., 1996). Participants reached an average score of 13.6 ± 1.4 (mean ± SD). The experiments were conducted according to the Helsinki declaration. The ethics committee at the medical faculty of the Technical University of Dresden approved the study design. Subjects were recruited by putting flyers on campus. They provided written informed consent.

Study design-

Subjects were pretested for odor identification score, followed by threshold testing and lateralization test. After checking for their normal olfactory and trigeminal sensitivity subject's underwent fMRI scanning. During fMRI measurements, odorous stimuli were presented using a portable and computer-controlled olfactometer (Sommer et al., 2012), embedded in a 2 L/ min constant airflow. Each functional run was comprised of 11 OFF or ''baseline" blocks and 10 ON blocks. From the main block design (Fig. 1, publication 2), 10 blocks of ON and OFF phases were analyzed to increase stimulus related BOLD signals.

Odors delivered intranasal during the ON session for 8 seconds and odorless air for 12 seconds during the OFF session. Each subject underwent four functional runs with one type of odor presented in one run. Across subjects, the sequence of these runs was randomized using a Latin Square. After each run, subjects were asked to rate the intensity and pleasantness for the odor presented. The intensity scale ranged from zero (unnoticeable) to 10 (very strong) and pleasantness ranged from zero (extremely unpleasant) to 10 (extremely

pleasant) with five being neutral. Subjects communicated orally through the scanner intercom system.

Odor stimuli-

We selected four odors for testing (provided by Takasago, Paris, France): Peppermint (order number ABX321352), Spearmint (ABX321351A), Strawberry (ABX321354A), and Cherry (ABX321603). These odors resemble flavors used in chewing gum. Peppermint and spearmint are minty, majorly trigeminal odors, whereas cherry and strawberry are non-minty, olfactory odors.

Psychophysical testing-

In the pilot study, intensities of all the presented stimuli were checked using visual analog scales so that they were perceived as isointense ($F [3, 76] = 2.11$, $p = 0.10$). Pure odors with no dilution were used. The isointense levels of odors were used for the lateralization task (Frasnelli et al., 2011; Hummel et al., 2003) which was performed to gauge trigeminal sensitivity for the 4 odors used.

In addition, odor thresholds were obtained for each of the four odors. Participants were repeatedly exposed to high and low odor concentrations (score range 1–8) (Laing & Doty, 2003). Subjects receive triplets of odors presented in glass bottles (4 ml liquid odor; bottles with 50 ml volume, diameter of opening 4 cm) and have to discriminate one bottle with odorous solution from two others containing the diluent propylene glycol (Sigma-Aldrich, Deisenhofen Germany; order number 398039). This test was done to identify the least distinguishable concentrations for each of the four odors used (Croy et al., 2009). All performed tests were based on a forced choice paradigm.

fMRI data acquisition and analysis-

Functional brain images were acquired on a 3-T MRI scanner (Siemens Verio, Erlangen, Germany) using a 32- channel head coil. 248 functional images were collected per individual using a T2 single-shot echo-planar imaging (EPI) sequence: TR = 869 ms, TE = 38 ms, 58˚ flip angle, no interslice gap, 210 x 210 mm field of view. A high-resolution structural T1 image was acquired using a 3D magnetization prepared gradient rapid acquisition gradient echo (MPRAGE) sequence (TR = 2000 ms, TE = 1.95 ms, 256* 256 mm field of view, voxel size 1x1x1 mm).

From the 10 ON and OFF phases used for analysis, we compared ON phases for presented stimuli and checked for group wise contrasts ON trigeminal versus ON olfactory for N = 40. Lateralization score, intensity, hedonics and threshold test scores were used as covariates. Whole brain activations significant at FWE corrected < 0.05 and p uncorrected < 0.001 with cluster size (k) > 10 voxels are reported. For clusters having multiple peaks, one with the

highest t- value is chosen. MNI coordinates are presented at x, y and z, L – left hemisphere, R – right hemisphere. Significant brain regions were labeled with AAL3 toolbox (http:// www.gin.cnrs.fr/en/tools/aal-aal2/) (Rolls et al., 2020).

Statistics-

Analyses were performed separately for each odor. In addition, to have an elaborated view about how individuals respond towards olfactory and trigeminal stimuli and how neural processing differs for the two sensory channels, we grouped the minty-trigeminal stimuli peppermint and spearmint under 'Trigeminal Group' whereas the non-minty olfactory stimuli strawberry and cherry were grouped under 'Olfactory Group'.

Analysis of psychophysical data: Paired sample t-test was performed to compare the intensity, hedonics, lateralization and threshold test scores for trigeminal (peppermint + spearmint) and olfactory (cherry + strawberry) groups for the total study population and for differences between groups (GC vs N'GC). For analysis, IBM SPSS version 27 (SPSS Inc., Chicago, Ill., USA) was used. The significance level for all statistical tests was set at $p < 0.05$ unless specified. Results presented as mean ± standard deviation (SD). Chi-square test was done comparing males and females in both groups.

Analysis of MRI results-

Task-driven general linear model approach (GLM) using Statistical Parametric Mapping (SPM) software was used for analysis of olfaction/ trigeminal-based fMRI. SPM12 was used to analyze the functional MRI data, which is a MATLAB (The Mathworks Inc., Natick, MA, USA) based software (Welcome Trust Centre for Neuroimaging, London, UK). On single-odor level, one sample t-test was performed for the ON condition. Furthermore, for group level, analysis contrast images from individuals were selected for a random effect analysis and paired t-tests were per-formed, between and within groups (GC vs N'GC). Results are presented as mean ± standard deviation (SD).

Contributions in publications

Publication 1: Conceptualization, methodology, conduct of experiments, data analysis, project administration, writing manuscript.

Publication 2: Conceptualization, methodology, conduct of experiments, data analysis, project administration, writing manuscript.

Publication 3: Conceptualization, methodology, conduct of experiments, data analysis, project administration, writing manuscript.

Publication 1 (First study) A novel technique for olfactory bulb measurements.

Abstract of publication 1

Background

To introduce new ways to calculate OB volumes, checking their validity and comparing them to already established technique i.e. OB volumetric based on manual segmentation of OB boundaries.

Methods

Two approaches were used to calculate OB volumes (1) Manual Segmentation using plan metric manual contouring; (2) Box-frame method, calculating the parameters based on a box placed around the OB.

Results

We calculated OB volumes using both techniques and found comparable outcomes. High inter-observer reliability was found for volumes calculated by both observers. For manual segmentation, Cronbach's alpha (α) was 0.91 and 0.93 for right and left OB volume, respectively, whereas for the box-frame method αwas 0.94 and 0.90 for right and left OB, respectively.

Conclusions

The simple box-frame method of OB volume calculation appears reliable. Its results are comparable to an established technique.

PLOS ONE

RESEARCH ARTICLE

A novel technique for olfactory bulb measurements

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Abstract

Background

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Two approaches were used to calculate OB volumes (1) Manual Segmentation using planimetric manual contouring; (2) Box-frame method, calculating the parameters based on a box placed around the OB.

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Conclusions

The simple box-frame method of OB volume calculation appears reliable. Its results are comparable to an established technique.

Introduction

The olfactory bulb (OB) is a highly significant structure in the processing of olfactory information. It is the first relay station from the peripheral olfactory system to higher order processing of olfactory information. In animals, OB continuously replace its local GABAergic interneurons which signifies [1–3] continuous generation of new neurons throughout lifetime [4].

Competing interests: The authors have declared that no competing interests exist.

From the sub-ventricular zone (SVZ), the OB receives progenitor cells through the rostral migration stream, which have the property of differentiation [4]. These newly born adult cells further integrate into an already existing OB neural network, hence adapting its function [5].

Less is known about the plastic nature of the OB in humans. Its regenerative property in humans is still a topic of debate. A study by Bergmann et al., focusing on the age of OB neurons in humans concluded that age of the OB neurons equals the age of an individual and that less than 1% of OB neurons are replaced in one's entire lifetime [6]. However, other groups reported indications for major regenerative activity in the OB [7]. In addition, the influx of neurons from the SVZ to the OB had been described in humans [8] which compares to animals [2].

Humans have varied OB volumes, which had been hypothesized to depend on synaptic input from olfactory receptor neurons [9, 10] In healthy subjects, OB volume was found to positively correlate with measured olfactory function, and varying with age [11–13]. OB volume varies in subjects with different olfactory pathologies. For example, subjects with congenital anosmia may have under-developed or no OB, whereas reduced OB volume was reported in subjects with post-infectious and post-traumatic olfactory loss [14]. As an exception to this rule, Weiss et al. reported normal olfactory functioning in women who did not have clear and distinct OB [14–16].

The OB volume is of clinical importance to gauge olfactory function [13, 17, 18]. As reported, change in OB volume correlates well with odor threshold and odor identification [19]. Moreover, because assessment of OB volume requires manual delineation, it is time-consuming and needs specific training of observers. Hence, OB volume measurements are typically not used in routine examinations of patients with olfactory loss. This might change with the availability of tools allowing reliable but less investigator-biased and faster OB volume measurement. Hence, the aim of the present study was to introduce a new way to calculate OB volumes, examining (1) its test- retest reliability and (2) validity, comparing them to the established technique, i.e. OB volumetric based on manual segmentation of OB boundaries (3) checking usability of the new technique by experts and non- experts.

Methods

Subjects

To calculate OB volumes, 52 subjects underwent magnetic resonance imaging (MRI) of the brain. All participating subjects visited the Smell and Taste Clinic at the Department of Otorhinolaryngology, University Hospital Carl Gustav Carus (Dresden, Germany) and were clinically diagnosed with smell loss. The local Ethics Committee approved the study. All subjects provided written informed consent and were tested for their orthonasal olfactory functioning using the "Sniffin' Sticks" test battery [20] which comprises three olfactory tests: olfactory threshold for phenyl ethyl alcohol (a rose-like odor), odor discrimination and odor identification. These tests were used to categorize olfactory loss patients as being either functionally anosmic, hyposmic or normosmic [21].

MRI acquisition

MRI data were acquired on a 3 Tesla scanner (model Prisma; Siemens, Erlangen, Germany). For the T2 weighted sequence a 32-channel head coil was used. The scanning parameters were: repetition time (TR) = 1500 ms; echo time (TE) = 78 ms; flip angle = 150˚; slice thickness = 1mm; field of view matrix = 256 x 320.

Measurement of OB volume. OB volumes (shown in Fig 1B) were calculated using two methods.

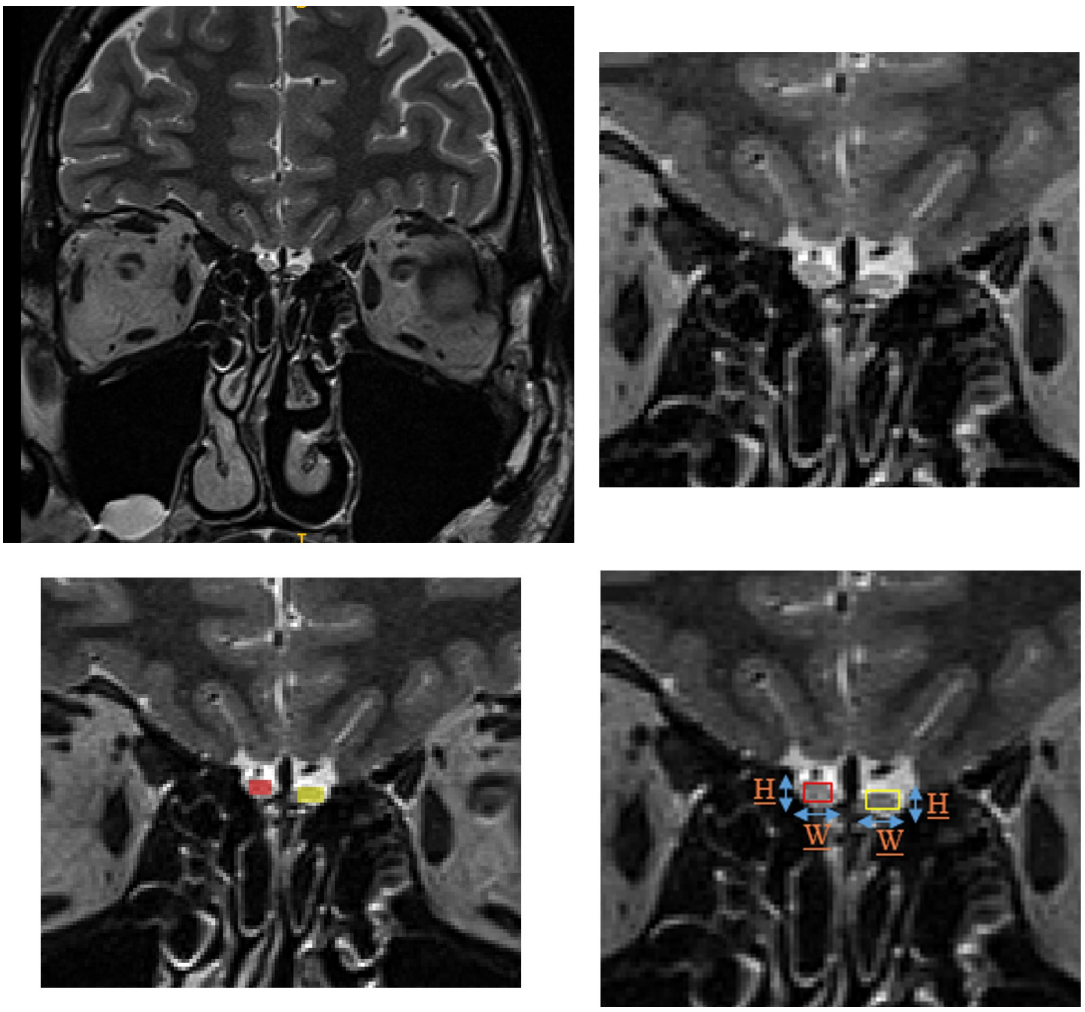

Fig 1. (A) Whole brain MR scan from a single subject. (B) right and left OB of the subject. (C) MS approach by plane manual contouring technique. (D) BF approach, with H = height and W = width depiction.

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Manual segmentation method (MS). AMIRA 3D visualization and modeling system (Visage Imaging, Carlsbad, USA) was used to calculate the volume of right and left OB using the planimetric manual contouring (PMC) technique (surface in mm^2) (Fig 1A and 1C). The OB sequence included acquisition of 1 mm thick T2- weighted fast spin images, in the coronal plane that covers middle and anterior portions of the skull base. A standardized PMC protocol was applied to all scans [22]. Firstly, number of slices with clear visibility of the OB were selected. On each successive slice of brain, contours on left and right side of OB were manually drawn. The proximal end of the OB was defined by the abrupt change in the diameter at the beginning of the olfactory tract [22, 23].Two trained observers blind to the diagnosis and clinical characteristics of the subjects, calculated the volumes (in mm^3).

Box- frame method (BF). ITK-SNAP (version 3.8.0, University of Pennsylvania & University of Utah, www.itksnap.org) [24] was used for the alternative calculations of OB volumes. Firstly, the number of slices with distinct visibility of the OB was noted down. Further, the slice having the most visible voxels for both right and left side was chosen as the standard slice (in most cases it was the central slice). As the OB shape varies between individuals, we framed

a box on it as shown in Fig 1A and 1D. Annotations were drawn on the standard slice using Image annotation tool. With the help of this tool, we calculated the width (w) and height (h) by physically drawing a line between two extreme points of OB. For calculation of box volume, the length (l) was calculated by selecting the total number of slices which showed clear and distinct OB, multiplied by the slice thickness (1mm) ($V = l^{*}w^{*}h$, in mm^3). Two expert observers (AJ, XY), blind to the subject's condition calculated the volumes of right and left OBs. When the difference exceeded 10%, a third expert observer calculated the volumes again. After input of the third observer, two closest volumes with less than 10% difference were selected.

The idea for proposing the BF approach was also its usability by non-experts in neuroimaging. Accordingly, we checked its performance by non- expert observers who belonged to a different background with no imaging experience. They were well explained how the technique works and

Table 1. Subject characteristics shown as mean ± standard deviation [SD] or number of subjects [N (%)].

Age (in years)	56 ± 14
Male/ female ratio	15/ 32
Causes of olfactory loss	
patients with idiopathic olfactory loss	N = 8 {17%}
patients with congenital olfactory dysfunction	N = 3 {6%}
patients with post- viral olfactory loss	N = 36 {77%}
OB results using the Manual Segmentation:	
Volume of right OB (Observer 1) (in mm^3)	21.52 ± 11.42
Volume of right OB (Observer 2) (in mm^3)	19.25 ± 10.67
Volume of left OB (Observer 1) (in mm^3)	22.73 ± 13.11
Volume of left OB (Observer 2) (in mm^3)	20.44 ± 12.11
OB results using the Box- Frame method (expert)	
Volume of right OB (Observer 1) (in mm^3)	34.34 ± 18.46
Volume of right OB (Observer 2) (in mm^3)	32.96 ± 17.51
Volume of left OB (Observer 1) (in mm^3)	32.38 ± 17.53
Volume of left OB (Observer 2) (in mm^3)	31.52 ± 17.41
OB results using the Box- Frame method (non-expert)	
Volume of right OB (Observer 1) (in mm^3)	33.65 ± 17.78
Volume of right OB (Observer 2) (in mm^3)	39.24 ± 22.10
Volume of left OB (Observer 1) (in mm^3)	42.12 ± 24.46
Volume of left OB (Observer 2) (in mm^3)	42.71 ± 27.23
Olfactory test scores	
TDI score	17.91 ± 7.86
Threshold score	2.57 ± 2.54
Discrimination score	7.87 ± 3.40
Identification score	7.64 ± 3.49
Duration of smell loss	
0–2 years	33
2–5 years	8
5–10 years	2
>10 years	4
Categorisation of participants	
Functional anosmia	23
Hyposmia	21
Normosmia	3

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were asked to do the measurements in all of the subject population. Following the same rules, when the difference exceeded 10%, a third non-expert observer calculated the volumes again.

Out of the total 52 subjects, five subjects were excluded due to unclear OBs and lack of subject's information and finally, volumes of 47 subjects were analyzed and compared for left and right OB volumes. Out of them, 36 subjects had reduced olfactory functioning due to an infection in the upper respiratory tract (URTI), eight were diagnosed with idiopathic olfactory loss (ID) and three had congenital anosmia.

Statistics

The Statistical Package for Social Sciences version 25.0 (IBM SPSS 25.0, Chicago, IL, USA) was used for statistical analysis. Table 1 shows the characteristic information for all subjects (means ± SD). A paired t-test was done to compare volumes of right and left OB as calculated by observers 1 and 2 using both methods. Furthermore, using Pearson correlation, inter-observer reliability was investigated for the volumes calculated by MS (AMIRA) and BF (ITK-SNAP) method. The level of significance was set at 0.05.

Results

Mean volumes for right and left OB as measured by 2 observers using MS and BF-methods varied significantly ($p<0.05$) with MS producing smaller volumes (Fig 2). Number of slices chosen by the 2 observers did not vary significantly for both methods. The mean number of slices for MS and BF methods were 6.3 and 6.8 respectively.

Positive correlation was found between OB volumes calculated by observer 1 and 2 for both methods: For MS, $r = 0.84$, $p < 0.01$ (right OB) and $r = 0.86$, $p < 0.01$ (left OB). For BF, $r = 0.95$, $p<0.01$ (right OB) and $r = 0.89$, $p< 0.01$ (left OB) (Table 2 and Fig 3).

Also, positive correlations were found between MS and BF methods (taking the average volumes measured by observer 1 and 2). For right OB, $r = 0.73$, $p<0.01$ and for left OB, $r = 0.70$, $p<0.01$ (Table 2).

High inter-observer reliability was found for volumes calculated by observers 1 and 2. For MS method, Cronbach's alpha (α) was 0.91 and 0.93 for right and left OB volume, respectively, whereas for the BF method α was 0.98 and 0.95 for right and left OB, respectively.

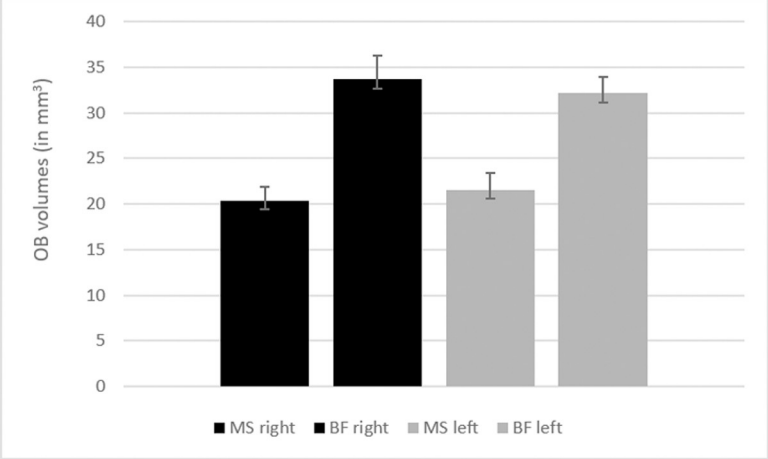

Fig 2. Averaging from measurements done by both the expert observers, OB volumes in Mean ± SEM, measured by both methods: MS right = 20.38 ± 1.54; BF right = 33.65 ± 2.59; MS left = 21.58 ± 1.8; BF left = 31.95 ± 2.5.

https://doi.org/10.1371/journal.pone.0243941.g002

Table 2. Correlations between right and left OB volumes obtained by expert observers (O1 = observer 1 and O2 = observer 2) and with different techniques (manual segmentation and box frame method).

		Coefficients of correlation (r)	level of significance (p)
O1 vs. O2: Manual Segmentation	Right OB	0.84	<0.01
O1 vs. O2: Box-frame	Right OB	0.95	<0.01
O1 vs. O2: Manual Segmentation	Left OB	0.86	<0.01
O1 vs. O2: Box-frame	Left OB	0.90	<0.01
Manual Segmentation vs Box- frame	Right OB	0.73	<0.01
Manual Segmentation vs Box- frame	Left OB	0.70	<0.01

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For BF approach, inter- observer reliability was checked for measurements done by experts and non- experts. The Cronbach's alpha (α) for right OB was 0.82 and 0.83 for left OB. The results advantages its usability by non- experts or less trained as well.

Discussion

In this study, we aimed to find an efficient, reliable yet less time-consuming method to calculate the OB volume. In fact, measurement time for the MS method was approximately 7–10 minutes whereas it takes only one minute for the BF method. Our study indicated that the BF approach provides reliable results which are in accordance with the results obtained from MS and when used by experts and non- experts.

So far, the MS of coronal slices is the most widely used method for volumetric measurements of the OB [25] Accuracy and reliability of MS method has been demonstrated clearly in previous studies [18, 26]. In the present study, we also followed up accuracy and reliability for the measurements made by the BF approach using ITK-SNAP software. This software was chosen for its user-friendly interface and free availability. However, many other software solutions could be used for this straight-forward technique. For the BF approach, intraclass coefficients of correlation between measurements of the two observers were at r = 0.96 for right OB and r = 0.89 for left OB. The results drawn from this new approach were comparable with the results obtained from MS approach with r = 0.84 for right OB and r = 0.86 for left OB.

The focus throughout the project was on the introduction of a method that can be clinically acceptable, with time demands being a major issue. This is important as OB volume is considered as a measure to evaluate the status of olfactory functioning. There has been evidence in support of how OB volume clinically describes the severity of olfactory loss. For example, in comparison to hyposmic patients, OB volumes were found to be smaller for anosmic subjects in olfactory loss, following infections of the upper respiratory tract or head trauma [27]. Importantly, OB volume also seems to be a predictor of recovery in patients with post-infectious olfactory loss [22]. Hence, the routine assessment of OB volume appears to be useful in patients with olfactory loss. This is more likely to be diagnostically implemented with the availability of a fast and convenient approach.

The present investigation also revealed that the internal consistency of measurements made with either method was excellent. Hence, it can be noted that the new BF method can be used as a clinically acceptable, efficient, reliable, easy and quick approach to calculate OB volumes. However, it has to be kept in mind that both MS and BF method remain subjective and voxel selection may vary depending on skills of the individual observers which requires some degree of training.

To conclude, the present results suggest that the BF method for OB volumetric is reliable and produces valid results, comparable to the results from MS. The new technique is a simple,

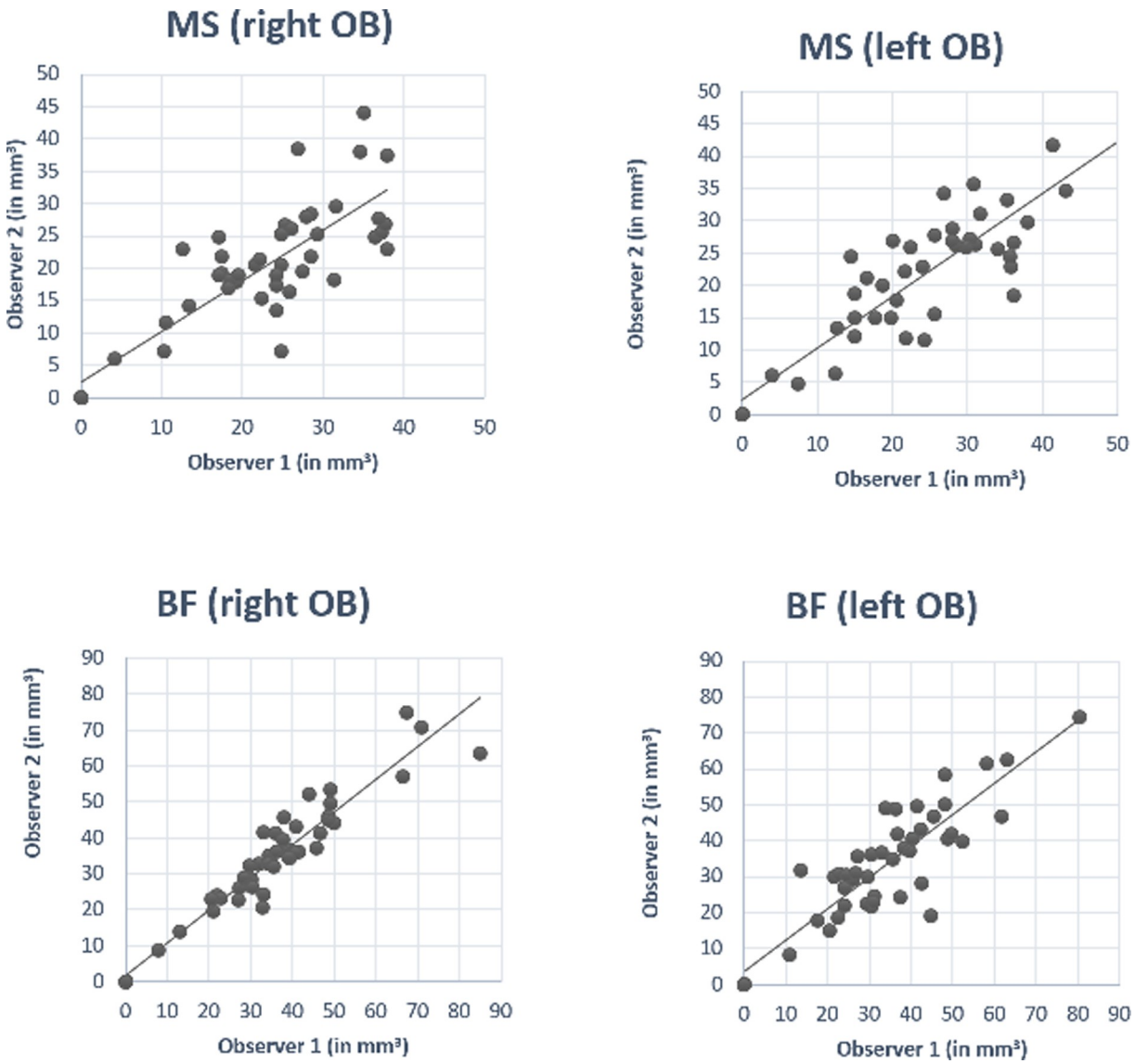

Fig 3. Positive correlation between volumes (in mm^3) measured by observer 1 and 2 for manual segmentation (MS) and box- frame methods (BF). For MS, (A) r = 0.84, p <0.01 (right OB volume) and (B) r = 0.86, p <0.01 (left OB volume). For BF, (C) r = 0.95, p<0.01 (right OB volume) and (D) r = 0.90, p< 0.01 (left OB volume).

https://doi.org/10.1371/journal.pone.0243941.g003

quick approach and may require less training than MS of the OB. It is hoped that this technique paves the road for the routine clinical assessment of OB volume in patients with olfactory loss.

Supporting information

S1 Data.
(XLSX)

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Author Contributions

Conceptualization: Akshita Joshi, Thomas Hummel.

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Formal analysis: Akshita Joshi, Thomas Hummel.

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Publication 2 (Second study): Neural processing of olfactory-related words in subjects with congenital and acquired olfactory dysfunction.

Abstract of publication 2

Background

Olfactory loss can be acquired (patients with a history of olfactory experiences), or inborn (patients without olfactory experiences/ life-long inability to smell). Inborn olfactory loss, or congenital anosmia (CA), is relatively rare and there is a knowledge gap regarding the compensatory neural mechanisms involved in this condition. The study aimed to investigate the top-down olfactory processing in patients with CA or idiopathic acquired anosmia (IA) in comparison to normosmia controls (NC) during expectancy and reading of odor-associated words.

Methods

Functional magnetic resonance imaging was used to assess brain activations in 14 patients with CA, 8 patients with IA, and 16 NC healthy participants during an expectancy and reading task. Words with strong olfactory associations (OW) (e.g. “banana”) or with little or no olfactory associations (CW) (e.g. “chair”) were used as stimuli and were presented in a block design. Analyses were conducted to explore the brain activation in response to OW expectancy or OW reading between groups (CW as baseline).

Results

During the expectancy condition of OW, IA and NC groups showed stronger activation in posterior OFC extending to right insula, caudate region and frontal medial OFC respectively. Whereas during the reading condition of OW, CA patients showed stronger activation in posterior OFC extending to the insula.

Conclusions

Increased activation of higher-order brain regions related to multisensory integration among CA patients suggests a compensatory mechanism for processing semantic olfactory cues.

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Neural processing of olfactory-related words in subjects with congenital and acquired olfactory dysfunction

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Olfactory loss can be acquired (patients with a history of olfactory experiences), or inborn (patients without olfactory experiences/life-long inability to smell). Inborn olfactory loss, or congenital anosmia (CA), is relatively rare and there is a knowledge gap regarding the compensatory neural mechanisms involved in this condition. The study aimed to investigate the top-down olfactory processing in patients with CA or idiopathic acquired anosmia (IA) in comparison to normosmia controls (NC) during expectancy and reading of odor-associated words. Functional magnetic resonance imaging was used to assess brain activations in 14 patients with CA, 8 patients with IA, and 16 NC healthy participants during an expectancy and reading task. Words with strong olfactory associations (OW) (e.g. "banana") or with little or no olfactory associations (CW) (e.g. "chair") were used as stimuli and were presented with a block design Analyses were conducted to explore the brain activation in response to OW expectancy or OW reading between groups (CW as baseline). During the expectancy condition of OW, IA and NC groups showed stronger activation in posterior OFC extending to right insula, caudate region and frontal medial OFC respectively. Whereas during the reading condition of OW, CA patients showed stronger activation in posterior OFC extending to the insula. Increased activation of higher-order brain regions related to multisensory integration among CA patients suggests a compensatory mechanism for processing semantic olfactory cues.

In humans, the causes for compete smell loss (anosmia) are due to either acquired or congenital causes. In contrast to acquired anosmia which the sense of smell is impaired later in the life, congenital anosmia (CA) is a rare condition which is characterized by a life-long lack of olfactory perception and the aplasia or hypoplasia of the olfactory bulb[1].

Stimulation with either odor molecules or olfactory associated non-chemical cues (e.g. pictures, words, metaphors) can activate the central olfactory system, representing the bottom-up and the top-down pathways for olfactory processing. For bottom-up process, odor molecules bind to olfactory receptors before olfactory signals are transmitted via olfactory bulb and are further processed in multiple olfactory related brain regions (e.g. piriform cortex, amygdala, orbitofrontal cortex, insula, hippocampus, anterior cingulate cortex)[2–4]. On the other hand, during top-down processing, the retrieval of cognitive information related to an odor occurs without the existence of a physical stimulus[5]. These top-down activations involve the olfactory-related as well as higher-order brain regions[2,6–9].

Patients with olfactory dysfunction demonstrate decreased brain activation in response to odor stimulation, indicating a disrupted bottom-up olfactory process[10,11]. Moreover, several brain imaging studies have suggested alterations of the top-down olfactory process among patients with olfactory loss. For example, Flohr et al.[12] found that patients with acquired smell loss are unable to vividly image odors with a given odor-associated cue, and exhibited enhanced brain activation in the dorsal lateral prefrontal cortex and the precuneus regions mainly

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involved in working memory. Using blocks of words with strong olfactory associations, Han et al.[13] investigated the reading of odor related words among a group of patients with acquired olfactory loss. Specifically, during the word priming, patients had increased activation of the lexical-semantic related areas during expectation of words with olfactory association. Combined, these studies suggested that patients with olfactory loss have changed neural responses in the olfactory cortex during processing of olfactory information. However, research on the neural processing of olfactory information among CA are limited.

To date only a few studies have investigated the structural and functional alterations in CA. While reduced gray matter volume in olfactory related brain regions are found in acquired anosmia, CA is associated with increased gray matter volume in the primary olfactory area and the orbitofrontal cortex[14,15]. One recent study showed that CA exhibited audio-visual multisensory enhancement, which suggested a compensation for complete lack of olfactory input[16]. If and to what extent brain responses during top-down olfactory processes are altered in CA is unknown. The current study aimed to investigate brain processing of odor-related words in CA and compare that to patients with acquired idiopathic anosmia (IA), and normosmic controls (NC). We hypothesized that IA and NC subjects show more activations in olfactory associated areas because of their pre-existing olfactory associated semantic knowledge whereas activations in CA subjects were expected to be significantly lower as compared to others because of their complete lack of olfactory experience[17].

Materials and methods

Participants. Participants were recruited from the resident of Dresden area (control participants) and the Smell and Taste Clinic, Department of Otorhinolaryngology, University Hospital Carl Gustav Carus, Dresden (patients). All participants received the "Sniffin' Sticks" olfactory test[18]. A composite odor threshold, odor discrimination and odor Identification score (TDI score) was used to classify normal olfaction (TDI > 30.5) and anosmia (TDI < 16.5). In order to ascertain anosmia in CA patients, olfactory event related potentials were recorded, and in none of the patient's olfactory event related potentials were detected[19]. CA subjects were diagnosed with lack or hypoplasia of olfactory bulb and a life-long olfactory dysfunction without other known etiology. Patients with idiopathic anosmia (IA) were those patients with no cause for their olfactory dysfunction was found after detailed clinical investigations (including medial history questionnaires, psychophysical olfactory testing, olfactory pathways morphology assessment)[20]. In addition, participants completed the German version of Beck Inventory [ranging from normal state (1–10) to extreme depression (over 40) II][21] and the Montreal Cognitive assessment (ranging from 0 to 30)[22] for assessing the level of depression and executive functions, respectively.

A total of 40 participants took part in the study. Of those, eighteen were control participants with normal olfaction (NC, mean age 49.2 years; SD 12.2; 10 females), 14 were patients with congenital anosmia (CA, mean age 37.4 years, SD 18.9; 7 females) and 8 patients with idiopathic anosmia (IA, mean age 56.4 years; SD 10.8; 4 females, disease duration ranging between 9 and 108 months). The study was approved by the Ethics committee of the medical faculty at the Technical University of Dresden. The experiment was conducted according to the Helsinki declaration. All participants provided written informed consent.

Study design. For our experimental design, 36 words with strong olfactory association (OW) and 36 words with little or no olfactory association i.e. control words (CW) were presented to the participants lying in the scanner. Apart from the 24 new words as displayed in Table 1, for convenience of later analysis some words were randomly repeated to have a block time of 20 s each.

We chose the words with higher olfactory association as reported by Han et al.[13]. Briefly, 50 words with olfactory association and 50 words with little or no olfactory association were screened and rated by experts (PH, TH, JA, IC). Through a pilot study, 18 normosmics were asked to rate the randomly presented OW and CW words for the degree of olfactory association using a numerical scale ranging from 0 to 5. Combining with the ratings from expert selection, CW had a mean score of 0.4 (SD 0.3) whereas OW had a mean rating score of 3.2 [(SD 0.9); $t(17) = 13.5$, $p < 0.001$][13].

The participants were instructed to covertly read the instructions and words. Cueing prior to word blocks was adopted to guide participants to (1) focus on the olfactory aspects of the displayed words (2) induce an expectation for the following words; and (3) to clearly separate the OW from the CW blocks. Olfactory related semantic differences were chosen as a criterion to differentiate between conditional activation. However, no control on the word frequency was marked on. The word length (e.g. number of characters in each word) was taken into consideration during selection, however, no statistical analysis was performed on this. Specifically, the experimental run contained 24 blocks in total with 12 blocks each of OW and CW; displayed in an alternating pattern. For each block, the expectation was induced with a slide showing for 2.5 s with the term 'Words with smell' (German: 'Wörter mit Duft') or "Words with no smell" (German: 'Wörter ohne Duft') followed by a 1-s fixation cross, making the expectation task for 3.5 s. The reading phase included three OW or CW presented for 2.5 s each, with 1-s intervals between words, making the reading task for 10.5 s. During inter-block intervals, a fixed cross was shown for 6 s. Each block was of 20 s. The order of the words within each block was randomized among participants. In the complete experiment we had 36 OW + 36 CW words in total scan time of 480 s = 8 min ((12 + 12) × 20 s/block). A simplified diagram of the fMRI design is depicted in Fig. 1.

Imaging data acquisition and preprocessing. Functional and structural brain images were acquired on a 3-T MRI scanner (Siemens Prisma, Erlangen, Germany) equipped with an 8-channel head coil. A total of 220 functional images were collected per individual using a T2 single-shot echo-planar imaging (EPI) sequence: TR = 2000 ms, TE = 40 ms, 90° flip angle[13], voxel size 3 × 3 × 3.75 mm, no interslice gap, 192 × 192 mm field of view. A high-resolution structural T1 image was acquired using a 3D magnetization prepared gradient rapid

Olfactory associated words	Non-olfactory associated control words
Fisch (fish)	Nadel (needle)
Popcorn (popcorn)	Stein (stone)
Zimt (cinnamon)	Schlüssel (key)
Karamel (caramel)	Teller (plate)
Senf (mustard)	Aufzug (elevator)
Leder (leather)	Schloß (lock)
Vanille (vanilla)	Kugel (bullet)
Zigarre (cigar)	Schere (scissors)
Wein (wine)	Brille (glasses)
Käse (cheese)	Halsband (collar)
Rose (rose)	Schachspiel (chess)
Ananas (pineapple)	Stuhl (chair)
Gummi (rubber)	Ventilator (fan)
Knoblauch (garlic)	Bildscirm (screen)
Anis (aniseed)	Spiegel (mirror)
Pfirsich (peach)	Hefter (stapler)
Menthol (menthol)	Wasser (water)
Schokolade (chocolate)	Handy (mobile)
Gras (grass)	Batterie (battery)
Orange (orange)	Eimer (bucket)
Erdbeere (strawberry)	Uhr (clock)
Kaffee (coffee)	Tasche (bag)
Banane (banana)	Tür (door)
Schweiß (sweat)	Glas (glass)

Table 1. Words shown to the participants in the scanner (words in German, with English translation in brackets).

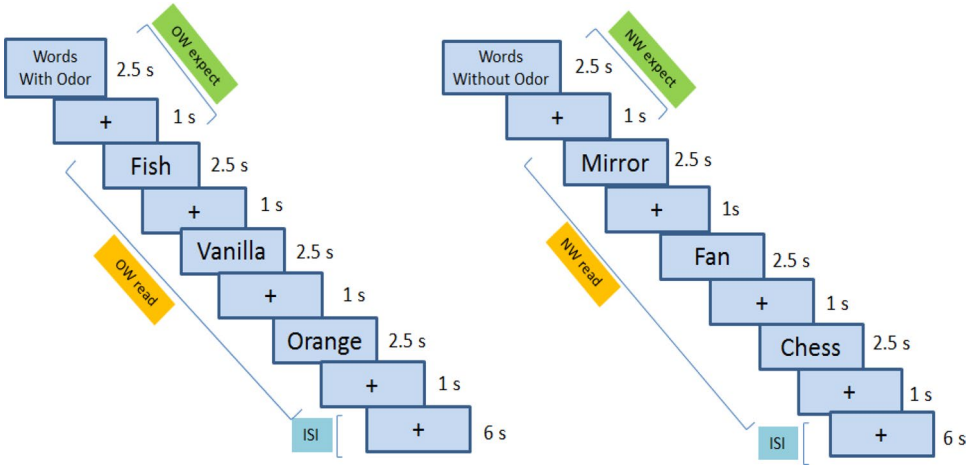

Figure 1. fMRI experimental block design with expectancy task (3.5 s) and reading task (10.5 s); "+" as fixation cross; "ISI" as inter-stimulus interval (6 s) and "s" as seconds.

acquisition gradient echo (MPRAGE) sequence (TR = 2530 ms, TE = 2.34 ms, 256 × 256 mm field of view, voxel size 1 × 1 × 1 mm).

SPM12 (statistical parametric mapping) was used to analyze the functional MRI data, which is a MATLAB (The Mathworks Inc., Natick, MA, USA) based software from Welcome Trust Centre for Neuroimaging, London, UK. Default settings were used for pre-processing of data which included—realignment, unwarping, co-registration, segmentation, smoothing and normalization. For all the subjects, head movement artifacts were further removed using ArtRepair software (version 4, Stanford University)[9], after which neuroimaging data of one control subject was discarded due to excessive movement. In the end, data set included functional images of 14 CA, 8 IA and 16 NC participants.

	NC (N = 16)	CA (N = 14)	IA (N = 8)	Comparison
Age (years)	49.2 ± 12.2[a]	37.4 ± 18.9[b]	56.4 ± 10.8[a]	p < 0.05
Female/male (n)	10/6	7/7	4/4	n.s
Odor threshold	8.6 ± 2.4 [a]	1.5 ± 1.5 [b]	0.4 ± 0.2 [b]	p < 0.001
Odor discrimination	12.8 ± 2.2 [a]	5.7 ± 1.7 [b]	5.6 ± 3.6 [b]	p < 0.001
Odor identification	14.2 ± 1.7 [a]	4.8 ± 2.0 [b]	3.9 ± 2.6 [b]	p < 0.001
TDI score	35.6 ± 3.8 [a]	12.0 ± 2.7 [b]	10.7 ± 6.0 [b]	p < 0.001
Taste sprays	3.7 ± 0.6	4.0 ± 0.0	4.0 ± 0.0	n.s
MoCA	27.0 ± 2.1	26.6 ± 2.8	25.4 ± 1.7	n.s
BDI	2.5 ± 2.4 [a]	2.8 ± 2.9 [a]	7.0 ± 7.2 [b]	p < 0.05

Table 2. Socio-demographical and psychophysical information for normal control (NC), congenital anosmia (CA) and the idiopathic anosmia (IA) groups. Comparison p values indicate main effect of ANOVA, superscripts with different letters (a, b) Indicate significant difference in post-hoc comparisons. *TDI* combined odor threshold, discrimination and identification score, *MoCA* Montreal cognitive assessment test. *BDI* Beck depression inventory test, *n.s.* not significant.

fMRI analysis. On the single-subject level, the conditions for OW expect and OW read were calculated as follows: $OW_{expect} = (OW - NW)_{expect,}$ and $OW_{read} = (OW - NW)_{read}$. Further, on the group level the contrast images from each individual were subjected to a random effect analysis to test specific research questions: (1) one-way ANOVA analysis was used to test between-group differences regarding OW expect; (2) the one-way ANOVA analysis was used to test between-group differences regarding OW read. Age, sex, and BDI scores were controlled in the models in the SPM 2nd level model. Significant brain activation was searched on the whole-brain level. To control for multiple statistical testing within the entire brain, we maintained a cluster-level false-positive detection rate at $p < 0.05$ using an initial voxel-level threshold of $p < 0.001$ with a cluster extent (k) empirically determined by Monte Carlo simulations (n = 1,000 iterations), by means of AlphaSim procedure[23]. This was done using the REST toolbox (https://www.restfmri.net/forum/REST_V1.7)[24]. A minimum cluster size (number of contiguous voxels) was determined for each specific contrast to achieve a cluster-level Family-Wise Error corrected $p < 0.05$, and were reported as part of the results. Significant brain regions were labelled and reported with the AAL toolbox[25] . The activation levels (contrast estimates) in significant clusters were plotted for each group (NC, IA, CA) using the plot function in SPM.

Statistical analyses for behavioral data. Behavioral and socio-demographic measurements ("Sniffin' Sticks" test score; BDI score; MCAT score) were analyzed using IBM SPSS version 2.4 (SPSS Inc., USA, Chicago) using one-way ANOVA, including age and sex as co-variables of no interest. The significance level for all the statistical tests was set at $p < 0.05$ unless specified. Results are represented as means ± standard deviation (SD).

Results

Characteristics of participants. The socio-demographical and psychophysical measurements for each group were shown in Table 2. Age of CA was significantly smaller compared to the other two groups. Patients with IA had highest BDI scores compared to NC and CA groups. The sex distribution, taste spray score or the Montreal cognitive assessment test score were not different between groups.

fMRI results. *Difference between control and patient groups during expectation of OW.* During expectation of OW, significant main effect of group was observed in the right posterior OFC extending to insula, the left posterior OFC, left caudate and anterior cingulate cortex (ACC) (Fig. 2; Table 3). The pairwise between-group comparison showed stronger activation of the frontal medial OFC extending to left ACC among NC compared to CA participants, and stronger activation of the posterior OFC extending to insula among IA compared to CA patients. Besides, the IA patients demonstrated significant stronger activation in the bilateral caudate as compared to NC participants during OW expectation (Table 3).

Differences in brain activation between control and patient groups during reading of OW. By applying the corrected threshold ($p < 0.001$ and $k > 18$ voxels), there was no significant activation of the main effect during reading OW. We further compared NC vs CA, and IA vs CA, separately. With a corrected threshold ($p < 0.001$ with cluster size > 43 voxels), the CA patients showed significantly stronger activation of the posterior OFC extending to the insular cortex compared to NC participants (peak MNI coordinates 36 16 − 16, T = 4.48, k = 92). There was also stronger activation of the left occipital cortex in CA as compared to IA patients (peak MNI coordinates − 34 − 82 36, T = 3.89, k = 85). No other significant activation was observed from the between-groups comparisons.

Discussion

The current study investigated neural processing of words with olfactory associations in patients with life-long olfactory loss (congenital anosmia, CA) in comparison with a group of control participants with normal olfaction (NC) and a group of patients with acquired olfactory dysfunction (idiopathic anosmia, IA). Most importantly, the

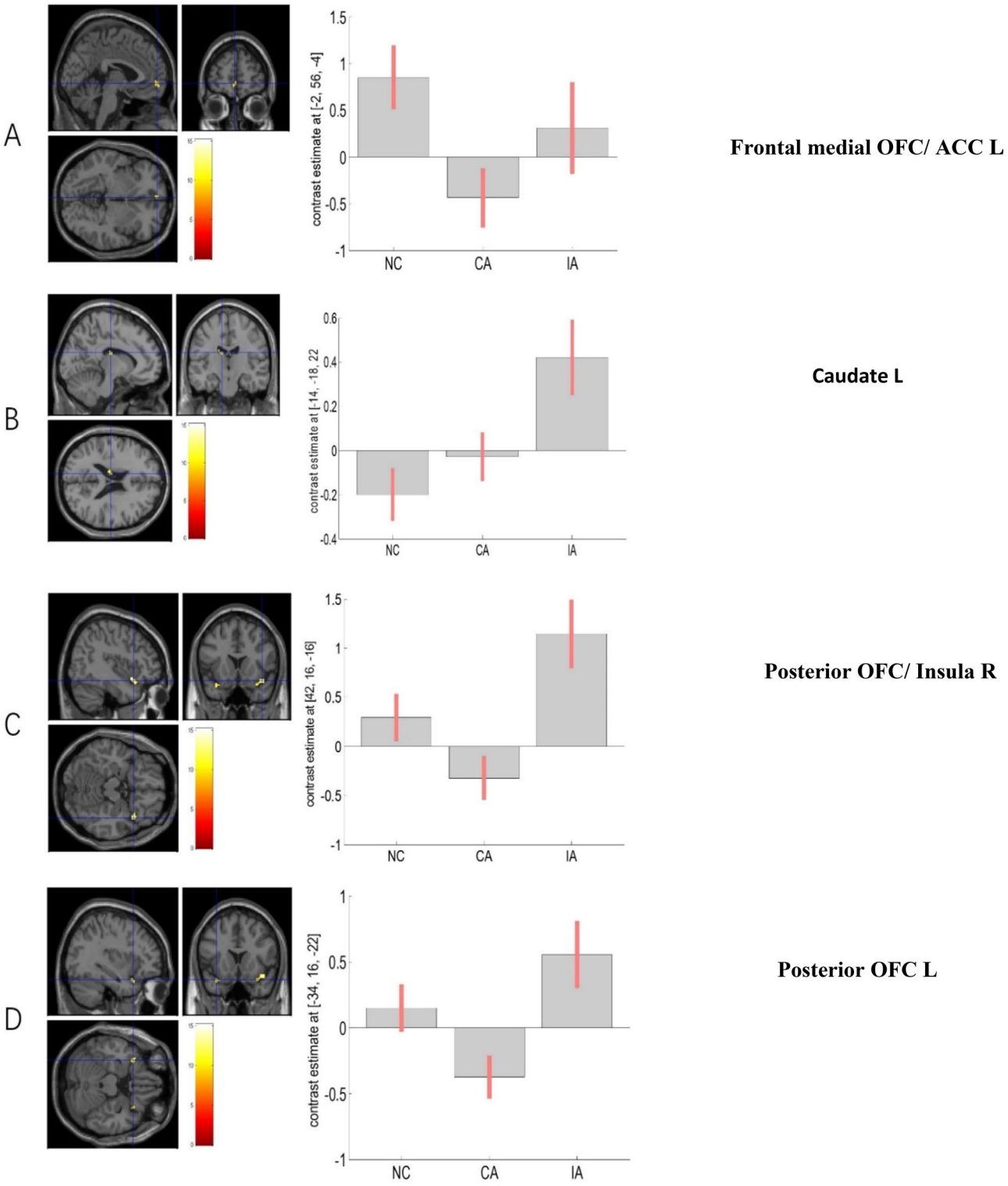

Figure 2. Neural responses showing the main group effect during OW expectation in (**a**) left frontal medial OFC; (**b**) left caudate; (**c**) posterior OFC/right insula; (**d**) left posterior OFC. Brain maps were threshold at $p_{uncorrected} \leq 0.001$ in combination of a cluster size determined using the Monte Carlo simulations (n = 1,000 iterations) following the AlphaSim procedure, and visualized on a template (ch2better.nni) provided in SPM12. Bar charts display the contrast estimates for the illustrated regions.

CA group, having never sensed any olfactory stimuli, showed stronger activations in the posterior OFC, extending to insular cortex compared to NC participants during OW reading. The activation in the OFC region is similar

	k	F/T value	x y z			Region
Main effect	63	15.14	42	16	− 16	Posterior OFC/Insula R
	20	12.65	− 34	16	− 22	Posterior OFC L
	37	11.48	− 14	− 18	22	Caudate L
	27	10.12	− 2	56	− 4	Frontal medial OFC/ ACC L
NC > CA	295	4.50	− 2	56	− 4	Frontal medial OFC/ ACC L
IA > NC	178	4.79	− 14	− 18	22	Caudate L
	40	4.17	22	− 24	24	Caudate R
IA > CA	117	5.43	42	16	− 16	Posterior OFC/Insula R
	46	4.72	− 34	16	− 22	Posterior OFC L

Table 3. Between-group comparisons during expectation of OW. Whole brain F or T maps were thresholded at uncorrected $p < 0.001$ and cluster size $k > 10$ voxels; For clusters with multiple peaks only the highest T value is reported; MNI coordinates are presented in x, y, and z. L, left hemisphere; R right hemisphere. Brain regions labelled with AAL toolbox (https://www.gin.cnrs.fr/en/tools/aal-aal2/).

to what has been observed in both healthy controls and patients with acquired olfactory loss where reading of olfactory associated words led to similar activations[7,13]. During expectation of OW, the IA patients demonstrated stronger activation in the posterior OFC extending to insula as compared to the CA patients. The OFC is relevant for the processing and integration of information from different sensory modalities[26]. Also, activation in the OFC supports the interaction between the odor related word cues and their respective odor percepts[27]. In comparison to NC, IA patients when expecting the olfactory associated words also showed major activations in caudate, which can be interpreted for its involvement in executive processes[28] specifically, in goal-directed actions. Moreover, in comparison to CA, NC group showed activations extending to ACC, which indicate its key role in attention[29,30] and in working memory tasks[31.] NC, IC, and CA seem to use distinct strategies when it comes to anticipating words with olfactory associations.

During OW reading, stronger activation of a very similar cluster in the posterior OFC extending to insula was found among CA as compared to NC groups. As stated above, this region is involved in multisensory integrative processing, that receive information from the olfactory, gustatory and visual sources[32–34]. Unlike the expectation condition where no direct odor-related cues were shown, displaying OW could initiate neural processing of word related semantic and olfactory information more efficiently. Although CA patients have a life-long deprivation of olfactory perception, their knowledge regarding other chemical inputs (e.g. gustatory and trigeminal input) as well as the semantic meaning of the OW remains intact. Besides, CA patients have been shown to exhibit slightly enhanced abilities for non-chemical multisensory (e.g. audio-visual) integration compared to people with intact olfaction[8], indicating an existing compensatory mechanism. Therefore, stronger activation during OW reading among the CA group may reflect the process of multisensory integration, involving semantic comprehension. The exact process behind the increased activation among CA remains to be explored. Stronger activation was also found in the occipital cortex among CA compared to IA during reading of OW, possibly indicated an enhanced attention paid to the olfactory-related words among CA patients.

A number of fMRI studies have reported similar patterns of brain activation during olfactory memory tasks cued by non-odorous objects such as images or words[6,35,36] and during the processing of physical olfactory stimuli. Brain activity during representation of sensory stimulus without direct external stimulus (mental imagery) has been studied in various modalities including visual, auditory and tactile. In general, regions associated with mental imagery were found to be those regions associated with perception in the same sensory modality[37,38]. In the present case one would expect participation of primary and secondary olfactory areas, given that these participate in olfactory perception[39,40]. The presently observed activation of OFC when reading the OW, can be related to the odor imagery approach as shown in previous studies. There activation in the right OFC, associating imagery with the perception of physically present odors was related to the experienced realness or "vividness" of an olfactory image[40,41]. In our study, given the visual sources, integration of visual and olfactory information occurs in OFC, where the odor percepts were linked to their respective names. Djordjevic et al.[2] also reported activation of the insular cortex as a result of odor imagery. Neuroimaging studies suggest that a number of factors could modify activation of these olfactory brain regions. Among these possible effects are: increased respiratory amplitude, due to sniffing[42], attentional demands[43], lexico-semantic processing of words[7], or cross modal associative learning[44]. All in all, olfactory top-down processing has a significant role in encoding or recalling of learned information[45], which results in anticipation of an odor or processing of odor-associated cues[13]. Therefore, based on the present results it appears that there is overlap of neural processing in terms of both bottom-up and top-down olfactory representation.

There are a few limitations applied to the current study. First, the small sample size. Given the scarcity of CA cases, studies on this group of patients are typically small (i.e. less than 20 patients). Second, the breathing was not monitored during the MRI scan. The possible alteration of breathing in patients[46] may introduce noise as variable that affect the observed brain responses[10,39]. Thirdly, we did not explore the association of the presented words with foods. Such an association might explain some of the overlapping activations in the 3 groups of patients; fourthly, for reasons of study design, olfactory words and control words were not evaluated for their valence and their association with edibility which also might impact the processing of words.

In conclusion, our results demonstrate different neural responses during expectation and reading of words with strong olfactory associations among people with acquired anosmia, congenital anosmia and normosmia. Increased activation of the higher-order brain regions related to multisensory integration among CA during reading of olfactory related words may suggest a compensatory mechanism for processing of semantic olfactory cues.

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Author contributions

A.J. and P.H. have equal contribution in writing the manuscript, performing tasks and analysing the data, V.F. helped in analysis, revised the paper and gave important feedback, M.L. contributed/revised the manuscript, T.H. made the study possible, finalized the experiments, helped in subject recruitment and gave final inputs to the manuscript.

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Competing interests

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Publication 3 (Third study) Habitual Exposure to Trigeminal Stimuli and Its Effects on the processing of Chemosensory Stimuli.

Abstract of publication 3

Objective

Our objective was to compare brain responses to trigeminal and olfactory stimuli in frequent and non-frequent gum chewers in order to explore whether habitual exposure to trigeminal stimuli affects their central-nervous processing.

Methods

In healthy subjects, fMRI brain scans were obtained for 20 frequent gum chewers (GC) and 20 non-frequent gum chewers (N'GC), in response to four odorous stimuli; 2 'trigeminal' (peppermint and spearmint) and 2 non-trigeminal or 'olfactory' (cherry and strawberry). During measurements, subjects reported intensity and pleasantness ratings for all stimuli. In addition, a test for general trigeminal sensitivity test (lateralization test) and an odor threshold test was performed. Brain activations in response to individual odors were investigated for the total study population followed by group wise (GC and N'GC) analysis separately for responses to trigeminal (peppermint + spearmint) and olfactory (cherry + strawberry) odors.

Results

(1) The GC group exhibited higher trigeminal sensitivity compared to the N'GC group. (2) Olfactory odors activated bilateral insular cortex and amygdala. Apart from olfactory areas (amygdala, insular cortex), trigeminal odors also produced activations in right thalamus and right substantia nigra. (3) In the GC group, olfactory odors produced higher bilateral insular cortex activation than in N'GC group, but no such differences were observed for trigeminal odors.

Conclusion

GC subjects appeared to be more responsive to trigeminal chemosensory stimuli. However, this did not directly translate into differences in central-nervous activations to trigeminal stimuli; instead, the use of chewing gum was associated with stronger brain activation towards olfactory stimuli.

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Habitual Exposure to Trigeminal Stimuli and Its Effects on the Processing of Chemosensory Stimuli

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Abstract—Our objective was to compare brain responses to trigeminal and olfactory stimuli in frequent and non-frequent gum chewers in order to explore whether habitual exposure to trigeminal stimuli affects their central-nervous processing. In healthy subjects, fMRI brain scans were obtained for 20 frequent gum chewers (GC) and 20 non-frequent gum chewers (N′GC), in response to four odorous stimuli; 2 'trigeminal' (peppermint and spearmint) and 2 non-trigeminal or 'olfactory' (cherry and strawberry). During measurements, subjects reported intensity and pleasantness ratings for all stimuli. In addition, a test for general trigeminal sensitivity test (lateralization test) and an odor threshold test was performed. Brain activations in response to individual odors were investigated for the total study population followed by group wise (GC and N′GC) analysis separately for responses to trigeminal (peppermint + spearmint) and olfactory (cherry + strawberry) odors. (1) The GC group exhibited higher trigeminal sensitivity compared to the N′GC group. (2) Olfactory odors activated bilateral insular cortex and amygdala. Apart from olfactory areas (amygdala, insular cortex), trigeminal odors also produced activations in right thalamus and right substantia nigra. (3) In the GC group, olfactory odors produced higher bilateral insular cortex activation than in N′GC group, but no such differences were observed for trigeminal odors. GC subjects appeared to be more responsive to trigeminal chemosensory stimuli. However, this did not directly translate into differences in central-nervous activations to trigeminal stimuli; instead, the use of chewing gum was associated with stronger brain activation towards olfactory stimuli.

Key words: smell, odor, FMRI, cooling, olfactory, chewing gum.

INTRODUCTION

We experience thousands of odors from our surroundings which predominantly involve activation of two major chemosensory systems; the olfactory system which mediates odor perception, and the trigeminal system which leads to sensations such as tickling, burning, stinging, touch, pressure and temperature (Albrecht et al., 2010; Hummel and Frasnelli, 2019). Both systems contribute to the odor percept. This interaction between the two systems is also important in terms of flavor (Pellegrino et al., 2017b).

A pure olfactory stimulus typically activates both the primary and secondary olfactory areas such as insular cortex, piriform cortex, and amygdala. Whereas odors which interact with both the olfactory and trigeminal systems (bimodal odors) induce widespread activations in regions including insular cortex, somatosensory cortex, thalamus, hypothalamus, caudate nucleus, orbitofrontal cortex and brain stem (Savic, 2002; Boyle et al., 2007; Albrecht et al., 2010; Pellegrino et al., 2017b; Lötsch et al., 2020). Functional overlap between the two systems includes activations of the piriform cortex, medial orbitofrontal cortex, and the secondary somatosensory cortex (Boyle et al., 2007; Hummel et al., 2009).

Adaptation is a major characteristic of the olfactory (Pellegrino et al., 2017a). Less is known about the trigeminal modality. Repeated exposure to trigeminal stimuli may result in increased sensitivity at peripheral and central-nervous levels (Dalton et al., 2006; Oleszkiewicz et al., 2018). Specifically, short-term exposure to bimodal stimuli results in increased sensitivity towards a selective trigeminal stimulus CO_2 (Oleszkiewicz et al., 2018). However, this idea is challenged by the desensitization which is found in response to repeated exposure to capsaicin (Van Gerven et al., 2017).

Little is known about the neural responses towards long-term exposure to trigeminal stimulation. We used habitual consumption of foods with trigeminal stimulants

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as a model to investigate effects of long-term trigeminal stimulation. A reason behind choosing the use of chewing gum is because it is one of the most popular pastimes in the younger population (Sasaki-Otomaru et al., 2011). In the present study, we aimed to study possible changes of the chemosensory systems in response to prolonged stimulation to trigeminal stimuli. Brain activations were compared between frequent chewing-gum users (GC) and non-frequent chewing-gum users (N′GC) in response to both trigeminal and olfactory odors using functional brain imaging. For testing, we included the minty flavor of chewing gums, namely peppermint and spearmint as ‘‘trigeminal odor” eliciting a cooling and even slightly painful sensation (McKemy et al., 2002; Hansen, 2017) and non-minty, olfactory stimuli such as strawberry and cherry.

EXPERIMENTAL PROCEDURES

Participants

Forty healthy subjects (m = 22, f = 18) with a mean age 25 ± 3 years (age range 18–40 years) were recruited in the study. Subjects received a structured medical history (Welge-Luessen et al., 2013), which, among others, included questions on demographics, smoking and drinking habits, medications, current disorders, family history of any neurodegenerative disease and general nasal health status. GC and N′GC subjects were identified based on a questionnaire asking about their mint consumption patterns. Subjects in the GC group (n = 20) chewed gums at least twice a day; used mint toothpaste, consumed peppermint tea or related foods frequently. N′GC (n = 20) consumed little or no chewing gums, mint toothpaste or any other mint related food items.

All subjects reported a normal sense of smell which was ascertained using an odor identification test (with maximum score of 16) from the ‘‘Sniffin’ Sticks” olfactory test battery (Oleszkiewicz et al., 2018). This test is performed within a forced choice paradigm where subjects have to identify 16 odors at supra- threshold concentrations using flash cards with four descriptors each (Cain and Stevens, 1989; Kobal et al., 2000). Participants reached an average score of 13.6 ± 1.4 (mean ± SD).

The experiments were conducted according to the Helsinki declaration. The ethics committee at the medical faculty of the Technical University of Dresden approved the study design. Subjects were recruited by putting flyers on campus. They provided written informed consent.

Study design

Subjects were pretested for odor identification score, followed by threshold testing and lateralization test. After checking for their normal olfactory and trigeminal sensitivity subject’s underwent fMRI scanning.

During fMRI measurements, odorous stimuli were presented using a portable and computer-controlled olfactometer (Sommer et al., 2012), embedded in a 2 L/min constant airflow. Each functional run was comprised of 11 OFF or ‘‘baseline” blocks and 10 ON blocks. From the main block design (Fig. 1), 10 blocks of ON and OFF phases were analyzed to increase stimulus related BOLD signals.

Odors were delivered intranasally during the ON session for 8 seconds and odorless air for 12 seconds during the OFF session. Each subject underwent four functional runs with one type of odor presented in one run. Across subjects, the sequence of these runs was randomized using a Latin Square. After each run, subjects were asked to rate the intensity and pleasantness for the odor presented. The intensity scale ranged from zero (unnoticeable) to 10 (very strong) and pleasantness ranged from zero (extremely unpleasant) to 10 (extremely pleasant) with five being neutral. Subjects communicated orally through the scanner intercom system.

Odor stimuli

We selected four odors for testing (provided by Takasago, Paris, France): Peppermint (order number ABX321352), Spearmint (ABX321351A), Strawberry (ABX321354A), and Cherry (ABX321603). These odors resemble flavors used in chewing gums. Peppermint and spearmint are minty, majorly trigeminal odors, whereas cherry and strawberry are non-minty, olfactory odors.

Psychophysical testing

In the pilot study, intensities of all the presented stimuli were checked using visual analog scales so that they were perceived as isointense (F [3, 76] = 2.11, p = 0.10). Pure odors with no dilution were used. The isointense levels of odors were used for the lateralization task (Hummel et al., 2003; Frasnelli et al., 2011) which was performed to gauge trigeminal sensitivity for the 4 odors used.

In addition, odor thresholds were obtained for each of the four odors. Participants were repeatedly exposed to high and low odor concentrations (score range 1–8) (Laing and Doty, 2003). Subjects receive triplets of odors presented in glass bottles (4 ml liquid odor; bottles with 50 ml volume, diameter of opening 4 cm) and have to discriminate one bottle with odorous solution from two others containing the diluent propylene glycol (Sigma-Aldrich, Deisenhofen Germany; order number 398039). This test was done to identify the least distinguishable concentrations for each of the four odors used (Croy et al., 2009). All performed tests were based on a forced choice paradigm.

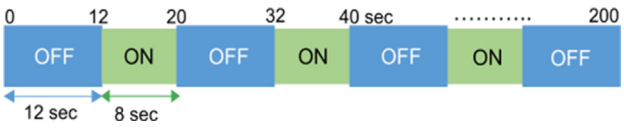

Fig. 1. Experimental design. fMRI design of odor presentation; ON session (10 repeats) each of 8 seconds and OFF session (11 repeats) each of 12 s.

fMRI data acquisition and analysis

Functional brain images were acquired on a 3-T MRI scanner (Siemens Verio, Erlangen, Germany) using a 32– channel head coil. Total of 248 functional images were collected per individual using a T2 single-shot echo-planar imaging (EPI) sequence: TR = 869 ms, TE = 38 ms, 58° flip angle, no interslice gap, 210 × 210 mm field of view. A high-resolution structural T1 image was acquired using a 3D magnetization prepared gradient rapid acquisition gradient echo (MPRAGE) sequence (TR = 2000 ms, TE = 1.95 ms, 256 × 256 mm field of view, voxel size 1 × 1 × 1 mm).

From the 10 ON and OFF phases used for analysis, we compared ON phases for presented stimuli and checked for group wise contrasts ON $_{trigeminal}$ versus ON $_{olfactory}$ for $N = 40$. Lateralization score, intensity, hedonics and threshold test scores were used as covariates. Whole brain activations significant at FWE corrected < 0.05 and p uncorrected < 0.001 with cluster size (k) > 10 voxels are reported. For clusters having multiple peaks, one with the highest t- value is chosen. MNI coordinates are presented at *x*, *y* and *z*, L – left hemisphere, R – right hemisphere. Significant brain regions were labeled with AAL3 toolbox (http://www.gin.cnrs.fr/en/tools/aal-aal2/) (Rolls et al., 2020).

Statistics

Analyses was performed separately for each odor. In addition, to have an elaborated view about how individuals respond towards olfactory and trigeminal stimuli and how neural processing differs for the two sensory channels, we grouped the minty-trigeminal stimuli peppermint and spearmint under 'Trigeminal Group' whereas the non-minty olfactory stimuli strawberry and cherry were grouped under 'Olfactory Group'.

Analysis of psychophysical data: Paired sample t-test was performed to compare the intensity, hedonics, lateralization and threshold test scores for trigeminal (peppermint + spearmint) and olfactory (cherry + straw berry) groups for the total study population and for differences between groups (GC vs N′GC). For analysis, IBM SPSS version 27 (SPSS Inc., Chicago, Ill., USA) was used. The significance level for all statistical tests was set at $p < 0.05$ unless specified. Results presented as mean ± standard deviation (SD). Chi-square test was done comparing males and females in both groups.

Analysis of MRI results: Task-driven general linear model approach (GLM) using Statistical Parametric Mapping (SPM) software (Penny et al., 2011) was used for analysis of olfaction/trigeminal based fMRI. SPM12 was used to analyze the functional MRI data, which is a MATLAB (The Mathworks Inc., Natick, MA, USA) based software (Welcome Trust Centre for Neuroimaging, London, UK). On single-odor level, one sample *t*-test was performed for the ON condition. Furthermore, for group level, analysis contrast images from individuals were selected for a random effect analysis and paired *t*-tests were performed, between and within groups (GC vs N′GC). Results are presented as mean ± standard deviation (SD).

RESULTS

The demographic data along with the group wise test scores for each odor are shown in Table 1. Results are presented as mean ± standard deviation. Subjects from GC and N′GC groups were matched for age. The two groups differed significantly in number of males and females χ (1) = 4.91, p = 0.03. The groups did not differ significantly in terms of odor identification score.

Compared to the N′GC group, the GC group scored higher in the lateralization task for all four stimuli ($t \geq 1.78$, $p < 0.04$) (Table 1). However, there were no significant group differences for odor thresholds, ratings of odor intensity and pleasantness (except for cherry which was rated more intense by the N′GC group ($t = 3.12$, $p = 0.006$). When grouping the odors into trigeminal (peppermint + spearmint) and olfactory stimuli (cherry + strawberry) a similar picture emerged with lateralization scores being higher in the GC group for both types of stimuli ($t > 4.30$, $p < 0.001$). No significant differences were found for the odor thresholds and ratings of intensity and pleasantness.

fMRI results

*Brain activation for individual odors across all participants (**N** = 40).* One sample *t*-test (separately for the

Table 1. Demographic data of participants, separately for chewing gum users (GC) and participants not using mint products (N′GC) (*N* = number of subjects, m = male, f = female, n.s = no significant difference; *p* value for comparison between the two groups)

n = 40	GC (*n* = 20)	N′GC (*n* = 20)	*P*-value
Age (in years)	25.5 ± 2.6	26.4 ± 3.3	n. s.
Sex (m/f)	14/6	7/13	0.03
Identification score/16	13.7 ± 1.6	13.4 ± 1.1	n. s.
Lateralization scores/20			
Peppermint	14.6 ± 1.9	13.0 ± 2.3	0.03
Spearmint	15.0 ± 2.5	11.5 ± 2.6	<0.001
Cherry	13.7 ± 3.1	11.5 ± 3.0	0.02
Strawberry	14.4 ± 2.4	10.5 ± 2.2	< 0.001
Threshold Scores/8			
Peppermint	6.7 ± 1.0	6.8 ± 0.8	n.s.
Spearmint	6.9 ± 1.0	6.8 ± 0.9	n.s.
Cherry	6.5 ± 0.9	6.6 ± 0.9	n.s.
Strawberry	6.5 ± 0.5	6.3 ± 0.8	n.s.
Intensity/10			
Peppermint	6.7 ± 2.2	6.8 ± 1.6	n. s.
Spearmint	6.3 ± 2.1	6.5 ± 1.3	n. s.
Cherry	6.4 ± 2.8	8.1 ± 1.3	0.006
Strawberry	7.8 ± 1.6	8.1 ± 1.3	n. s.
Pleasantness/10			
Peppermint	7.5 ± 1.7	6.6 ± 2.1	n.s.
Spearmint	5.9 ± 1.9	6.0 ± 1.7	n.s.
Cherry	5.2 ± 2.7	5.5 ± 3.0	n.s.
Strawberry	5.6 ± 2.6	5.3 ± 2.5	n.s.

Table 2. Brain regions, involving all participants, activated for the 4 individual odors at FWE $p_{corrected} < 0.05$ and cluster size $k > 10$ voxels, $N = 40$; MNI coordinates presented in *x*, y, and z; L, left hemisphere; R right hemisphere

Odor (ON)	k	*T* value	*x*	*y*	*z*	Region
Peppermint	157	9.17	24	2	–18	Amygdala R
	390	8.36	40	4	–8	Insula R
	452	8.35	−38	0	–12	Insula L
	256	7.64	10	–2	2	Thalamus R
	106	7.36	−24	–2	−16	Amygdala L
	24	7.60	12	–20	−12	SN R
Spearmint	276	7.49	46	20	–4	Insula R
	333	6.71	−42	14	–10	Insula L
	55	6.62	−22	0	–16	Amygdala L
	56	6.23	24	0	–16	Amygdala R
	31	5.66	12	0	6	Thalamus R
Cherry	22	6.09	−40	2	–8	Insula L
Strawberry	79	7.90	−24	0	–14	Amygdala L
	100	7.83	24	2	–18	Amygdala R
	81	7.32	−38	2	–8	Insula L
	33	6.74	38	6	–8	Insula R

four odors) was done to investigate general cerebral activation patterns in response to the four individual odors. Amygdala and insular cortex were the common areas activated by both olfactory and trigeminal stimuli whereas thalamus along with substantia nigra (SN) showed significant activations in the presence of trigeminal odors, peppermint and spearmint. Peppermint odor activated strong thalamic cluster than spearmint odor (Table 2, Fig. 2).

Comparison between responses to trigeminal and olfactory stimuli across all participants. Compared to olfactory stimuli the trigeminal stimuli, across the total study population ($N = 40$), produced activation in left SN and left thalamus at $p_{uncorrected} < 0.001$ for contrast $ON_{Trigeminal} > ON_{olfactory}$ whereas the reverse comparison showed no activations (Fig. 3, Table 3).

Comparisons between activations to trigeminal and olfactory odors separately for GC and N′GC groups. GC group did not show any significant differences between activations to trigeminal and olfactory stimuli (contrast $GC_{trigeminal}$ versus $GC_{olfactory}$). In contrast, N′GC group showed stronger activation in right and left insula for trigeminal stimuli compared to olfactory stimuli (contrast $N'GC_{trigeminal} > N'GC_{olfactory}$, $p_{uncorrected} < 0.001$) (Fig. 4, Table 4).

Comparisons of trigeminal and olfactory activations between groups GC and N′GC. No differences in the brain activations were found for GC and N′GC group in response to trigeminal odors. However, the GC group exhibited higher activations in response to olfactory odors compared to the N′GC group in bilateral insular cortex ($GC_{olfactory} > N'GC_{olfactory}$ at $p_{uncorrected} < 0.001$) (Fig. 5, Table 5).

DISCUSSION

Frequent chewing gum users (GC) localized the trigeminal odors better than the N′GC group. This indicated that the frequent, habitual use of chewing gum was associated with increased chemosensory trigeminal sensitivity. However, in terms of fMRI, they did not show higher central-nervous activations in response to trigeminal odors. It is worth saying that although GC group derives reward or pleasure from the mint odor or from the trigeminal stimulation, no evidence supporting this (dopaminergic activation) was found. In contrast, the N′GC group exhibited enhanced bilateral activations in the insular cortex to trigeminal odors indicating that the trigeminal odors were more meaningful and arousing to them compared to the GC group. This activation in the insular cortex may also relate to gustatory associations. Because of the habitual use of an oral stimulus, chewing gum, this might also serve as an explanation why the GC group had enhanced olfactory activation in comparison to the N′GC group.

Oleszkiewicz et al. (2018) showed that exposure to trigeminal stimuli improves sensitivity towards chemosensory stimuli. The present results appear to confirm these findings with subjects from the GC group having higher scores when localizing trigeminal odors peppermint and spearmint. Another explanation of GC group lateralizing peppermint better than the N′GC group could possibly be due to its repeated sensory exposure whereas for spearmint odor, GC group find its trigeminal association with mint; as for pepper; (Han et al., 2018) whereas N′GC group did not. In addition, Han et al., 2020 also showed that subjects with relatively high use of minty chewing gums exhibited higher responses to chemosensory stimuli compared to subjects with a lower frequency of minty chewing gum use.

Yet, there are contradictory findings when it comes to long-term exposure. Dalton et al. (2006) give one such example of mixed effects where subjects when exposed to the irritant acetic acid over a period of approximately 2 weeks, showed decreased intensity ratings in combination with decreased amplitudes of responses obtained from the nasal respiratory epithelium. Conversely, intensity ratings for the control irritant acetone increased in this study in combination with increased amplitudes of

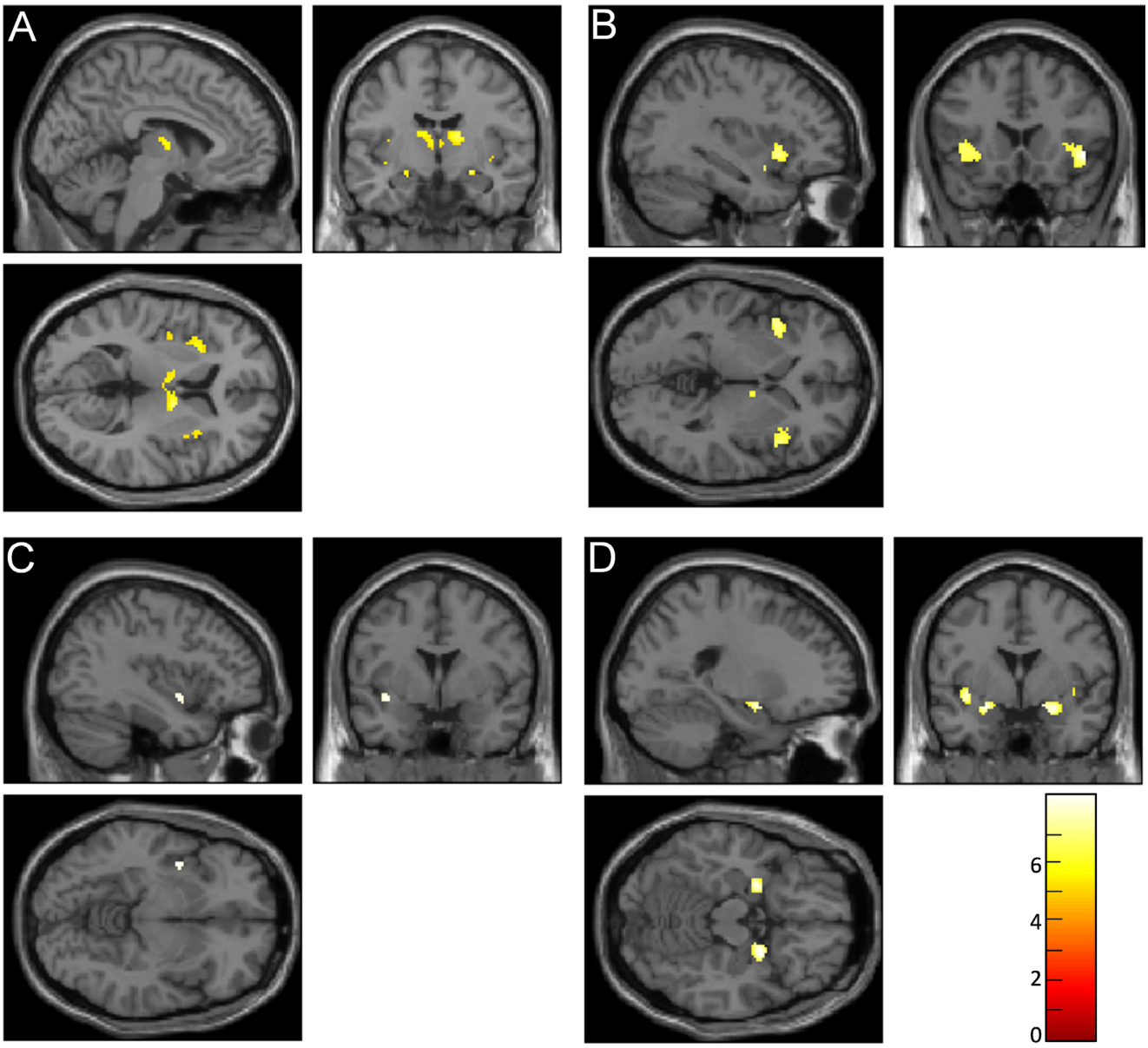

Fig. 2. BOLD responses for individual odors. *N* = 40; brain maps thresholded at FWE $p_{corrected} < 0.05$. Here A = Peppermint odor; B = Spearmint odor; C = Cherry odor; D = Strawberry odor, calibration bars represent threshold values.

responses from the nasal epithelium. This indicated that effects of exposure to chemosensory irritants are not uniform.

When we looked at brain activations in response towards individual odors, we found overlapping neural activations for the olfactory and trigeminal stimuli perceived. Olfactory stimuli cherry and strawberry produced significant activations in the insular cortex and amygdala, which are activated in response to olfactory stimuli. Here the insular cortex acts as an integration area for olfactory, gustatory and trigeminal stimulation (Savic, 2002). However, trigeminal odors peppermint and spearmint also show activations in thalamus and substantia nigra.

Especially with regard to the thalamic activation, the present results confirm previous work indicating that thalamic activation is a crucial part of the processing of nasal trigeminal sensations. Reasons for this include the (1) increased arousal produced by trigeminal stimuli and (2) the somatosensory nature of the trigeminal stimuli (Pellegrino et al., 2017a; Han et al., 2018).

Our study supports previous findings, showing that bimodal odors (having both olfactory and trigeminal properties) are processed in brain regions such as amygdala (Roussos and Hirsch, 2014), insular cortex, and thalamus (Pellegrino et al., 2017b). ‘‘In other words, overlapping activations in the olfactory areas that is amygdala and insula, were seen for all the four odorous stimuli (Fig. 2table 2). Whereas bimodal odors, peppermint and spearmint that interact with both the olfactory and trigeminal systems also showed activations in the thalamus. The thalamus constitutes a major part of the

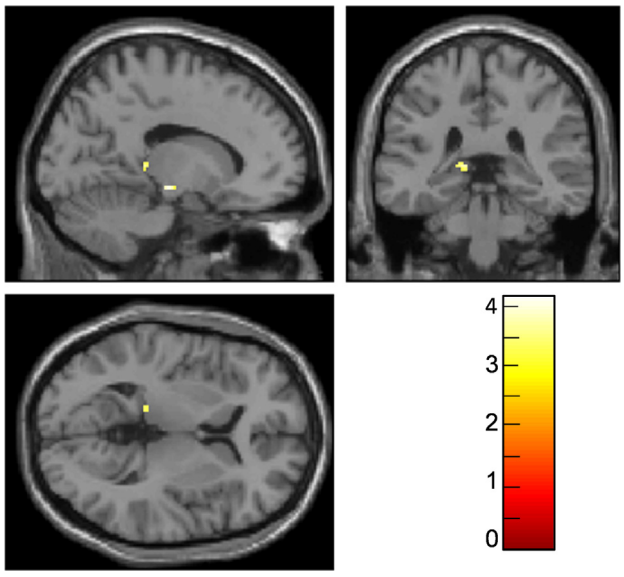

Fig. 3. Trigeminal vs. Olfactory condition. *N* = 40 for contrast ON $_{trigeminal}$ > ON $_{olfactory}$; p $_{uncorrected}$ < 0.001; cluster level 'K' > 10 voxels; calibration bars represent threshold values.

Table 3. Brain regions, involving all participants, activated for contrast ON $_{Trigeminal}$ > ON $_{olfactory;\ N=}$ 40; p $_{uncorrected}$ < 0.001 and cluster level 'K' > 10 voxels; L = left hemisphere and R = right hemisphere; MNI coordinates presented in *x*, *y*, *z*

K	*T* value	*x*	*y*	*z*	Region
16	4.09	−12	–20	−12	SN L
15	3.64	−12	–34	4	Thalamus L

trigeminal pathway which is involved in mediating attention, chemosensory perception and learning (Courtiol and Wilson, 2015). Apart from the thalamus, the right

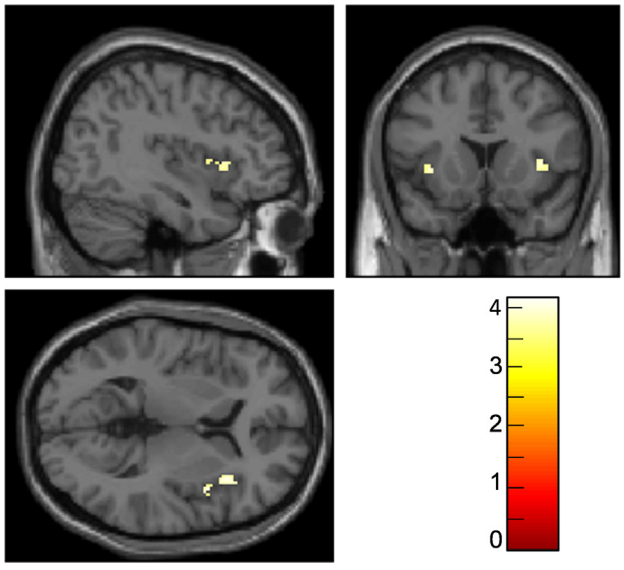

Fig. 4. Trigeminal vs. Olfactory condition: N′GC group. Brain activations in bilateral insular cortex for contrast N′GC $_{trigeminal}$ > N′GC $_{olfactory}$; p $_{uncorrected}$ < 0.001; cluster level 'K' > 10 voxels, calibration bars represent threshold values.

Table 4. Brain regions activated for contrast N′GC $_{trigeminal}$ > N′GC $_{olfactory;}$ *N* = 20 each, p $_{uncorrected}$ < 0.001 and cluster level 'K' > 10 voxels; L = left hemisphere and R = right hemisphere; MNI coordinates presented in *x*, *y*, *z*

K	T value	*X*	*y*	*z*	Region
100	3.82	38	18	4	Insula R
18	3.63	−36	16	0	Insula L

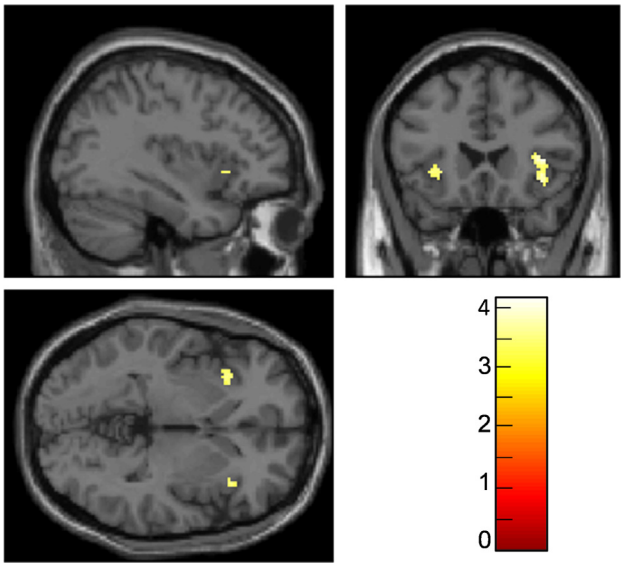

Fig. 5. Olfactory condition: GC versus N′GC group. Brain activations in bilateral insular cortex for contrast GC $_{olfactory}$ > N′GC $_{olfactory;}$ p $_{uncorrected}$ < 0.001; cluster level 'K' > 10 voxels, calibration bars represent threshold values.

Table 5. Brain regions for comparisons between GC and N′GC groups - contrast GC $_{olfactory}$ > N′GC $_{olfactory\ at}$ p $_{uncorrected}$ < 0.001 and at cluster level 'K' > 10 voxels, L = left hemisphere and R = right hemisphere; MNI coordinates presented in *x*, *y*, *z*

K	*T* value	*X*	*y*	*z*	Region
91	4.12	38	24	6	Insula R
38	3.48	−30	22	0	Insula L

substantia nigra was activated in response to the peppermint stimulus. The substantia nigra receives sensory input from the trigeminal system (Harper et al., 1979) and is involved in the perception of trigeminal stimuli (Starr et al., 2011)."

Although trigeminal and olfactory systems have different peripheral pathways, they share brain activations (Frasnelli et al., 2007) in areas such as OFC, insula, somatosensory cortex and amygdala (Boyle et al., 2007; Albrecht et al., 2010). Furthermore, activations in these areas are typically more pronounced for trigeminal stimuli as compared to olfactory stimuli. Our results support this as we see stronger activations in insula and amygdala for the mixed trigeminal-olfactory stimuli peppermint and spearmint in comparison to the

olfactory stimuli cherry and strawberry. These results can be elegnatky explained by the overlapping of neural networks for the trigeminal and olfactory systems (Bensafi et al., 2008; Boyle et al., 2007; Hummel et al., 2005)." In conclusion, GC subjects appeared to be more responsive and sensitive towards trigeminal chemosensory stimuli. However, this did not translate into differences in central-nervous activations to trigeminal stimuli, but the GC group exhibited stronger activation towards olfactory stimuli. Instead, non-frequent chewing gum users showed enhanced trigeminal activation in bilateral insular cortex. Therefore, although the N′GC group was less sensitive, the trigeminal stimuli obviously were more arousing and meaningful to the N′GC group because of their lower degree of exposure in daily life.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

A. Joshi: Data curation, Project administration. **D. Thaploo:** Software, Project administration. **X. Yan:** Data curation, Methodology. **Y. Zang:** Data curation, Methodology. **J. Warr:** Conceptualization. **T. Hummel:** . **:** Conceptualization, Project administration, Supervision, Validation.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2021.07.006.

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Discussion and Outlook

In our first study (publication 1), in this study, we aimed to find an efficient, reliable yet less time-consuming method to calculate the OB volume. In fact, measurement time for the MS method was approximately 7–10 minutes whereas it takes only one minute for the BF method. Our study indicated that the BF approach provides reliable results which are in accordance with the results obtained from MS and when used by experts and non- experts.

So far, the MS of coronal slices is the most widely used method for volumetric measurements of the OB (V. Gudziol et al., 2009). Accuracy and reliability of MS method has been demonstrated clearly in previous studies (Mueller et al., 2005; Yousem et al., 1997). In the present study, we also followed up accuracy and reliability for the measurements made by the BF approach using ITK-SNAP software. This software was chosen for its user-friendly interface and free availability. However, many other software solutions could be used for this straight-forward technique. For the BF approach, intraclass coefficients of correlation between measurements of the two observers were at r = 0.96 for right OB and r = 0.89 for left OB. The results drawn from this new approach were comparable with the results obtained from MS approach with r = 0.84 for right OB and r = 0.86 for left OB.

The focus throughout the project was on the introduction of a method that can be clinically acceptable, with time demands being a major issue. This is important, as OB volume is considered as a measure to evaluate the status of olfactory functioning. There has been evidence in support of how OB volume clinically describes the severity of olfactory loss. For example, in comparison to hyposmic patients, OB volumes were found to be smaller for anosmic subjects in olfactory loss, following infections of the upper respiratory tract or head trauma (Han, Croy, et al., 2020). Importantly, OB volume also seems to be a predictor of recovery in patients with post-infectious olfactory loss (Rombaux et al., 2012). Hence, the routine assessment of OB volume appears to be useful in patients with olfactory loss. This is more likely to be diagnostically implemented with the availability of a fast and convenient approach.

The present investigation also revealed that the internal consistency of measurements made with either method was excellent. Hence, it can be noted that the new BF method can be used as a clinically acceptable, efficient, reliable, easy, and quick approach to calculate OB volumes. However, it has to be kept in mind that both MS and BF method remain subjective and voxel selection may vary depending on skills of the individual observers which requires some degree of training.

To conclude, the present results suggest that the BF method for OB volumetric is reliable and produces valid results, comparable to the results from MS. The new technique is a simple,

quick approach and may require less training than MS of the OB. It is hoped that this technique paves the road for the routine clinical assessment of OB volume in patients with olfactory loss.

In our second study (Publication 2), we investigated neural processing of words with olfactory associations in patients with life-long olfactory loss (congenital anosmia, CA) in comparison with a group of control participants with normal olfaction (NC) and a group of patients with acquired olfactory dysfunction (idiopathic anosmia, IA). Most importantly, the CA group, having never sensed any olfactory stimuli, showed stronger activations in the posterior OFC, extending to insular cortex compared to NC participants during OW reading. The activation in the OFC region is comparable to what has been observed in both healthy controls and patients with acquired olfactory loss where reading of olfactory associated words led to similar activations (González, Barros-Loscertales, Pulvermüller, Meseguer, Sanjuán, Belloch, & Avila, 2006; Han, Croy, et al., 2020). During expectation of OW, the IA patients demonstrated stronger activation in the posterior OFC extending to insula as compared to the CA patients. The OFC is relevant for the processing and integration of information from different sensory modalities (Kringelbach & Rolls, 2004). In addition, activation in the OFC supports the interaction between the odor related word cues and their respective odor percepts (Olofsson et al., 2014). In comparison to NC, IA patients when expecting the olfactory associated words also showed major activations in caudate, which can be interpreted for its involvement in executive processes (Seger & Cincotta, 2005) specifically, in goal-directed actions. Moreover, in comparison to CA, NC group showed activations extending to ACC, which indicate its key role in attention (Botvinick, 2007; Pessoa, 2008) and in working memory tasks (*The Prefrontal Cortex - 5th Edition*). NC, IC, and CA seem to use distinct strategies when it comes to anticipating words with olfactory associations.

During OW reading, stronger activation of a very similar cluster in the posterior OFC extending to insula was found among CA as compared to NC groups. As stated above, this region is involved in multisensory integrative processing, that receive information from the olfactory, gustatory and visual sources (Bonnici et al., 2016; Fournel et al., 2017; Seghier, 2013). Unlike the expectation condition where no direct odor-related cues were shown, displaying OW could initiate neural processing of word related semantic and olfactory information more efficiently. Although CA patients have a life-long deprivation of olfactory perception, their knowledge regarding other chemical inputs (e.g. gustatory and trigeminal input) as well as the semantic meaning of the OW remains intact. Besides, CA patients have been shown to exhibit slightly enhanced abilities for non-chemical multisensory (e.g. audio-visual) integration compared to people with intact olfaction (Plailly et al., 2012), indicating an existing compensatory mechanism. Therefore, stronger activation during OW reading among the CA group may reflect the process of multisensory integration, involving semantic comprehension. The exact process behind the increased activation among CA remains to be explored. Stronger activation

was also found in the occipital cortex among CA compared to IA during reading of OW, possibly indicating an enhanced attention paid to the olfactory-related words among CA patients.

A number of fMRI studies have reported similar patterns of brain activation during olfactory memory tasks cued by non-odorous objects such as images or words (Arshamian et al., 2013b; Gottfried, 2010b; Lehn et al., 2013) and during the processing of physical olfactory stimuli. Brain activity during representation of sensory stimulus without direct external stimulus (mental imagery) has been studied in various modalities including visual, auditory and tactile. In general, regions associated with mental imagery were found to be those regions associated with perception in the same sensory modality (Halpern, 2001; Kosslyn et al., 2001). In the present case one would expect participation of primary and secondary olfactory areas, given that these participate in olfactory perception (Sobel et al., 1998; Zald & Pardo, 2000). The present observed activation of OFC when reading the OW, can be related to the odor imagery approach as shown in previous studies. There activation in the right OFC, associating imagery with the perception of physically present odors was related to the experienced realness or "vividness" of an olfactory image (Zald & Pardo, 2000; Zatorre & Jones-Gotman, 2000). In our study, given the visual sources, integration of visual and olfactory information occurs in OFC, where the odor percepts were linked to their respective names. Djordjevic et al. (Djordjevic et al., 2005b) also reported activation of the insular cortex as a result of odor imagery. Neuroimaging studies suggest that a number of factors could modify activation of these olfactory brain regions. Among these possible effects are: increased respiratory amplitude, due to sniffing (Kleemann et al., 2009), attentional demands (Geisler & Murphy, 2000), lexico-semantic processing of words (González, Barros-Loscertales, Pulvermüller, Meseguer, Sanjuán, Belloch, & Avila, 2006), or cross modal associative learning (Royet et al., 2013). In all, olfactory top-down processing has a significant role in encoding or recalling of learned information (Rolls, 2011a), which results in anticipation of an odor or processing of odor-associated cues (Han, Croy, et al., 2020). Therefore, based on the present results it appears that there is overlap of neural processing in terms of both bottom-up and top-down olfactory representation.

A few limitations apply to the current study. First, the sample size is small. However, given the scarcity of CA cases, studies on this group of patients are typically not large (i.e., less than 20 patients). Second, the breathing was not monitored during the MRI scan. The possible alteration of breathing in patients (H. Gudziol et al., 2010) may introduce noise as variable that affect the observed brain responses (Kareken et al., 2004; Sobel et al., 1998). Thirdly, we did not explore the association of the presented words with foods. Such an association might explain some of the overlapping activations in the three groups of patients; fourthly, for reasons of study design, olfactory words and control words were not evaluated for their valence and their association with edibility which also might impact the processing of words.

In conclusion, our results demonstrate different neural responses during expectation and reading of words with strong olfactory associations among people with acquired anosmia, congenital anosmia and normosmia. Increased activation of the higher-order brain regions related to multisensory integration among CA during reading of olfactory related words may suggest a compensatory mechanism for processing of semantic olfactory cues and is an expression of adaptive changes in response to changes of olfactory function.

In our third study (Publication 3), frequent chewing gum users (GC) localized the trigeminal odors better than the N'GC group. This indicated that the frequent, habitual use of chewing gum was associated with increased chemosensory trigeminal sensitivity. However, in terms of fMRI, they did not show higher central-nervous activations in response to trigeminal odors. It is worth saying that although GC group derives reward or pleasure from the mint odor or from the trigeminal stimulation, no evidence supporting this (dopaminergic activation) was found. In contrast, the N'GC group exhibited enhanced bilateral activations in the insular cortex to trigeminal odors indicating that the trigeminal odors were more meaningful and arousing to them compared to the GC group. This activation in the insular cortex may also relate to gustatory associations. Because of the habitual use of an oral stimulus, chewing gum, this might also serve as an explanation why the GC group had enhanced olfactory activation in comparison to the N'GC group.

(Oleszkiewicz et al., 2018) showed that exposure to trigeminal stimuli improves sensitivity towards chemosensory stimuli. The present results appear to confirm these findings with subjects from the GC group having higher scores when localizing trigeminal odors peppermint and spearmint. Another explanation of GC group lateralizing peppermint better than the N'GC group could possibly be due to its repeated sensory exposure whereas for spearmint odor, GC group found its trigeminal association with mint; as for pepper; (Han et al., 2018) whereas the N'GC group did not. In addition, (Han, Croy, et al., 2020)also showed that subjects with relatively high use of minty chewing gums exhibited higher responses to chemosensory stimuli compared to subjects with a lower frequency of minty chewing gum use.

Yet, there are contradictory findings when it comes to long-term exposure. (Dalton et al., 2006) gave one such example of mixed effects where subjects when exposed to the irritant acetic acid over a period of approximately 2 weeks, showed decreased intensity ratings in combination with decreased amplitudes of responses obtained from the nasal respiratory epithelium. Conversely, intensity ratings for the control irritant acetone increased in this study in combination with increased amplitudes of responses from the nasal epithelium. This indicated that effects of exposure to chemosensory irritants are not uniform and may depend on the stimulant, the duration of exposure, the interval between exposures, context or the concentration of the stimulant.

When we looked at brain activations in response towards individual odors, we found overlapping neural activations for the olfactory and trigeminal stimuli perceived. Olfactory stimuli cherry and strawberry produced significant activations in the insular cortex and amygdala, which are activated in response to olfactory stimuli. Here the insular cortex acts as an integration area for olfactory, gustatory and trigeminal stimulation (Savic et al., 2002). However, trigeminal odors peppermint and spearmint also showed activations in thalamus and substantia nigra.

Especially with regard to the thalamic activation, the present results confirm previous work indicating that thalamic activation is a crucial part of the processing of nasal trigeminal sensations. Reasons for this include the (1) increased arousal produced by trigeminal stimuli and (2) the somatosensory nature of the trigeminal stimuli (Han et al., 2018; Pellegrino, Sinding, et al., 2017).

Our study supports previous findings, showing that bimodal odors (having both olfactory and trigeminal properties) are processed in brain regions such as amygdala (Roussos & Hirsch, 2014), insular cortex, and thalamus (Pellegrino, Drechsler, et al., 2017). In other words, overlapping activations in the olfactory areas that is amygdala and insula, were seen for all the four odorous stimuli (Fig. 2, table 2, publication 2). Whereas bimodal odors, peppermint and spearmint that interact with both the olfactory and trigeminal systems also showed activations in the thalamus. The thalamus constitutes a major part of the trigeminal pathway, which is involved in mediating attention, perception and learning (Courtiol & Wilson, 2015). Apart from the thalamus, the right substantia nigra was activated in response to the peppermint stimulus. The substantia nigra receives sensory input from the trigeminal system (Harper et al., 1979) and is involved in the perception of trigeminal stimuli. Most odorants not only stimulate olfactory receptor neurons but also activate the intranasal trigeminal nerve. The simultaneous activation of the olfactory and the trigeminal system leads to an interaction in the brain. Therefore, assessment of the trigeminal impact of odorants may be difficult in subjects with a normal sense of smell. To obtain a deeper insight into both, mechanisms of changes in trigeminal sensitivity in anosmic patients and interactions between the olfactory/trigeminal systems in healthy subjects, 21 patients with isolated congenital anosmia (ICA) were investigated in this series of explorative, hypothesis-generating experiments and compared with 35 healthy controls. Trigeminal sensitivity was measured by psychophysical (lateralization task, intensity ratings) and electrophysiological (trigeminal event-related potential, negative mucosal potential) means. ICA patients were found to have higher peripheral activation than controls. On central levels, however, similar responsiveness to trigeminal stimuli was found in ICA patients when compared with healthy subjects. The results of the study are discussed by proposing a model of mixed sensory adaptation/compensation in the interactions between olfactory and the trigeminal system.

Although the trigeminal and olfactory systems have different peripheral pathways, they share brain activations (Frasnelli et al., 2007) in areas such as OFC, insula, somatosensory cortex and amygdala (Albrecht et al., 2010; Boyle et al., 2007). Furthermore, activations in these areas are typically more pronounced for trigeminal stimuli as compared to olfactory stimuli. Our results support this as we see stronger activations in insula and amygdala for the mixed trigeminal-olfactory stimuli peppermint and spearmint in comparison to the olfactory stimuli cherry and strawberry. These results can be elegantly explained by the overlapping of neural networks for the trigeminal and olfactory systems Bensafi et al., 2008; Boyle et al., 2007; Hummel et al., 2005). In conclusion, GC subjects appeared to be more responsive and sensitive towards trigeminal chemosensory stimuli. However, this did not translate into differences in central-nervous activations to trigeminal stimuli, but the GC group exhibited stronger activation towards olfactory stimuli. Instead, non-frequent chewing gum users showed enhanced trigeminal activation in bilateral insular cortex. Therefore, although the N'GC group was less sensitive, the trigeminal stimuli obviously were more arousing and meaningful to the N'GC group because of their lower degree of exposure in daily life.

Summary in German

Hintergrund

Der Geruchssinn spielt eine wichtige Rolle in unserem täglichen Leben, während sein Fehlen erhebliche Auswirkungen auf das Leben von Menschen mit Geruchsstörungen hat, einschließlich Veränderungen in ihrer geistigen, sozialen und körperlichen Gesundheit. Der Verlust des Geruchssinns kann eine Vorstufe zu schweren neurodegenerativen Erkrankungen wie Parkinson und Alzheimer sein, und kann mit depressiven Symptomen einhergehen. Daher sollten Menschen mit Riechverlust adäquat untersucht und behandelt werden. In den drei zu einer Arbeit zusammengefassten Veröffentlichungen wurde die MRT zur Untersuchung der Riechfunktion und ihrer Plastizität eingesetzt, vor allem bei Patienten mit Riechstörungen. Publikation 1 befasste sich mit der Verbesserung bestehender Methoden zur Bewertung des Volumens des Bulbus olfactorius (OB) hinsichtlich der strukturellen Bewertung der Riechfunktion. Publikation 2 befasste sich mit der funktionellen Plastizität des olfaktorischen Systems bei Patienten mit angeborener und erworbener Anosmie, wenn der olfaktorische Input fehlt. Publikation 3 befasste sich mit der Plastizität des chemosensorischen Systems am Beispiel der gewohnheitsmäßigen Exposition zu trigeminalen Gerüchen.

Methoden

In Publikation 1 wurden 52 Probanden einer 3-T-MRT Untersuchung des Gehirns unterzogen. Alle Probanden wurden mit der "Sniffin' Sticks"-Testbatterie auf ihre orthonasale Riechfunktion hin getestet. Mit Hilfe der AMIRA®-Software berechneten zwei geschulte Beobachter das OB-Volumen mit einem manuellen Segmentierungsverfahren, der planimetrischen manuellen Konturierung (PMC) (Fläche in mm^3). Mit ITK-SNAP®-Software verwendeten die gleichen Beobachter die neue Methode "box-frame" zur Berechnung des OB-Volumens. Zunächst wurde die Anzahl der Schichten (Länge) mit deutlicher Erkennbarkeit des OB notiert. Bei der Box-Methode wurde angenommen, dass Höhe und Breite der Markierungen in einem Winkel von 90° zueinander stehen. Das Volumen wurde als Vielfaches von L x B x H (Scheibendicke in mm^3) berechnet. Bei divergenten Befunden wurde ein dritter Beobachter herangezogen, und die zwei am nächsten liegenden Volumina mit weniger als 10 % Unterschied zur weiteren Betrachtung ausgewählt.

In Publikation 2 wurden 40 Probanden mit 3-T-fMRT untersucht. Davon waren 18 gesunde Probanden, 14 waren Probanden mit kongenitaler Anosmie und 8 hatten eine idiopathische Anosmie. Den Probanden wurden 36 Wörter mit starker olfaktorischer Assoziation (OW) und 36 Kontrollwörter mit geringer oder keiner olfaktorischen Assoziation (CW) präsentiert. Die Teilnehmer wurden angewiesen, die Anweisungen und Wörter zu lesen. Vor den Wortblöcken wurden die Teilnehmer darauf hingewiesen, sich auf die olfaktorischen Aspekte der angezeigten Wörter zu konzentrieren, um eine Erwartung für im Folgenden gezeigten Wörter

zu wecken und um die OW- von den CW-Blöcken klar zu trennen. Geruchsbezogene semantische Unterschiede wurden als Kriterium für die Unterscheidung zwischen den Aktivierungen gewählt. Wir verglichen vor allem Aktivierungsphasen, in denen OW erwartet wurden mit denjenigen, in denen OW gelesen wurden.

In Publikation 3 nahmen 40 gesunde Probanden an einer fMRT-Untersuchung teil. Ein Teil der Probanden kaute regelmäßig Kaugummi mit Minzegeschmack (GC, n = 20), ein anderer Teil verwendete nie bzw. sehr selten Kaugummi oder andere Lebensmittel mit Minzgerüchen, z.B. Pfefferminztee (N'GC, n = 20). Mit Hilfe eines computergesteuerten Olfaktometers wurden den Probanden in vier separaten Sitzungen zwei „trigeminale Gerüche“ (Pfefferminze und Minze) und zwei „olfaktorische Gerüche“ (Kirsche und Erdbeere) verabreicht. Nach jeder Sitzung bewerteten die Probanden die Intensität und die Angenehmheit der angebotenen Gerüche.

Ergebnisse

In Publikation 1 berechneten wir die OB-Volumina mit beiden Techniken und fanden vergleichbare Ergebnisse. Für die von beiden Beobachtern berechneten Volumina wurde eine hohe Korrelation festgestellt. Für die manuelle Segmentierung betrug Cronbachs α 0,91 bzw. 0,93 für das rechte bzw. linke OB-Volumen, während für die Box-Frame-Methode α 0,94 bzw. 0,90 für das rechte bzw. linke OB-Volumen betrug.

In Publikation 2 zeigten die Teilnehmer mit idiopathischer und congenitaler Anosmie während der Erwartung der OW eine stärkere Aktivierung im posterioren OFC, die sich bis zur rechten Insula, dem Caudatum und dem fronto-medialen OFC erstreckte. Während des Lesens der OW zeigten Teilnehmer mit congenitaler Anosmie eine stärkere Aktivierung im posterioren OFC, die bis zur Insula reichte.

In Publikation 3 zeigte die GC-Gruppe eine höhere trigeminale Empfindlichkeit im Vergleich zur N'GC-Gruppe. Olfaktorische Gerüche aktivierten den bilateralen insulären Kortex und die Amygdala. Neben den olfaktorischen Bereichen (Amygdala, insulärer Kortex) führten trigeminale Gerüche auch zu Aktivierungen im rechten Thalamus und der rechten Substantia nigra. In der GC-Gruppe führten olfaktorische Gerüche zu einer stärkeren bilateralen Aktivierung des insularen Kortex als in der N‘GC-Gruppe, während für trigeminale Gerüche keine derartigen Unterschiede beobachtet wurden. GC-Probanden schienen auf trigeminale chemosensorische Reize empfindlicher zu reagieren.

Schlussfolgerungen

Mit der Veröffentlichung 1 konnten wir eine neue zuverlässige Methode vorstellen, die plastische Veränderungen auf der Ebene des OB auf effiziente Weise messbar macht. Die Methode ist zeitsparend und erfordert nur einen geringen technologischen Aufwand, was in

die klinische Routine bedeutsam ist. Damit können strukturelle plastische Veränderungen des zentralnervösen Riechsystems zu diagnostischen Zwecken effektiv genutzt werden.

In Publikation 2 fanden wir funktionelle Plastizität bei Patienten mit angeborener und erworbener Anosmie. Dieser Ansatz zeigte eine Aktivierung in den sekundären Geruchsregionen wie dem posterioren OFC, die sich bei Menschen mit angeborener Anosmie im Vergleich zu Riechgesunden bis zur Insula ausdehnte. Diese Aktivität ist am ehesten im Zusammenhang mit multisensorischer Integration zu sehen, was wiederum auf kompensatorische Mechanismus für die Verarbeitung semantischer Geruchsinformationen bei fehlendem Riechvermögen schließen lässt.

In Publikation 3 untersuchten wir die Plastizität des chemosensorischen Systems bei gewohnheitsmäßiger Exposition zu trigeminalen Gerüchen. Gegenüber selektiv olfaktorischen Aktivierungen gibt es Überlappungen aber auch deutliche Unterschiede in der Peripherie und im ZNS, wie trigeminale Gerüche verarbeitet werden. Erwartungsgemäß schienen Teilnehmer mit habituellem Minzgebrauch empfindlicher auf trigeminale chemosensorische Reize zu reagieren. Dies führte jedoch nicht zu Unterschieden in der zentralnervösen Aktivierung für trigeminale Reize. Vielmehr erschienen trigeminale Gerüche für die Gruppe mit geringem Minzkonsum bedeutungsvoller und erregender. In der Summe zeigen die Arbeiten, dass das chemosensorische System außerordentlich plastisch ist, auf stuktureller und funktioneller Ebene und wir uns ständig an unsere Umwelt anpassen.

Summary in English

Background

Sense of smell or olfaction has a major role in our daily life whereas its absence has a major consequence on life of people with olfactory dysfunction including changes in their mental, social and physical health. Smell loss is a precursor of major neurodegenerative disorders such as Parkinson's and Alzheimer's disease and negligence in treatment might increase risk of major diseases. Therefore, people need to be aware and smell loss needs to be acknowledged with proper treatment.

In the three publications mentioned, MRI was used to investigate olfactory function and its plasticity, mostly in patients with olfactory dysfunction. Publication 1 focused on the improvement of existing methods to assess OB volume as a structural assessment of olfactory function. Publication 2 focused on the functional plasticity of olfactory system in patients with congenital and acquired anosmia when in absence of olfactory input. Publication 3 focused on the plasticity of chemosensory system using habitual exposure of trigeminal odors as an example.

Hypothesis

In publication 1, we aimed at introducing a new efficient, faster way to calculate OB volume. We examined 1) its test-retest reliability and 2) validity, comparing them to the established technique, i.e. OB volumetric based on manual segmentation of OB boundaries (3) checking usability of the new technique by experts and non- experts. Change in OB volume correlates well with the odor functioning, however, assessment of it requires volume delineation and is time consuming and also requires specific training. Because of this, it is not yet included in routine examination. This might change with the availability of a reliable but less investigator based and faster method.

In publication 2 using fMRI, we aimed to investigate top- down brain processing of odor-related words in CA and compare that to patients with acquired IA and NC. We hypothesized that IA and NC subjects show more activations in olfactory associated areas because of their pre-existing olfactory associated semantic knowledge whereas activations in CA subjects were expected to be significantly lower as compared to others because of their complete lack of olfactory experience. To date, there are very few studies focusing on the functional alterations in congenital anosmic patients.

In publication 3, using fMRI, we used habitual consumption of foods with trigeminal stimulants as a model to investigate effects of long-term trigeminal stimulation. A reason behind choosing

the use of chewing gum is because it is one of the most popular pastimes in the younger population. we aimed to study possible changes of the chemosensory systems in response to prolonged stimulation to trigeminal stimuli. Brain activations were compared between GC and N'GC in response to both trigeminal and olfactory odors using functional brain imaging. For testing, we included the minty flavor of chewing gums, namely peppermint and spearmint as ''trigeminal odor" eliciting a cooling and even slightly painful sensation (McKemy et al., 2002) and non-minty, olfactory stimuli such as strawberry and cherry. We hypothesized reduced habituation effect in frequent gum chewers.

Methods

In publication 1, 52 subjects underwent 3 T MRI of the brain. All subjects were tested for their orthonasal olfactory functioning using the "Sniffin' Sticks" test battery. Using AMIRA software, two trained observers calculated the OB volume using manual segmentation approach that is the planimetric manual contouring (PMC) technique (surface in mm3). Using ITK-SNAP, two trained observers used the new method "box-frame" to calculate OB volume. Firstly, the number of slices (length) with distinct visibility of the OB was noted. Using box approach, marking annotations height and width were assumed to be at 90 degree angle to each other. Volume was calculated as multiple of l*w*h* slice thickness mm3. After input of the third observer, two closest volumes with less than 10% difference were selected.

In publication 2, forty subjects underwent 3 T fMRI scanning. Out of them, 18 were healthy subjects, 14 were congenital anosmic subjects and 8 were idiopathic. Thirty-six words with strong olfactory association (OW) and 36 control subjects (less or no olfactory association) were presented to the subjects. Participants were instructed to covertly read the instructions and words. Cueing prior to word blocks was adopted to guide participants to (1) focus on the olfactory aspects of the displayed words (2) induce an expectation for the following words; and (3) to clearly separate the OW from the CW blocks. Olfactory related semantic differences were chosen as a criterion to differentiate between conditional activation. We focused on OW expect and OW read. We did one way ANOVA to test between group differences regarding OW expect and OW read.

In publication 3, forty healthy subjects participated in fMRI study. GC, n = 20 and N'GC, n = 20 were identified based on a questionnaire about their mint consumption patterns. Using computer-controlled olfactometer, subjects received two trigeminal odors peppermint and spearmint and two olfactory odors cherry and strawberry in four separate sessions. After each session, subjects rated intensity, pleasantness for the delivered odors.

Results

In publication 1, we calculated OB volumes using both techniques and found comparable outcomes. High inter-observer reliability was found for volumes calculated by both observers.

For manual segmentation, Cronbach's alpha (α) was 0.91 and 0.93 for right and left OB volume, respectively, whereas for the box-frame method αwas 0.94 and 0.90 for right and left OB, respectively.

In publication 2, during the expectancy condition of OW, IA and NC groups showed stronger activation in posterior OFC extending to right insula, caudate region and frontal medial OFC respectively. Whereas during the reading condition of OW, CA patients showed stronger activation in posterior OFC extending to the insula.

In publication 3, The GC group exhibited higher trigeminal sensitivity compared to the N'GC group. (2) Olfactory odors activated bilateral insular cortex and amygdala. Apart from olfactory areas (amygdala, insular cortex), trigeminal odors also produced activations in right thalamus and right substantia nigra. (3) In the GC group, olfactory odors produced higher bilateral insular cortex activation than in N0GC group, but no such differences were observed for trigeminal odors. GC subjects appeared to be more responsive to trigeminal chemosensory stimuli.

Conclusions

With publication 1 we introduced a new reliable method that allows us to track plastic changes at the level of OB in a very efficient way. This method is time efficient, requires less technicality which might pave its way into routine clinical workup for better assessment in patients with olfactory loss.

With publication 2, we found functional plasticity in patients with congenital and acquired anosmia when in absence of olfactory input. The top-down approach gave a deeper understanding of higher order brain activations in people with olfactory loss. This approach successfully showed activation in the secondary olfactory regions such as posterior OFC extended to insula in congenital anosmic patients compared to healthy controls. This activity is related to multisensory integration suggesting a compensatory mechanism for processing of semantic olfactory cues.

With publication 3, we accessed plasticity of the chemosensory system with habitual exposure to trigeminal odors. There exist peripheral and central differences in the way trigeminal odors are processed. Behaviorally - as expected - frequent mint consumers appeared to be more responsive and sensitive towards trigeminal chemosensory stimuli. However, this did not translate into differences in central-nervous activations to trigeminal stimuli. Rather, trigeminal odors were more meaningful and arousing for the group with non-frequent mint consumption.

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