fMRI study of olfactory processing in mice under three anesthesia protocols: Insight into the effect of ketamine on olfactory processing

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ABSTRACT

Functional magnetic resonance imaging (fMRI) is a valuable tool for studying neural activations in the central nervous system of animals due to its wide spatial coverage and non-invasive nature. However, the advantages of fMRI have not been fully realized in functional studies in mice, especially in the olfactory system, possibly due to the lack of suitable anesthesia protocols with spontaneous breathing. Since mice are widely used in biomedical research, it is desirable to evaluate different anesthesia protocols for olfactory fMRI studies in mice. Dexmedetomidine (DEX) as a sedative/anesthetic has been introduced to fMRI studies in mice, but it has a limited anesthesia duration. To extend the anesthesia duration, DEX has been combined with a low dose of isoflurane (ISO) or ketamine (KET) in previous functional studies in mice. In this report, olfactory fMRI studies were performed under three anesthesia protocols (DEX alone, DEX/ISO, and DEX/KET) in three different groups of mice. Isoamyl-acetate was used as an odorant, and the odorant-induced neural activations were measured by blood oxygenation-level dependent (BOLD) fMRI. BOLD fMRI responses were observed in the olfactory bulb (OB), anterior olfactory nuclei (AON), and piriform cortex (Pir). Interestingly, BOLD fMRI activations were also observed in the prefrontal cortical region (PFC), which are most likely caused by the draining vein effect. The response in the OB showed no adaptation to either repeated odor stimulations or continuous odor exposure, but the response in the Pir showed adaptation during the continuous odor exposure. The data also shows that ISO suppresses the olfactory response in the OB and AON, while KET enhances the olfactory response in the Pir. Thus, DEX/KET should be an attractive anesthesia for olfactory fMRI in mice.

1. Introduction

Functional magnetic resonance imaging (fMRI) (Ogawa et al., 1990) can non-invasively measure neural activations in the central nervous system (CNS) with wide spatial coverage, and it has been proven to be a powerful tool for investigating neural processing in animals. Due to the extreme sensitivity of fMRI to subject motion, the majority of animal fMRI studies have been performed under anesthesia (Adamczak et al., 2010; Baltes et al., 2011; Bosshard et al., 2010; Grandjean et al., 2014; Guilfoyle et al., 2013; Jonkers et al., 2011; Muegler et al., 2003; Nair and Duong, 2004; Poplawsky et al., 2015; Poplawsky and Kim, 2014; Reimann et al., 2018; Sanganahalli et al., 2016; Schroeter et al., 2014; Thompson et al., 2018). Anesthetics such as isoflurane (ISO), α-chloralose, propofol, urethane, xylazine, dexmedetomidine (DEX), or etomidate have been used in mice fMRI studies. Among these anesthetics, urethane has been used for olfactory fMRI studies in mice (Xu et al., 2003, 2005), but is not suitable for longitudinal studies due to its toxicity (Matsuura and Downie, 2000). DEX, an α2-adrenoreceptor agonist which can provide sedation, has been used in longitudinal fMRI studies of visual and somatosensory processing in mice (Adamczak et al., 2010; Niranjani et al., 2016). However, the anesthesia duration with DEX alone in mice is relatively short (~1 h) (Adamczak et al., 2010). Furthermore, a study in rats shows that DEX alone as anesthesia causes epileptic activities, and that a low dose of ISO (~0.3%) suppresses such epileptic activities (Fukuda et al., 2013). Low dose of ISO (0.2%–0.5%) or ketamine (KET) have been added to DEX for functional studies in mice to improve the anesthesia duration in previous studies (Grandjean et al., 2014; Luo et al., 2012; Schroeter et al., 2017). If DEX/ISO or DEX/KET is used for olfactory fMRI study in mice, the impacts of ISO or KET on olfactory processing need to be investigated.

KET is a well-established supplementary anesthetic and has been used in both humans and animals (Domino et al., 1965; Shim et al., 2018).
addition to be used as a supplementary anesthetic, KET with a subanesthetic dose elicits symptoms similar to those observed in schizophrenia (Duncan et al., 1998; Kandratavicius et al., 2015). Thus, subanesthetic KET in animals has been used as an animal model for schizophrenia drug discovery. If subanesthetic KET modulates olfactory processing, the modulation may be used as a biomarker to test the reversal effect of potential therapeutic compounds for schizophrenia (Monte et al., 2013). Previous studies have tested the effects of subanesthetic KET on the olfactory processing in the OB of rats by c-fos mRNA and electrophysiological recordings (Jacobson et al., 1990; Wilson et al., 1996), but the results are not conclusive. Surprisingly, there are no reports about the effect of KET on olfactory processing in the high olfactory structures such as Pir. To understand effects of KET on the olfactory system is significant not only for anesthetia selection in functional studies, but also for schizophrenia drug discovery.

Neuronal activity leads to a series of physiological events, including localized increases in cerebral blood flow, cerebral blood volume, and cerebral metabolic rate of oxygen (CMRO₂). These physiological responses cause an alteration in blood deoxyhemoglobin level, which can be detected by blood oxygen level dependent (BOLD) fMRI (Ogawa and Lee, 1990). For BOLD fMRI, changes of the deoxyhemoglobin level in the blood of capillaries/venules in activated regions act as the physiological source for BOLD fMRI signals.

In this work, BOLD fMRI studies were performed under three anesthesia protocols (DEX alone, DEX/ISO, and DEX/KET) in three different groups of mice to investigate odorant-induced olfactory processing. The similarities between the responses from these three groups demonstrate the reliability of the observed fMRI activations. The differences in the responses among these groups provide information for assessing the impacts of ISO and KET on olfactory processing.

2. Experimental procedures

2.1. Animal preparation

All animal procedures were approved by the Institutional Animal Care and Use Committee of our institution. Thirty female C57BL/6 mice from Taconic (3 studies, 10 mice/study), with ages ranging between 15 and 24 weeks, and weights ranging between 19 and 24 g, were studied. The animals were initially anesthetized with isoflurane (ISO) (5% for induction and 2.5% during set-up). Oxygen-enriched medical air (a mixture of medical air and O₂ with the ratio of 1 : 0.3) was used as the induction and 2.5% during set-up). The animals were initially anesthetized with isoflurane (ISO) (5% for induction and 2.5% during set-up). Oxygen-enriched medical air (a mixture of medical air and O₂ with the ratio of 1 : 0.3) was used as the induction and 2.5% during set-up). Oxygen-enriched medical air (a mixture of medical air and O₂ with the ratio of 1 : 0.3) was used as the induction and 2.5% during set-up).

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2.2. Odor stimulation

Olfactory activations were induced by the odorant isoamyl-acetate. The odorant was delivered to animals in the respiratory gas. The gas (oxygen-enriched medical air) with or without the odorant was continuously delivered to the nosecone throughout the experiment. The gas flow was split into two pathways, one of which received odorized gas through a bubbling bottle containing the odor solution. The two pathways joined together prior to entering the nosecone. During the odor stimulation period, the odor was introduced by opening two valves (Part No.: 075P2NC24-02SQ, BIO-CHEM FLUIDICS) at the inlet and outlet of the bubbling bottle through a control signal from the MRI sequence. In our study, the gas delivered to nosecone contained half bubbled gas (see Fig. 1A in Zhao et al. (2015)). The estimated concentration of isoamyl-acetate was ~2870 ppm.

2.3. fMRI experiments

All MRI measurements were performed on a 7-T, 30-cm bore Bruker Biospec 70/30 USR system. An actively-decoupled mouse brain coil from RAPID (RAPID Biomedical GmbH, Germany) as the receiver was positioned on the top of the mouse brain. An actively-decoupled 72-mm diameter volume coil was used as the RF transmitter. Magnetic field homogeneity was optimized by automatic global shimming. Images in three directions (axial, sagittal, and coronal) were acquired by FLASH sequence. The sagittal image was used for fMRI slice selection. fMRI data were acquired in sixteen consecutive coronal slices with the slice thickness of 0.7 mm. These slices covered the brain region from the rostral edge of the olfactory bulb to the caudal edge of the visual cortex. Single-shot gradient-echo echo planar imaging (GE EPI) sequence was used to acquire the fMRI data: echo time (TE) = 11 ms, repetition time (TR) = 4 s, flip angle = 90°, matrix size = 64 × 64, field of view = 20 × 20 mm², and in-plane resolution of 0.31 × 0.31 mm². Each fMRI measurement included a 40-sec baseline, 40-sec stimulation, and 80-sec recovery, having 10 (baseline) + 10 (stimulation) + 20 (recovery) volume acquisitions with a repetition time of 4 s (i.e., images of sixteen slices within one volume were acquired in 4 s). The total time for one fMRI measurement was ~180 s (~3 min), including 12 s for dummy scans to allow the MRI signal to stabilize, 160 s for data acquisition, and ~8 s for data storage. Thirty fMRI measurements were targeted for each mouse during the 1.5-hr fMRI data acquisition session.

2.4. Data analyses

Data was processed using Stimulate (Strupp, 1996) (http://www.cmrr.umn.edu/downloads/stimulate.html) and custom MATLAB routines (Version 7.12.0, Mathworks, Natick, MA). EPI images from one mouse were used as template images, and EPI images from the other mice were realigned to the template images using maximum cross correlation with rigid body spatial transformations. The data from the fMRI measurements of all animals in each group were averaged to map their respective olfactory fMRI activations.
Statistical t-value maps were computed by comparing the fMRI data during the control and stimulation periods on a voxel-by-voxel basis. The control periods included the 40-s pre-stimulus baseline and the period from 40-s after stopping the stimulation to the end of the data acquisition. The stimulation period included the period from 8 s after stimulus onset to the end of stimulation. To detect activations, a statistical threshold of $p < 0.01$ and a contiguous cluster size of 4 were simultaneously used (Forman et al., 1995). Based on the assumption that brain regions of true neural activity tend to induce fMRI signal changes over contiguous pixels, using Monte Carlo simulations, Forman, et al. have established the per-pixel probability of detecting false positive pixels as a function of statistical threshold and cluster size for neuroimaging study (Forman et al., 1995). The per-pixel false positive probability corresponding to the statistical threshold of 0.01 and the cluster size of 4 is 0.000004 (the 2nd element in the 4th row of Table 1 in Forman et al. (1995)). With this per-pixel false positive probability, the probability of false activations in a single slice with the matrix size of $64 \times 64$ is $(1 - 0.000004)^{64^2} = (1-0.98) = 2\%$ For the statistically active pixels, percentage signal changes $(100\% \times \Delta S/S$, where $\Delta S$ is the stimulation-induced signal change and $S$ is the baseline signal intensity) were calculated. To show fMRI activations, color-encoded percentage signal changes were overlaid on the EPI images. BOLD fMRI signal increases were displayed with hot colors (red/yellow).

For quantitative analysis of fMRI activations, four regions of interest (ROI) were defined. The fMRI data from all three groups were averaged together to calculate the activation maps. Based on these activation maps, three regions of interest (ROI) were defined: (1) Olfactory bulb (OB) consisting of the activated pixels in the olfactory bulb region; (2) Anterior olfactory nuclei (AON) consisting of the activated pixels in the region where the anterior olfactory nuclei are located; (3) Prefrontal cortical region (PFC) consisting of the activated pixels in the prefrontal cortical region close to the OB. In addition to these three ROIs, the ROI of the piriform cortex (Pir) was defined based on the activation maps from the DEX/KET group. It consists of the activated pixels in the region where the piriform cortex is located.

For each fMRI measurement in each animal, the time courses of the voxels in each ROI were averaged. To observe temporal characteristics of olfactory fMRI responses during continuous odor exposure, the time courses from all olfactory fMRI measurements in each ROI were averaged. The strength of the fMRI response was calculated by averaging the fMRI signals during the 40-s stimulation period.

To examine the consistency of the observed fMRI responses during the repeated stimulations, the time courses from 5 consecutive fMRI measurements were averaged. The data from animals which succeeded the whole 1.5-hr fMRI session yielded equivalent 6 responses from 30 fMRI measurements. The strength of each response was quantified by averaging the amplitudes of fMRI signals during the stimulation period.

### 2.5. Statistics

ANOVA was used to examine the stability in the respiration rates during the fMRI session. The impact of respiration rate on the response strength was analyzed by linear least squares regression analysis (Press et al., 1992). The differences in the olfactory responses of different groups were examined with the two-tailed student t-test.

### 3. Results

#### 3.1. Anesthesia durations and respiration rates under the three anesthesia protocols

For the DEX group, 5 out of the 10 mice remained sedated throughout the 2-hr experimental session, while the other 5 mice moved before the end of the 2-hr experimental session. For the DEX/ISO group, 7 out of the 10 mice remained anesthetized throughout the 2-hr experimental session, while the other 3 mice moved before the end of the 2-hr experimental session. For DEX/KET group, 8 out of the 10 mice remained anesthetized throughout the 2-hr experimental session, while the other 2 mice moved before the end of the 2-hr experimental session. Fig. 1 shows the respiration rates during the fMRI session for each individual mouse. Sudden drops are observed in several mice in the DEX group and the DEX/KET group (Fig. 1A & C). Similar sudden drops in respiration rate have also been observed in the previous study with DEX as anesthesia (Fig. 1C in Adamczak et al. (2010)). There are no significant differences in the respiration rates between different groups (t-test, all $p > 0.25$).

#### 3.2. Spatial locations of the olfactory fMRI activations under the three anesthesia protocols

For each anesthesia protocol, the data from the fMRI measurements of all animals within the group were averaged to map the olfactory activations. Fig. 2 shows the olfactory fMRI activation maps for the three groups. fMRI activations are observed in the OB and PFC in all three groups. In the AON, fMRI activations are robustly observed in the DEX and DEX/KET groups. In the Pir, fMRI activations are robustly observed only in the DEX/KET group. Similar fMRI activations in the Pir of mice have been reported in the previous work with xylazine/KET as anesthesia (Muir et al., 2019). In the OB, higher activations (yellow) are located in the parenchyma close to both the OB surface and the midline. Since the midline is where the medial surfaces of the left and right OB are located, the activation pattern depicted in the OB indicates that stronger BOLD fMRI activations are concentrated in superficial and shallow layers of the OB. The activation patterns in the OB from the three anesthesia protocols are similar, indicating that the fMRI activations in the OB are reproducible. Eklund et al. (2016) suggests that the standard to validate true fMRI activations is the reproducibility of the activations.

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**Fig. 1.** Respiration rates during fMRI session under the three anesthesia protocols. For each anesthesia protocol, the thin lines represent data from individual mice, and the thick black lines represent the averaged data. The respiration rates range from ~90 to ~210 breaths/min among the different mice, and are relatively stable in each individual mouse during the fMRI session. There is no significant difference in the respiration rates during the fMRI session for all three groups (ANOVA analysis. DEX group, $F_{19,213} = 0.16$, $p = 1.0$; DEX/ISO group, $F_{29,233} = 0.05$, $p = 1.0$; DEX/KET group, $F_{29,251} = 1.30$, $p = 0.15$).
reproducibility observed in our study proves that the fMRI activations in the OB truly represent the neural activations-induced BOLD response during the odor stimulation.

### 3.3. Temporal patterns of the fMRI activations during odor stimulation

To examine the temporal characteristics of olfactory fMRI responses during continuous odor exposure, the time courses of fMRI signals in each of the four ROIs from all animals in each group were averaged. Fig. 3B–E show the time courses from the three groups in the four ROIs. In the OB (Fig. 3B), fMRI activations during the continuous 40-s odor exposure do not decrease, suggesting that the activations in the OB do not exhibit a significant adaptation during the continuous 40-s odor exposure. Regarding the response strength, the data shows that the response under DEX/ISO is weaker than the response under either DEX or DEX/KET. In the AON (Fig. 3C), the temporal patterns of the fMRI activations are more interesting. A rebound can be observed after stopping the odor stimulation. Similar to the responses in the OB, the response strength under DEX/ISO is also weaker than the response under either DEX or DEX/KET. In the Pir (Fig. 3D), two peaks are observed in the responses with an initial peak following odor onset and a second peak following odor offset for all three anesthesia protocols. The first peak indicates fast adaptation during the odor stimulation, and the second peak is caused by post-inhibitory excitation rebound. Under DEX/KET, while the initial peak can be observed, the response remains elevated above the baseline during odor stimulation. Under DEX and DEX/ISO, however, the responses following the initial peak decrease to below baseline during odor stimulation. The negative response during odor stimulation in Pir has been observed in the previous rat olfactory fMRI study (Zhao et al., 2017), and can be explained by the features of sparse coding and global inhibition in the olfactory processing of the Pir (Poo and Isaacson, 2009). In the PFC (Fig. 3E), the trend of relative response strength of the three groups appears to follow the trend of those in the OB. The DEX/ISO group has the weakest response, while the DEX group has the strongest response.

Fig. 2. BOLD olfactory fMRI activation maps. The fMRI Activation maps with each anesthesia protocol were calculated from the olfactory fMRI data averaged over all mice with that protocol. They are displayed as percentage signal changes overlaid on the EPI images. (A) With DEX anesthesia, activations are observed in the OB (indicated by purple arrows), AON (indicated by green arrows), and PFC (indicated by white arrows). (B) With DEX/ISO anesthesia, activations are mainly observed in the OB (indicated by purple arrows), and PFC (indicated by white arrows). (C) With DEX/KET anesthesia, activations are observed in the OB (indicated by purple arrows), AON (indicated by green arrows), PFC (indicated by white arrows), and Pir (indicated by blue arrows). The numbers in the corner of each image represent anterior-posterior coordinates with respect to Bregma (Paxinos and Franklin, 2013).

Fig. 3. Temporal changes in the mean fMRI signal intensity over the fMRI time course with each anesthesia protocol.
3.4. Comparison between the olfactory fMRI responses under the three anesthesia protocols

To compare the differences between the responses under the three different anesthetic protocols, the strength of the fMRI response was calculated by averaging the fMRI signals during the stimulation period (indicated by red bars in Fig. 3B–E) in the four ROIs for each individual animal. Fig. 3F shows the response strengths for each group in the four ROIs. The response strengths under DEX/ISO in the OB, AON, and PFC are significantly lower than the response strengths under either DEX or DEX/KET. This indicates that isoflurane as low as 0.3% can significantly suppress odorant-induced responses in the olfactory system. Under DEX/KET, the response strength in the Pir is significantly higher than the response strengths under either DEX or DEX/ISO. This indicates that ketamine enhances the response strength in the Pir of mice.

3.5. Relationship between respiration rates and the olfactory fMRI responses

In this study, respiration rates during the fMRI data acquisition period were recorded for each mouse (Fig. 1). A high respiration rate may elevate the olfactory response in olfactory sensory neurons (ORNs), increase the excitatory drive to mitral cells in the OB, and enhance the olfactory response in the OB and downstream structures (e.g., AON and Pir). Respiration rate may also affect the neurovascular coupling by changing the blood gas condition (e.g., pO2 and pCO2 in blood). To evaluate the effect of respiration rate on the fMRI response, the averaged respiration rate during the fMRI session for each individual mouse was calculated. In Fig. 3G, the respiration rate is plotted against the response strength. There are no clear correlations between the respiration rates and the response strengths for each individual group.
3.5. Consistency of olfactory responses to the repeated odor stimulations

To examine the consistency of olfactory responses to the repeated odor stimulations, fMRI data from the mice which had completed the 1.5-hr fMRI session were analyzed. The 1.5-hr experiment period was divided into six 15-min periods. The time courses of fMRI signals from the 5 consecutive fMRI measurements in each 15-min period were averaged. Fig. 4 shows these time courses. In all four ROIs, responses during different periods with the same anesthesia protocol are similar. The temporal patterns of the responses during the different time periods for each olfactory ROI are similar to the temporal patterns shown in Fig. 3B–E. This consistency proves that the observed responses to the repeated odor stimulations are repeatable. The repeatability of the fMRI activations in temporal domain further indicates that the activations observed in our study are true activations (Eklund et al., 2016).

3.7. Adaptation to the repeated odorant stimulations in OB

The data from all three studies were used to examine the temporal characteristics of the response in the OB to repeated odorant stimuli. The strengths of the fMRI responses in the OB (shown in Fig. 4A) were investigated by averaging the fMRI signal amplitudes during the stimulation period. Fig. 5A, B, & C shows the amplitudes of the fMRI responses in the OB for the three studies. The differences between these amplitudes are not significant in all three studies: (ANOVA analysis. DEX: F_{9,50} = 0.91, p = 0.52; DEX/ISO: F_{4,25} = 1.54, p = 0.22; DEX/KET F_{2,12} = 0.25, p = 0.78). These results indicate that the fMRI responses to the repeated odor stimulations are stable, suggesting that the activation signal in the OB does not exhibit a significant adaptation effect during repeated odorant stimulation.

4. Discussion

4.1. Summary of findings

The major findings of this study are as follows: (1) three DEX-based anesthesia protocols (DEX, DEX/ISO, DEX/KET) can be used for olfactory fMRI study in mice. DEX/ISO and DEX/KET extend the anesthesia duration compared to DEX alone. (2) Robust BOLD fMRI activations during odorant stimulation are observed in the OB, and in the PFC region...
DEX-anesthetized mice are observed in broader regions compared to odorant stimulation. Interestingly, olfactory fMRI activations in our study in neuronal cultures shows that processing is minimal. While a previous in-vitro electrophysiological agonist. Previous researches suggest that the impact of DEX on olfactory response in the Pir shows significant adaptation. (5) KET enhances the olfactory response in the Pir.

4.2. DEX-based anesthesia protocols for olfactory fMRI study in mice

Three DEX-based anesthesia protocols were tested for the olfactory fMRI study in mice. DEX is a highly selective α2-adrenergic receptor agonist. Previous researches suggest that the impact of DEX on olfactory processing is minimal. While a previous in-vitro electrophysiological study in neuronal cultures shows that α2-adrenergic receptor agonists affect neural transmission between mitral cells and granule cells (Trombley and Shepherd, 1992), a behavioral study shows that the modulation of general α-adrenergic receptor function in the OB does not affect odor discrimination in rats (Doucette et al., 2007). A literature search shows that reports on the effects of α2-adrenergic receptor agonists on olfactory processing by in-vivo electrophysiological recording are limited, indicating that the involvement of the α2-adrenergic system in olfactory processing should be minimal. Our study demonstrates that odorant-induced olfactory responses can be observed in the major olfactory structures (i.e., OB, AON, and Pir) under DEX anesthesia, albeit the olfactory response in the Pir shows significant adaptation during the odorant stimulation. Interestingly, olfactory fMRI activations in our DEX-anesthetized mice are observed in broader regions compared to those from a previous fMRI study in conscious mice (see Fig. 7 in Chen et al. (2019)). This difference is likely caused by that fMRI data from anesthetized animals is immune from noises caused by inevitable motion in conscious animals. Overall, DEX should be a better choice as the anesthesia for olfactory fMRI studies. Unfortunately, the mice under DEX have limited anesthesia duration.

To extend anesthesia duration, protocols combining DEX with low dose of ISO and low dose of KET were tested. As expected, these combinations extended the anesthesia duration. DEX/ISO combination has been used in previous fMRI studies in mice (Grandjean et al., 2014; Schroeter et al., 2017). ISO is a popular anesthetic due to its ease of use and fast recovery (Masamoto et al., 2007). A disadvantage for ISO is that it causes confounding effects on olfactory fMRI studies. ISO affects the baseline cerebral blood flow and neurovascular coupling, which would confound fMRI quantification (Petrinovic et al., 2016). In addition, ISO also suppresses neural activity (Liu et al., 2011). For olfactory studies, ISO itself acts as an odorant (Vincis et al., 2012). A previous optical imaging study shows that ISO as an odorant induces specific glomerular activations, and as an anesthesia suppresses neural responses induced by other odorant stimulations (Vincis et al., 2012). Due to these confounding factors, ISO dose should be as low as possible if it is used for olfactory fMRI studies as an anesthetic. In previous mice studies with DEX/ISO, the doses of ISO are 0.4%-0.5% (Belloy et al., 2018; Grandjean et al., 2014; Schroeter et al., 2017), and the equivalent dose of DEX is 0.15 mg/kg bolus + 0.3 mg/kg/hr infusion (Belloy et al., 2018). In our study, the dose of DEX is higher than the dosage used in those studies, but the dose of ISO in our study is 0.3%, which is lower than the dosage used in those studies. As shown in Fig. 3F, ISO as low as 0.3% can still significantly suppress the odorant-induced fMRI responses in the OB and AON. Such suppression can be attributed to two factors (Vincis et al., 2012). First, constant delivery of ISO may induce adaptation and/or desensitization of olfactory receptor neurons due to its odorant property. Second, ISO suppresses odorant-induced neural activities in the olfactory system.

KET in combination with either DEX or xylazine as an anesthesia has previously been used in mice studies (Muir et al., 2019; Shim et al., 2018; Vazquez et al., 2013). The KET doses in those studies are high: 75-100 mg/kg initial bolus plus repeated injection. Such high KET doses are defined as anesthetics. In our study the KET dose is 8 mg/kg bolus plus 16 mg/kg/hour infusion. With the 2-hr study, the total KET delivered to the animal is 40 mg/kg at the end of the study. Such a low dose is defined as a subanesthetic dose (Irifune et al., 1997). As shown in Figs. 2 & 3F, the subanesthetic KET enhances the olfactory response in the Pir. The enhanced olfactory response in the Pir can be used as a biomarker to test the reversibility of potential therapeutic compounds for schizophrenia (Monte et al., 2013). Thus the DEX/KET anesthesia protocol should be a natural choice for olfactory fMRI to support schizophrenia drug discovery.

4.3. Ketamine enhances the olfactory response in the Pir

In this study, we measured the odorant-induced fMRI responses under DEX alone and under DEX/KET in two different groups of mice. The effect of KET on olfactory processing can be assessed by comparing the fMRI response under DEX/KET with the fMRI response under DEX alone. As shown in Fig. 3D & F, the fMRI response in the Pir of the DEX/KET group is significantly higher than the fMRI response in the Pir of the DEX group, indicating that KET enhances the fMRI response in the Pir. Since fMRI measures neural activity-induced hemodynamic responses, the strength of the fMRI response depends on both neural activations and neurovascular coupling (Attwell and Iadecola, 2002; Murphy et al., 2011). KET may cause physiological changes in cardiovascular and respiratory systems which can alter the neurovascular coupling. KET itself as a pharmacological agent may also alter the neurovascular coupling. The observed KET enhancement on the fMRI response in the Pir can be attributed to either KET’s enhancement of neural activations, or its possible enhancement of neurovascular coupling. Our data suggests that the KET enhancement on the fMRI response in the Pir is due to the first
possibility, it enhances neural activations in the Pir. First, if the observed enhancement is from the effect of KET on the neurovascular coupling, we would expect to observe similar enhancements in the fMRI responses from other structures such as the OB or AON, since the effect of KET on neurovascular coupling is similar across these brain structures. Our data shows no enhancements in the OB and AON (Fig. 3F). Second, if the observed KET effect is from its effect on the neurovascular coupling, we would expect that the response under DEX/KET should have a similar temporal pattern as the response under DEX. As shown in Fig. 3D, the fMRI response in the Pir of the DEX group is biphasic. It has the first positive peak which is followed by a negative response during the odor stimulation period. In contrast, the fMRI response in the Pir of the DEX/KET group remains elevated above the baseline, and does not show the biphasic response during the odor stimulation period. Therefore, the observed KET effect in Pir is from its neural effect, and KET enhances the olfactory response in the Pir.

The KET enhancement can be attributed to the antagonistic action of NMDA glutamate receptors on the dendrodendritic synapses between the principle neurons and interneurons in the Pir. The olfactory fMRI response is from the neural activities in the principle neurons (pyramidal cells). The pyramidal cells receive inhibition from local interneurons (Poo and Isaacson, 2009; Suzuki and Bekkers, 2012), and the glutamate NMDA system is involved in the inhibition (Stokes and Isaacson, 2010) (Franks et al., 2011). With the DEX/KET protocol, KET interrupts the glutamate NMDA transmission from pyramidal cells to the interneurons by blocking the NMDA receptors on the dendrites of interneurons. Thus, NMDA receptor-mediated activations in interneurons are blocked (Franks et al., 2011; Luna and Pettit, 2010; Stokes and Isaacson, 2010), and the inhibition to pyramidal cells by interneurons is stopped. Without the interneuron-induced inhibition, the suppression on the neural activities in the pyramidal cells during the stimulation period is attenuated, and the enhanced olfactory fMRI response is observed.

In addition to the Pir, NMDA receptors also play a role in the dendrodendritic synapses between principle neurons (mitral cells) and interneurons in the OB (Arevian et al., 2008; Urban and Sakmann, 2002). It is expected that KET should also enhance the olfactory response in the OB of mice by blocking the inhibition from interneurons. Surprisingly, no KET enhancement on the response in the OB was observed in this study (Fig. 3B). A similar observation has been reported by c-fos method (Wilson et al., 1996). The lack of KET enhancement in the OB may be due to the lower significance of interneuron-induced inhibition in the OB as compared to that in the Pir. As demonstrated in Wilson (1998); Zhao et al. (2017), the olfactory adaptation in the Pir is much more significant than the olfactory adaptation in the OB. Since olfactory adaptation is due to the inhibition from interneurons (Zhao et al., 2017), the interneuron-induced inhibition in OB is not as significant as that in the Pir. Thus, KET’s blocking effect on inhibition in the OB is not as prominent as that in the Pir. Therefore, the KET enhancement on the olfactory response can be observed in the Pir, not in the OB.

4.4. Adaptations of the olfactory responses in the OB and Pir of mice

Olfactory adaptation, characterized by an attenuation of responses to continuous or repeated stimulations, has been observed in the OB and higher olfactory structures of rats (Chaudhury et al., 2010; Schafer et al., 2005; Wilson, 1998, 2000; Zhao et al., 2016, 2017). However, studies on olfactory adaptation in the OB and higher olfactory structures of mice are scarce. In this study, the odor stimulation paradigm is repeated 40-sec continuous odorant exposures with an inter-stimulus-interval (ISI) of ~3 min. With this stimulation paradigm, the olfactory adaptations to both continuous odorant exposure and repeated stimulations can be examined. In the OB of mice, no substantial adaptations are observed either during the continuous 40-sec odor exposure (Fig. 3B) or during the repeated odor stimulations (Figs. 4A and 5). While this lack of adaptation in the OB of mice conflicts with previous studies in rats in which adaptation to repeated stimulations had been robustly observed (Wilson, 1998; Zhao et al., 2016, 2017), it is consistent with findings from NHPs (Zhao et al., 2015). There are no adaptations to both continuous odor exposure and repeated odor stimulations in the OB of NHPs. Since it has been suggested that the glutamate accumulation and release in the dendrodendritic synapses between the principle neurons and interneurons play a major role in the olfactory adaptation (Zhao et al., 2017), this difference in the adaptation to the repeated odor stimulations between different species suggests that there may be a difference in the glutamate accumulation process in the dendrodendritic synapses of OB.

In the Pir of mice, the temporal pattern of the olfactory response to the continuous odor stimulation displays a two-peak pattern (Fig. 3D) which is similar to observations from both fMRI and electrophysiological recording in rats (Wilson, 1998; Zhao et al., 2016). The first peak is due to the initial response to the odor on-set, then the response attenuates due to the adaptation. The degrees of adaptation are different among different anesthesia protocols. Under DEX and DEX/ISO, the adaptations are more significant that negative responses following the initial peak are observed during odor stimulation (Fig. 3D). A similar negative response during odor stimulation has previously been observed in the Pir of rats (Zhao et al., 2017), and is caused by a substantial adaptation from the feature of global inhibition in the olfactory processing of Pir (Poo and Isaacson, 2009).

The second response peak following the odor off-set is caused by post-inhibitory excitation rebound. Since the ‘excitation rebound’ has been proven to be a property of neurons that occurs after prolonged periods of stimulus-induced inhibition (Butovas and Schwarz, 2003; Grenier et al., 1998), the prominent post-inhibitory excitation rebound provides the other evidence that the adaptation during the continuous odorant exposure is caused by the inhibition in the principle neurons in the Pir.

4.5. BOLD fMRI activations in the PFC are caused by draining vein effect

As shown in Fig. 2, reliable BOLD fMRI activations are observed in the PFC region under all three anesthesia protocols. These BOLD fMRI activations are either from the odorant-induced neural activations in the PFC, or from the draining vein effect. Previous studies show that the PFC is connected to the Pir (Carmichael et al., 1994; Datiche and Cattarelli, 1996). Thus, odorant-induced neural activations in the Pir could be transmitted to the PFC to induce the neural activations in the PFC. If this is true, neural activations in the PFC should follow the neural activations in the Pir, and the temporal pattern of the BOLD fMRI response in the PFC should be similar to the temporal pattern of the BOLD fMRI response in the Pir. As shown in Fig. 3D and E, the response in the Pir has two peaks, while the response in the PFC does not. Since there is no similarity between the response in the Pir and the response in the PFC, the BOLD fMRI activations observed in the PFC are not caused by the neural activations originated from the Pir. Another possibility is that the neural activations in the OB can be transmitted to the PFC, but there is no evidence suggesting a direct neural connection between the OB and PFC. Thus, the observed BOLD fMRI activations cannot be explained by transmitted neural activations from the OB. The most likely source for the BOLD fMRI activations in the PFC is the draining vein effect. The PFC is close to the OB, and the hyper-oxygenated venous blood from the OB during odor stimulation is drained to the PFC, causing a BOLD fMRI signal increase in the PFC. Such misplaced BOLD fMRI activation from the draining vein effect is an intrinsic characteristic of BOLD fMRI.

As suggested by previous research (Kim and Ogawa, 2012; Turner, 2002), the prominence of the draining vein effect depends on the spatial extent of the neural activations, and the distance from the activated region. When the activated region is small, hyper-oxygenated blood from activation region is quickly diluted with blood from inactivated regions, effectively reducing the oxygenation level change in the venous blood of adjacent regions, and the draining vein effect does not cause significant misplaced BOLD fMRI activations. This is the case when BOLD fMRI is used to study small brain structures such as somatosensory cortex in mice (Adamczak et al., 2010; Shim et al., 2018). However, when a large brain
area responds to stimulus, relatively large amount of venous blood drained from activated area flows into relatively distant downstream vessels without sufficient dilution. In addition to fMRI activations in the original activated region, misplaced BOLD fMRI activations would also be observed in regions where draining veins are located. This is the case for our study. The OB is a relatively large brain structure in mice. The hyper-oxygenated venous blood from the activated OB can be drained to the PFC without sufficient dilution, and thus BOLD fMRI activations are observed in the PFC. As shown in Fig. 3B and E, the relative strengths of the fMRI activations in the PFC of the three groups follow the relative strengths of the fMRI activations in the OB of the three groups. The DEX/ISO group has the lowest responses in both the OB and PFC, while the DEX group has the highest responses in both the OB and PFC. The similarity between the relative response strengths in the PFC and OB of the three groups suggests that the BOLD fMRI activations in the PFC originate from the venous blood drained from the OB.

To further verify that the BOLD fMRI activations in the PFC are caused by the draining vein effect, cerebral blood volume (CBV)-weighted fMRI was performed in an additional group of mice (n = 8) with the DEX/ISO anesthesia. Ultra-small particles of iron oxide (USPIO) contrast agent (Feraheme, AMAG Pharmaceuticals, Cambridge, MA) was intravenously administered (5 mg/ml, 25 mg/kg, 5 ml/kg) during the preparation. An increase in CBV during odor stimulation would induce an increase in the content of the paramagnetic contrast agent, and consequently a decrease in CBV-weighted fMRI signal (Kim et al., 2013; Zhao et al., 2006). However, the draining vein effect would also cause an increase in the CBV-weighted fMRI signal in the PFC where the draining veins are located. Fig. 6 shows the results from the CBV-weighted fMRI study. As expected, the decreases in CBV-weighted fMRI signals indicating the increases in neural activations during the odor stimulation are observed in the OB (Fig. 6A and B). The increases in CBV-weighted fMRI signals are observed in the OB (Fig. 6A and B). The increases in CBV-weighted fMRI signals are observed in the OB (Fig. 6A and B). The increases in CBV-weighted fMRI signals are observed in the OB (Fig. 6A and B).
activation in the PFC from this study are caused by the draining vein effect.

5. Conclusions

We demonstrate that BOLD fMRI can be used to study the odorant-induced olfactory responses in the OB and higher olfactory structures such as the AON and Pir in mice with three DEX-based anesthesia protocols. We also show that KET enhances the olfactory response in the Pir. The KET-enhanced olfactory fMRI response in the Pir should be a valuable biomarker for Schizophrenia drug discovery.

CRediT authorship contribution statement

Fuqiang Zhao: Conceptualization, Methodology, Software, Data curation, Formal analysis, Writing – original draft. Xiangjun Meng: Conceptualization, Methodology. Sherry Lu: Conceptualization, Visualization, Investigation. Lynn A. Hyde: Conceptualization, Resources. Matthew E. Kennedy: Conceptualization, Resources. Andrea K. Houghton: Conceptualization, Resources. Jeffrey L. Evelhoch: Conceptualization, Resources. Catherine D.G. Hines: Conceptualization, Resources, Project administration.

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