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Layer-Dependent BOLD and CBV-weighted fMRI Responses in the Rat Olfactory Bulb

Alexander John Poplawsky^a and Seong-Gi Kim^{a,b,c,d}

^aNeuroimaging Laboratory, Department of Radiology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

^bNeuroimaging Laboratory, Department of Neurobiology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

^cCenter for Neuroscience Imaging Research, Institute of Basic Science (IBS), Sungkyunkwan University, Suwon 440-746, Republic of Korea

^dDepartment of Biological Science, Sungkyunkwan University, Suwon 440-746, Republic of Korea

Abstract

The olfactory bulb is a laminarized brain structure involved in odor sensation that has important implications to basic neuroscience research, like mechanisms for neurovascular coupling and early disease diagnosis. To investigate laminar-dependent responses to odor exposure, blood oxygenation level-dependent (BOLD) and cerebral blood volume weighted (CBVw) fMRI with iron oxide nanoparticle contrast agent were obtained with $110 \times 110 \times 500 \mu\text{m}^3$ resolution in urethane-anesthetized rats at 9.4 T. The baseline total CBV is largest at the olfactory bulb surface and midline, and decreases in the deeper layers, while a band of increased microvasculature density is observed at the glomerular, external plexiform and mitral cell layers. With odor exposure, CBVw fMRI is more sensitive and reproducible than BOLD. BOLD fMRI had the greatest activation on the bulb surface, midline, olfactory nerve and glomerular layers, while CBVw activation peaked in glomerular and external plexiform layers, but was still significant in mitral cell layer. Negative BOLD responses were observed in the bulb midline and near large blood vessels. CBVw laminar profiles are similar to the layer-dependent metabolic changes to the same odor exposure reported by previous glucose metabolism studies. Unique activation patterns for two different odor conditions were also differentiated with CBVw fMRI. Our study suggests that CBVw activation better represents the spatial location of metabolic activity in the olfactory bulb than BOLD.

Keywords

functional MRI; olfactory bulb; BOLD; cerebral blood volume (CBV); monocrySTALLINE iron oxide nanoparticles (MION); layer dependent

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Correspondence: Alex Poplawsky, University of Pittsburgh, McGowan Institute for Regenerative Medicine, 3025 East Carson Street, Pittsburgh, PA 15023, USA. ajp94@pitt.edu, Phone: +1 412-383-6696, Fax: +1 412-383-6799.

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Introduction

The olfactory bulb is a unique model system beneficial for studying neurovascular coupling, learning and plasticity, and the early diagnosis of neurological disorders, which is anatomically and functionally organized by the thin bulb layers (Lancet et al., 1982; Sharp et al., 1977; Shepherd, 1972). The primary odor pathway in the bulb begins with the olfactory receptor neuron axons propagating through the olfactory nerve layer (ONL) to form dense excitatory synapses with the apical dendrites of mitral cells in the glomerular layer (GL). These dendrites transmit through the external plexiform layer (EPL) to the mitral cell body layer (MCL) and mitral cell axons exit the bulb to cortical targets, such as the piriform cortex. In addition, inhibitory granule cells in the granule cell layer (GCL) receive centrifugal projections from the cortex and form dendro-dendritic synapses with mitral cells in EPL. In this way, different functional components of the olfactory neural circuit are anatomically organized to the different bulb layers, thus allowing the study of how distinct neuro-electrical events, such as excitatory or inhibitory processes, contribute to the hemodynamic response. The study of neurovascular mechanisms in the olfactory bulb is popular with optical imaging techniques (Chaigneau et al., 2003; Chaigneau et al., 2007; Gurden et al., 2006), but such studies are limited to layers near the bulb surface. Next, enhancement of odor discrimination to learned odor stimuli was shown to be accompanied by increased synaptic connections in discrete olfactory bulb glomeruli (Jones et al., 2008). This discovery provides an interesting fMRI model to study neural plasticity in a primary sensory brain region that can be easily evoked by odor in an anesthetized animal model; contrary to studying plasticity in more traditional brain regions, like the hippocampus, which are more difficult to evoke in an fMRI experiment. Finally, olfactory dysfunction is an early symptom of many diseases, such as multiple sclerosis, Parkinson's disease and Alzheimer's disease, that precedes the development of the more debilitating symptoms; and a greater understanding of *in vivo* odor function with fMRI can benefit such diagnostic applications. Therefore, high-resolution layer-dependent functional imaging of the entire bulb is highly desirable.

Layer-dependent metabolic changes to odor stimulation were historically examined with [^{14}C]-2-deoxy-D-glucose (2-DG) methods (Johnson et al., 1998; Lancet et al., 1982; Sharp et al., 1977). These studies showed that single odorants increased cellular metabolism in localized glomeruli (foci) and activation of different glomerular populations formed spatial patterns in GL that are unique to the odorant. In addition, line profiles through the bulb layers showed that cell metabolism varied with bulb depth. For example, a profile through an activation focus revealed increasing metabolism in ONL that peaks in GL and gradually decreased through the deeper layers (fig. 7E) (Sharp et al., 1977). However, 2-DG measurements are performed *ex vivo* and only allow examination of a single odorant for each animal. More recently, *in vivo* manganese-enhanced MRI measured odor-specific activation patterns in GL and MCL that are similar to 2-DG (Chuang et al., 2010; Chuang et al., 2009; Pautler and Koretsky, 2002), but were still limited to the testing of one odorant per MRI measurement. Other *in vivo* methods like optical intrinsic signaling (Rubin and Katz, 1999; Uchida et al., 2000) and fMRI (Kida et al., 2002; Liu et al., 2004; Martin et al., 2007; Schafer et al., 2005; Schafer et al., 2006; Xu et al., 2000; Xu et al., 2003; Yang et al., 1998) overcome these limitations but at the cost of measuring signal changes that originate from the vasculature; while only fMRI technology preserves measurement in the deeper bulb layers. Results from original BOLD fMRI studies, similar to 2-DG, measured focal activations that had odor-specific activation patterns and had the largest signal change in ONL and GL. However, unlike 2-DG, limited signal change was observed in EPL and little to no change in MCL and GCL (Kida et al., 2002; Xu et al., 2000; Yang et al., 1998). This discrepancy between the superficial and deeper bulb layers can be explained by i) the baseline CBV contribution to BOLD (Kim and Ogawa, 2012), ii) large signal contributions

from pial vessels, whereby magnetic field gradients from pial vessels contribute to the fMRI response in GL near the bulb surface (Kim and Ogawa, 2012), and/or iii) a mismatch between the location of metabolism and the hemodynamic response. Thus, it is critical to understand whether the BOLD response is directly related to the laminar-dependent baseline CBV in the olfactory bulb, and whether hemodynamic-based fMRI measures changes in MCL and GCL.

To examine afore-mentioned issues, we obtained BOLD and cerebral blood volume-weighted (CBVw) fMRI with $110 \times 110 \times 500 \mu\text{m}^3$ resolution and compared their sensitivity, reproducibility and spatial activation profiles across layers. The CBVw fMRI technique with injection of iron oxide nanoparticles was chosen for its decreased contributions from large blood vessel signal changes and enhanced sensitivity in capillaries proximal to metabolically active cells (Kim et al., 2013; Mandeville and Marota, 1999; Mandeville et al., 1998; Zhao et al., 2006). Baseline total blood and microvasculature enhanced volumes were also measured and compared to the layer-dependent BOLD and CBVw fMRI activation patterns. Finally, CBVw fMRI was used to measure the activation patterns for two odors to determine if unique stimuli can be functionally differentiated.

2. Materials and Methods

2.1 Animal Preparation and Odor Stimulation

Six male Sprague-Dawley rats (315–415 g) were studied with approval from the University of Pittsburgh Institutional Animal Care and Use Committee. Animals were induced with 5% and maintained with 2% isoflurane gas in medical air and O₂ gases during all surgery. The right femoral artery and vein were catheterized for physiological monitoring and administration of fluids, anesthetic and contrast agent, respectively. A 15-mm diameter section of scalp directly above the olfactory bulb was removed to increase the coil sensitivity and to prevent image wrapping artifacts for the $7 \times 7\text{-mm}^2$ field of view (FOV). A sub-cutaneous dose of carprofen (10 mg/kg) analgesia was administered before the anesthesia was switched from isoflurane to urethane (1.3 g/kg i.p. induction, followed by continuous 0.1 g/kg/hr i.v. maintenance). The animals under urethane anesthesia were free breathing and did not require intubation. Mean blood pressure was monitored through the arterial line and maintained between 70–130 mmHg (MP150, BioPac Systems Inc., Goleta, CA). In addition, the animals' rectal temperature was maintained at $37 \pm 1^\circ\text{C}$ using a warm water circulator and breathing rate was recorded with a pneumatic pillow sensor. A 0.9% saline and 5% dextrose supplemental fluid was administered intravenously at 2–3 ml/kg/hr. A single bolus of 15 mg Fe/kg Feraheme (ferumoxytol, AMAG Pharmaceuticals, MA) was intravenously injected for CBV weighting after the BOLD fMRI studies.

Odor stimulation was performed by a home-built apparatus with TTL-controlled solenoid valves (EW-98302-02 and -22, Cole-Parmer, Vernon Hills, IL) and one-way check valves (EW-98553-00, Cole-Parmer) (Fig. 1A). The solenoid valves diverted airflow (1 L/min) to one of three 500 mL Pyrex flasks (EW-34503-07, Cole-Parmer) containing 5% amyl acetate in mineral oil, 1% pyridine or 100% mineral oil. Each odor condition had dedicated Tygon tubing (EW-95631-05, Cole-Parmer) with one-way check valves (indicated by arrowheads) to prevent mixing. The dedicated lines converged to a single line ($\sim 1 \text{ cm}^3$ common volume) before purging into a nose cone sealed around the rat snout. The dedicated lines allowed for quick transitions between odor conditions despite the long distance the odor had to travel from the odor source ($\sim 5 \text{ m}$). A vacuum line at the opposite end of the nose cone removed the odors. This odor stimulation system was synchronized with MRI acquisition.

2.2 General Magnetic Resonance Imaging Procedures

All MRI experiments were performed on a 9.4-T/31-cm MR system interfaced by a DirectDrive console (Agilent Tech, Santa Clara, CA) and an actively shielded gradient coil with 40 G/cm peak gradient strength and 120 μ s rise time (Magnex, UK). The head of the rat was fixed in a non-magnetic head restraint with a bite bar and ear plugs. A home-built 1-cm inner diameter surface coil was positioned dorsal to the olfactory bulb for radio-frequency excitation and reception (see Fig. 1B). Preliminary spin-echo images of the entire bulb were acquired and used to select five 0.5-mm thick slices without gap for subsequent imaging studies with a 7×7-mm² FOV (see images in Fig. 1B). Both removal of the dorsal scalp and the diminished coil sensitivity in the ventral bulb minimized folding artifacts in the dorsal-ventral phase-encoding direction.

2.3 High Resolution Anatomical and Blood Volume Images

High resolution anatomical, T_2^* and T_2 -weighted images were acquired with a matrix size of 128×128 (55×55 μ m² in-plane resolution). Anatomical images were acquired with a fast spin-echo sequence in all six animals and imaging parameters of 5.0 s T_R , train of 4 echoes, 40.7 ms effective T_E and 24 averages. T_2^* -weighted images were acquired with a fast low-angle shot (FLASH) sequence with a 190 ms T_R , 6.0 ms T_E and 40 averages. T_2 -weighted images were acquired with a multi-echo, spin-echo sequence with a 3.0 s T_R , train of 8 echoes, T_E = 12.5, 25.1, 37.6, 50.1, 62.7, 75.2, 87.8 and 100.3 ms, and 10 averages. The high-resolution T_2^* and T_2 -weighted images were acquired before and approximately 5 and 150 minutes after MION injection (15 mg/kg) in four of the six animals, respectively.

2.4 fMRI Data Acquisition

The odor-evoked BOLD fMRI methods were adapted from previous studies in the olfactory bulb (Kida et al., 2002; Xu et al., 2000; Yang et al., 1998), followed by CBVw fMRI. The FLASH sequence was used to obtain high-quality images in and around large magnetic susceptibility areas surrounding the sinuses, where the echo planar imaging technique induces large image distortions. Imaging parameters were an in-plane resolution of 110×110 μ m² (64×64 matrix size), 125 ms T_R , and 8 s temporal resolution. A 10 kHz sampling bandwidth was chosen for its increased baseline signal-to-noise ratio (SNR). T_E was determined by the approximate T_2^* value measured at GL before and after MION injection; and was 18 ms for BOLD and 8 ms for CBVw fMRI, respectively. The RF flip angle was determined by acquiring the baseline fMRI images at increasing flip angles and selecting the one that had maximal signal within the olfactory bulb. Each fMRI run consisted of 15 baseline (120 s), 8 odor-evoked (64 s) and 15 recovery images (120 s). 100% mineral oil was used for baseline control. Amyl acetate was used as odor stimulation for both BOLD and CBVw fMRI in all six animals, while 1% pyridine was used for CBVw fMRI in four animals. Pyridine was introduced after the completion of all amyl acetate runs in CBVw experiments in order to keep the odor stimulus conditions similar to BOLD. 12–15 runs were repeated for each experimental condition and the number of runs for each condition was constant for each animal. The time between stimulation offset to onset of the next (inter-stimulus interval) was approximately 240 s and the time between the end of BOLD runs and the beginning of CBVw fMRI runs was approximately 45 minutes.

2.5 Data Analysis

Data were processed with home-written Matlab codes (MathWorks, Natick, MA). All imaging data were initially calculated on a voxel-by-voxel basis. Quantitative layer-dependent analysis was performed on a regions of interest (ROI) basis. For each ROI, one mean value was initially calculated for each animal and, subsequently, averaged across the different animals (reported as mean $\pm$ s.e.m., n = # of animals). Different ROI values were

compared with one-way ANOVA and post-hoc Student's t-tests. For layer-dependent blood volume post-hoc testing, layers were compared to GL because it contains the highest capillary density (Borowsky and Collins, 1989). Similarly, functional activation was compared to GL because the primary functional units of the olfactory bulb (glomeruli) are located here (Guthrie et al., 1993; Lancet et al., 1982; Ressler et al., 1994).

2.5.1 Selection of ROI for Olfactory Bulb Layers—In order to examine laminar-dependent signals, seven olfactory bulb layers were defined, based on the high-resolution anatomical images (Fig. 1C). The seven ROIs were manually drawn on all five slices for each animal: 1) Surface ROI including the bulb surface, midline and any pial vessels identified as signal hypo-intensities (Fig. 2B), 2) ONL, 3) GL, 4) EPL, 5) MCL, 6) GCL and 7) Core ROI including the subependymal layer, the intrabulbar part of the anterior commissure and the olfactory branch of the lateral ventricle. GL and MCL were defined in anatomical images by the outer and inner bands of hypo-intense signal, respectively, and Core by the inner-most hyper-intense band; while ONL, EPL and GCL were identified by their spatial relationship to these bands. Layer definitions were adapted from previous BOLD fMRI studies (Kida et al., 2002; Yang et al., 1998). Voxels in the ventral-most region of the bulb were not included in the ROI mask analyses due to the diminished coil sensitivity, and was characterized by an approximate 40% decrease in SNR relative to dorsal bulb. Also, due to matrix size difference between anatomical (128×128) and functional (64×64) images, four voxels in the anatomical image were manually assigned to a single ROI for the one spatially-corresponding 64×64 voxel. No voxel in the ROI mask was assigned to more than one ROI and some partial volume effects are expected at the ROI boundaries due to a limited spatial resolution.

2.5.2 R_2^* and R_2 Changes due to MION: Calculating Baseline Blood Volume Maps— R_2^* and R_2 were calculated from the high-resolution T_2^* and T_2 -weighted images, respectively, and their changes due to MION injection ($\Delta R_2^*_{\text{MION}}$ and ΔR_2_{MION}) were calculated. $\Delta R_2^*_{\text{MION}}$ was calculated by $\ln(S_{\text{pre}}/S_{\text{post}})/T_E$, where S_{pre} and S_{post} are the measured signals before and after MION injection, respectively. R_2 values were calculated by single exponential fitting of the 8 different T_E data, and ΔR_2_{MION} was then calculated from the difference of R_2 values before and after MION injection. Note that $\Delta R_2^*_{\text{MION}}$ is related to total CBV, while ΔR_2_{MION} is an index of micro-vascular CBV (Dennie et al., 1998; Kim et al., 2013; Tropès et al., 2001).

2.5.3 fMRI Data Analysis—All repeated runs were analyzed together except the reproducibility test in which even and odd runs were analyzed separately. No spatial or temporal smoothing was performed. Initially, fMRI time series were linearly detrended for each run. Then, control images (image numbers 3–15 and 33–38) and odor-evoked images (image numbers 17–23) for all fMRI runs were grouped together. The initial two images were excluded because of the transition period to reach steady state; as were pre- (image number 16) and post-stimulus (image numbers 24–32) transition periods. Transition periods were determined from activation time courses of preliminary studies. Then, i) a two-sample Student's t-test was performed to compare control vs. odor-evoked images or ii) the relative signal change calculated ($\Delta S/S_0 = S - S_0/S_0$, where S is the average of odor-evoked images and S_0 is the average of control images). For CBVw fMRI only, an increase in CBV decreases fMRI signals, resulting in negative $\Delta S/S_0$ and t-values. Thus, the sign of t-values for CBVw fMRI was reversed for direct comparison to BOLD.

For reproducibility studies, BOLD vs. CBVw and two odor comparisons, the spatial correlation coefficient (SCC) was calculated to quantify the degree to which two spatial pattern maps were similar or different (Cole, 1949; Xu et al., 2003). This correlation analysis considers commonly activated, as well as commonly inactivated pixels as

components of the maps' spatial signature. Thresholds were applied to the maps and each pixel was assigned a 1 (above threshold) or a 0 (below threshold). Individual, spatially corresponding pixels from two maps were compared (represented as Map1 $\cap$ Map2) and assigned to one of four categories: [1, 1], [0, 1], [1, 0] and [0, 0] according to their threshold values, respectively. The total sum of pixels in each category, $N_{[1,1]}$, $N_{[0,1]}$, $N_{[1,0]}$ and $N_{[0,0]}$, were then used to calculate the SCC values according to the following conditions:

$$1. \quad N_{[1,1]} N_{[0,0]} = N_{[1,0]} N_{[0,1]},$$

$$SCC = \frac{N_{[1,1]}N_{[0,0]} - N_{[1,0]}N_{[0,1]}}{(N_{[1,0]} + N_{[1,1]})(N_{[1,0]} + N_{[0,0]})}$$

$$2. \quad N_{[1,1]} N_{[0,0]} < N_{[1,0]} N_{[0,1]} \text{ and } N_{[1,1]} = N_{[0,0]},$$

$$SCC = \frac{N_{[1,1]}N_{[0,0]} - N_{[1,0]}N_{[0,1]}}{(N_{[1,0]} + N_{[1,1]})(N_{[0,1]} + N_{[1,1]})}$$

$$3. \quad N_{[1,1]} N_{[0,0]} < N_{[1,0]} N_{[0,1]} \text{ and } N_{[1,1]} > N_{[0,0]},$$

$$SCC = \frac{N_{[1,1]}N_{[0,0]} - N_{[1,0]}N_{[0,1]}}{(N_{[1,0]} + N_{[0,0]})(N_{[0,1]} + N_{[0,0]})}$$

P-values were calculated (Cole, 1949) and, when indicated by horizontal dashed lines in the figures, the two maps are significantly overlapped ($0.2 < SCC < 1.0$, $p < 0.01$) or significantly segregated ($-0.2 > SCC > -1.0$, $p < 0.01$). SCC data was averaged across animals and reported as mean $\pm$ s.e.m.

BOLD vs. CBVw fMRI: To compare the sensitivity and laminar-dependency, various thresholds were used with the minimum number of clustered active voxels equal to 2; i) t-values of 2 and 4, and ii) top 3%, 6% and 12% most activated voxels. Threshold included all positively and negatively active voxels. Representative time courses ($\Delta S/S_0$) were obtained from each ROI to determine the effects of habituation. To fairly compare time courses across ROIs, the 100 pixels with the most significant absolute t-values that were common to both BOLD and CBVw images were averaged for each ROI.

CBVw maps of two odors: Maps of two different odors (5% amyl acetate and 1% pyridine) were compared ($n = 4$ animals). A strict, top 3% threshold was used to examine the spatial location of activation foci, while a more liberal, top 12% threshold was used to determine differences in global activation. A paired Student's t-test was used to compare SCC values for reproducibility and two odor map comparisons for 3% and 12% thresholds, respectively.

Quantitative laminar-dependent analyses: Single line profiles were obtained across the olfactory bulb layers along BOLD and CBVw foci to compare with line profiles of 2-DG studies (Sharp et al., 1977). Two ROI-based analyses were also performed from maps with top 3%, 6% and 12% most significant t-values; i) distribution of active voxels normalized by the ROI volume fraction = (# of active voxels at each ROI/# of total active voxels)/(# of total voxels at each ROI/# of total voxels of all ROIs), and ii) averaged absolute t-value of active voxels. In addition, for each ROI, the averaged absolute t-values without any threshold were calculated.

3. Results

3.1 Baseline Blood Volume Maps

Since BOLD fMRI is sensitive to baseline CBV, the distribution of total CBV and microvascular-enhanced CBV was determined by $\Delta R_2^*_{\text{MION}}$ and $\Delta R_{2\text{ MION}}$ maps, respectively. Note that anatomical pre-MION R_2^* and R_2 contrasts were removed by subtraction in $\Delta R_2^*_{\text{MION}}$ and $\Delta R_{2\text{ MION}}$ calculations. A single representative olfactory bulb slice from one animal is shown in Fig. 2A–D. The high-resolution T_2^* -weighted image before MION injection (Fig. 2A) shows hyper-intensities (white, indicated by red arrows) located on the bulb surface and midline, which are due to inflow effects from large blood vessels. Regions of hypo-intensities (black) on the bulb surface and midline indicate increased magnetic susceptibility and are associated with large veins. These hyper- and hypo-intense regions (indicated by red arrows) co-localize with hypo-intensities in the anatomical T_2 -weighted images (Fig. 2B) and correspond to large $\Delta R_2^*_{\text{MION}}$ values (Fig. 2C), which supports that they are indeed large blood vessels. The $\Delta R_2^*_{\text{MION}}$ map also shows blood vessels that penetrate perpendicular to the bulb surface and midline that decrease in blood volume as they progress toward the core. Next, $\Delta R_{2\text{ MION}}$ maps show the largest change on the bulb surface and midline, which is due to contamination from intravascular R_2 changes and is not indicative of micro-vessel blood volume (Zhao et al., 2006). However, Fig. 2D shows a circular band of increased $\Delta R_{2\text{ MION}}$ that follows the contour of layers GL (green overlay), EPL and MCL (orange overlay). Interestingly, the micro-vascular enhancement is not observed in lateral ONL. To better visualize the blood volume fractions across layers, line profiles of $\Delta R_2^*_{\text{MION}}$ and $\Delta R_{2\text{ MION}}$ values (Fig. 2E) were plotted from the red ROI (Fig. 2C–D). The difference between the steep changes toward the Surface ROI in $\Delta R_2^*_{\text{MION}}$ and $\Delta R_{2\text{ MION}}$ within ONL is approximately 200–300 μm , possibly due to the extended susceptibility effects from large pial vessels. As expected from dorsal ONL studies of capillary density (Chaigneau et al., 2003; Lecoq et al., 2009), lateral ONL has low $\Delta R_{2\text{ MION}}$ similar to GCL and Core ROIs, indicating a low microvascular density. In addition, the profile shows a local maximum in both the total blood ($\Delta R_2^*_{\text{MION}}$) and micro-vascular enhanced volumes ($\Delta R_{2\text{ MION}}$) at GL, with sustained increases in $\Delta R_{2\text{ MION}}$ through EPL and MCL (Fig. 2E). These same layers were histologically shown to contain a high capillary density (Borowsky and Collins, 1989).

Laminar CBV distribution was further assessed with the ROI analysis across four animals in Fig. 2F. To reduce inter-animal variations and compare across layers, $\Delta R_2^*_{\text{MION}}$ and $\Delta R_{2\text{ MION}}$ were normalized by the corresponding values of GL for each animal. $\Delta R_2^*_{\text{MION}}$ is greatest in the Surface ROI and rapidly decreases with increasing bulb depth. For example, Surface ROI has $71.8 \pm 6.2\%$ more total CBV and GCL has $76.1 \pm 3.9\%$ less total CBV as compared to GL. It is noted that large vessels in Surface and ONL ROIs induce blood susceptibility gradients and may overestimate total blood volume in the superficial bulb layers. For $\Delta R_{2\text{ MION}}$ measurements, pial vessel susceptibility effects are likely insignificant from GL through Core ROIs. However, purely extravascular R_2 changes are indicative of microvascular blood volume, but intravascular R_2 changes also contribute in layers with large diameter vessels and volumes. Thus, Surface and ONL ROIs were removed from the microvascular blood volume analysis as they were previously shown to contain few capillaries in the dorsal bulb (Chaigneau et al., 2003) and changes are assumed to be mostly due to intravascular R_2 changes. The capillary density in other ONL regions, namely lateral and medial ONL, is assumed to be similar to dorsal ONL, which is supported by the $\Delta R_{2\text{ MION}}$ line profile in Fig. 2E. One-way ANOVA showed a significant difference between the remaining ROIs and post-hoc t-tests comparing them to reference layer GL showed significant differences in MCL, GCL and Core ROIs (Fig. 2F, red line); thus, showing a lower capillary volume in these regions compared to GL. For comparison, total blood volume ($\Delta R_2^*_{\text{MION}}$) one-way ANOVA indicated a significant difference between the

same five ROIs and post-hoc t-tests comparing them to reference layer GL showed significant differences in EPL, MCL, GCL and Core (Fig. 2F, black line). Our results show a layer-dependent vascular organization of the olfactory bulb in that large diameter vessels with large total blood volumes dominate the bulb surface, midline and ONL layers, but transition to primarily micro-vessel blood volumes in GL, EPL and MCL.

3.2 Sensitivity of BOLD vs. CBVw fMRI in the Olfactory Bulb

BOLD and CBVw fMRI were compared for their ability to detect signal changes caused by the 5% amyl acetate stimulation. To examine the sensitivity of BOLD and CBVw fMRI, functional t-value maps are shown with a very low threshold ($t > 1.5$) and with $t > 4$ for three imaging slices in three representative animals (Fig. 3A–B). Clearly, more hot color voxels were detected for CBVw fMRI compared to BOLD, indicating that CBVw fMRI is more sensitive, similar to previous studies in cortical areas (Mandeville et al., 2004; Mandeville et al., 1998; Van Camp et al., 2005; Zhao et al., 2006).

For BOLD images, scattered focal activations of highly significant t-values (red voxels), appear on the bulb surface and in the upper bulb layers, which are consistent with previous findings (Xu et al., 2000; Yang et al., 1998). In addition, negatively active BOLD changes (blue and cyan voxels) were observed mostly in the midline regions. Specifically, for BOLD, $46.3 \pm 5.7\%$ and $22.1 \pm 3.1\%$ (mean $\pm$ s.e.m., $n = 6$ animals) of active pixels ($t > 3$) were negative at Surface and ONL ROIs, respectively, compared to $19.3 \pm 2.6\%$ and $7.2 \pm 1.2\%$ for CBVw. With the increased sensitivity, CBVw maps show more discrete activation foci that are surrounded by a lesser, global activation (yellow voxels). CBVw activation is the greatest in GL and EPL, yet significant activation is consistently observed in deeper layers. These CBVw observations are consistent with 2-DG studies of odor exposure (Lancet et al., 1982).

Since 64-s odor exposures were used to obtain enough images with an 8-s temporal resolution, neural habituation may decrease the fMRI response with stimulus time. Time courses averaged from the 100 most activated pixels for each ROI that were common to BOLD and CBVw images (Fig. 4A and 4B) are shown in Fig. 4. Generally, larger CBVw changes were observed compared to BOLD responses. Note that the negative CBVw changes indicate a CBV increase. Across the six animals, BOLD signals increase with stimulation and have the greatest change in the Surface, ONL or GL ROIs that decreases with depth. Contrarily, CBVw signals decrease with stimulation due to local increases in CBV and have signal changes that peak in ONL, GL or EPL. In three of the six animals, the BOLD and CBVw change peaked around 32-s after odor onset and showed slight decreases in the final 32-s of stimulation (Fig. 4C–D), while the others plateaued without the signal decrease during stimulation. Slight fMRI signal decreases during the 64-s odor stimulation period is similar to previous BOLD fMRI reports for a 1-min odor presentation (Schafer et al., 2005; Xu et al., 2000), and may be attributed to stimulus habituation; while inter-animal differences may be due to varying effects of anesthesia on olfactory feedback inhibition (Kato et al., 2012).

3.3 Reproducibility of BOLD and CBVw Maps

Reproducibility was tested to determine if BOLD and CBVw fMRI are robust measures of odor activation. Even and odd runs were compared in each animal (Fig. 5A–B). Generally, the pattern of activations is similar between even and odd runs. Common BOLD activation is spurious and localized to the surface and midline (Fig. 5C), while common CBVw activation is clustered around foci (Fig. 5D). In order to quantify the similarity of even and odd activation maps, SCC values were calculated for pixels within the whole bulb and GL for $t > 2$ and $t > 4$ (Fig. 5E). One animal was not included in the reproducibility test due to

insubstantial contrast-to-noise after even and odd trial separation. All SCC values were greater than 0.2 for each location and threshold chosen, which suggests a significant reproducibility of the images ($p < 0.01$). In addition, CBVw reproducibility was significantly greater than BOLD for pixels within GL and a threshold of $t > 4$ ($p < 0.05$, $n = 5$ animals).

3.4 BOLD vs. CBVw Activation Sites

When two measurement methods have different sensitivity, it is difficult to directly compare their activation patterns. To overcome this shortfall, the same number of most active voxels was selected as thresholds with 3%, 6% and 12% most-significant t -values. Statistical maps for one representative slice are shown in Fig. 6A–B. For BOLD fMRI, activation is segregated to the bulb surface, midline and ONL at a higher, 3% threshold, but includes the deeper bulb layers like GL and EPL with the more inclusive 6% and 12% thresholds (Fig. 6A). Contrarily, CBVw fMRI activation is located to GL and EPL at the higher threshold, and has increased activation in these layers plus MCL at lower 6% and 12% thresholds (Fig. 6B). To determine similarity and difference between BOLD and CBVw activation, overlap maps are shown (Fig. 6C) and SCC values were computed for each ROI (Fig. 6D). These overlap maps confirm that BOLD activation is located to more superficial layers of the bulb, while CBVw activation is present in deeper layers. In addition, the SCC values in Fig. 6D suggest that BOLD and CBVw maps are segregated (negative SCC values), have little overlap (low, positive SCC values) and/or are randomly overlapped ($p > 0.01$) at the thresholds chosen.

3.5 Layer-dependent BOLD vs. CBVw Response in the Olfactory Bulb

To compare layer-dependent BOLD vs. CBVw responses, line profiles are drawn to measure across BOLD (L_{BOLD} in Fig. 7A) and CBVw foci (L_{CBV} in Fig. 7B). Laminar BOLD activation is spurious with peak activation in neural-specific (GL and EPL) and non-specific (Surface) layers (black in Fig. 7C–D), while CBVw profiles are more similar with activation peaks in ONL, GL and EPL (red in Fig. 7C–D). In addition, BOLD and CBVw profile trends are not similar along the same line, indicating a possible difference in vascular source. The CBVw profile in Fig. 7D is the most qualitatively similar to the metabolic response obtained from the 2-DG focus for a similar amyl acetate stimulus in rats (Fig. 7E, (Sharp et al., 1977)), in which the CBVw response peaks around GL and decreases, but remains significant, through MCL. For both BOLD and CBVw, the individual line profiles do not represent general laminar changes in the bulb and are dependent on the line position.

To avoid single line profile bias, laminar ROIs were used for further analyses to compare BOLD and CBVw fMRI. For the top 3%, 6% and 12% thresholded maps, the distribution of active voxels normalized by the ROI volume fraction (Fig. 8A) and average absolute t -value of active voxels were calculated (Fig. 8B). For comparison, the average t -value (Fig. 8C) of all voxels within each ROI was obtained. Normalized number of active voxels indicates the relative distribution of active voxels across layers (Fig. 8A), since the total number of voxels across the layers is different. One-way ANOVA analyses of BOLD and CBVw ROIs in Fig. 8A indicate laminar differences for both methods and at all thresholds. Post-hoc t -tests with GL as a reference consistently show similar activation patterns in Surface, ONL and GL for BOLD, while CBVw activation is similar in GL and EPL. Mean absolute t -values show the relative detection sensitivity across layers (Fig. 8B–C). Mean absolute t -values without threshold (Fig. 8C) have a similar trend to the normalized distribution of active pixels (Fig. 8A). One-way ANOVA indicates a laminar difference for t -values (Fig. 8C) and post-hoc t -tests show similarity of GL to most layers for BOLD, but CBVw activation in GL is most similar to EPL and, to a lesser extent, to MCL and ONL. In general, the BOLD response is highest at the Surface ROI, ONL and GL, and decreases with cortical depth. The highest CBVw response occurs at GL and EPL, and is still measurable in MCL; while GCL activity

is small for both methods, observed by the ROI analysis (Fig. 8) and low t-values (dark voxels, Fig. 3A). Clearly, the activation peak shifts to deeper layers for CBVw than for BOLD. The laminar t-value distribution of BOLD (Fig. 8B–C) is similar to the baseline total CBV gradient ($\Delta R_2^*_{\text{MION}}$) observed in Fig. 2F. Consistent with observations in line profiles, CBVw fMRI is similar to 2-DG results (see Fig. 7D–E), suggesting that the vascular source of the CBVw signal is near metabolically active cells.

3.6 CBVw Odor Maps of Two Different Odors

With high sensitivity and specificity to GL, CBVw fMRI was used to map spatial patterns of the olfactory bulb responding to two different odor conditions, 5% amyl acetate (Fig. 9A) and 1% pyridine (Fig. 9B). In both odor maps, foci (red voxel clusters indicated by arrows) are located in GL, but at different locations. A significant global difference between the two maps is observed in the ventro-medial bulb of the first two slices (indicated by white contour). To further compare the difference and similarity between the two odors, the same number of active voxels was selected. A strict, 3% threshold reveals individual activation foci that differ in their spatial location between the two odors (arrows), which is evident by a low SCC value (Fig. 9C). A more liberal, 12% threshold again reveals global activation differences in the ventro-medial portion of the bulb (white outline), which is commonly inactive in Pyr condition (Fig. 9D). Previous 2-DG activation patterns are similar to our fMRI data; amyl acetate activates dorsolateral and medial GL (Johnson et al., 1998), which is consistent to our observations indicated by blue arrows in Fig. 9A and C and to other animals reported (Fig. 3). Derivatives of pyridine (containing the pyridyl functional group) activate glomerular pairs located to the dorsal bulb (Johnson et al., 2005), which is also consistent with our data indicated by the white arrows in Fig. 9B and C.

In all four animals, SCC values were calculated (amyl acetate $\cap$ pyridine) for pixels within the whole bulb and GL only for both 3% and 12% threshold (Fig. 9E) and compared to the reproducibility of amyl acetate maps (even $\cap$ odd runs). In all cases, the mean SCC value was lower for the two odor comparison compared to the reproducibility test, suggesting that the maps of the two odors have less overlap than those of the same odor. In addition, at the higher threshold, these differences were significant ($p < 0.05$, $n = 4$, paired Student's t-test). It should be noted that the sensitivity for the reproducibility test was less than that of the two odor comparison due to even and odd trial separation. Our data suggest that CBVw fMRI distinguishes unique spatial activation patterns for the two different odor conditions.

4. Discussion

4.1 Blood Volume Fractions and Vascularization of the Olfactory Bulb

Our blood volume measurements agree with previous reports of the layer-dependent vascularization of the olfactory bulb (Borowsky and Collins, 1989; Chaigneau et al., 2003; Coyle, 1975). Large vessels identified on the bulb surface and midline (red arrows, Fig. 2A–C) likely include the four main pial arteries; specifically, the dorsal-medial and dorsal-lateral branches of the olfactofrontal artery that supply the dorsal bulb and the ventral-medial and ventral-lateral olfactory bulb arteries that supply the ventral bulb. These arteries form anastomotic networks and project ramifications perpendicular to the bulb surface to supply bulb layers with different vascular volumes (Coyle, 1975); similar to the penetrating vessels observed in Fig. 2C. The vascular composition of ONL in the dorsal olfactory bulb was previously shown to be predominantly composed of larger diameter vessels and to have few capillaries (Chaigneau et al., 2003). Our study suggests a similar vasculature organization in lateral ONL regions (see Fig. 2E).

Capillaries emerge and are the most dense in GL (Borowsky and Collins, 1989; Chaigneau et al., 2003), which agrees with the local $\Delta R_2^*_{\text{MION}}$ maximum at GL (Fig. 2E–F) and the

micro-vasculature enhancement beginning in this layer (green overlay, Fig. 2D). In addition, capillary density relative to GL was approximately 75% in EPL and 45% in GCL (Borowsky and Collins, 1989), which is similar to the respective $84\% \pm 6\%$ in EPL and $36\% \pm 3\%$ in GCL observed in our study (red line, Fig. 2F).

4.2 Odor Mapping with fMRI

Pioneering olfactory bulb BOLD fMRI studies have extensively characterized the ground work for *in vivo* odor mapping at high spatial resolutions. Consistent with our BOLD results, activation was greatest in ONL and GL (Yang et al., 1998). In addition, spatial activation patterns from a single odor were highly reproducible, comparable between animals, and unique spatial activation patterns were observed with different odors (Liu et al., 2004; Schafer et al., 2006; Xu et al., 2000; Xu et al., 2003), similar to our results. However, the large BOLD activity in ONL and GL has potentially multiple contributions. 1) The large activity, especially in ONL, is likely due to the contributions from pial vessel susceptibility gradients (Kim and Ogawa, 2012). 2) Since venous blood drains to the cortical surface via intracortical venules, upstream activity influences downstream regions of GL and ONL. 3) Especially at high spatial resolution, fMRI sensitivity is reduced and weighted by baseline CBV; thus, BOLD fMRI is highly sensitive to regions in and around large vessels. To indirectly support this notion, the overlap of BOLD and CBVw activation (Fig. 6C–D) is very small at thresholds that include greater BOLD activation in GL (6% and 12% thresholds). This suggests that the vascular signal source for the two methodologies is different at high spatial resolutions and that large vessel contamination must be considered further (see discussion below). Previously, focal activation isolated to GL was shown with BOLD fMRI at a spatial scale of a single glomerulus (Kida et al., 2002). However, direct comparison of high resolution fMRI and 2-DG is needed to determine if such functional changes are indeed neural-specific within the voxel resolution.

4.3 Negative BOLD Response

In this study, negative BOLD changes were most prominent at the bulb midline and surface (Fig. 3A–B). This phenomenon was less profound in CBVw images due to lower baseline signal or to hemodynamic changes occurring without concomitant CBV change. Negative BOLD sites often co-localize with the hypo-intense regions in T_2 -weighted anatomical images (blue arrows, Fig. 6A–B) that were previously shown to be large blood vessels (Fig. 2A–C). Therefore, based on their bulb location, possible sources of the negative BOLD are increased blood susceptibilities associated with large draining veins or by the functional reduction of the cerebral spinal fluid volume fraction (Jin and Kim, 2010). Further study is needed to determine if changing the physiological condition of the animal, such as using an alternate anesthesia or air-gas mixture, will eliminate the negative BOLD response.

4.4 Vascular filter function for BOLD and CBVw fMRI

Functional MRI signal changes originate from the vasculature and are, therefore, an indirect measure of neural activity. Mandeville and Marota (1999) previously showed that contrast-to-noise (CNR) of BOLD fMRI linearly increases with resting total blood volume, whereas, CNR of CBVw fMRI has a bell-shaped curve that initially increases but later peaks and decreases with further increasing blood volume. This vascular filter is sensitive to baseline blood volume and dose of MION. The higher dose reduces the larger signal intensity from nearby pial vessels, shifting the peak sensitivity to deeper layers. This phenomenon can explain our current study in which BOLD activation (black lines, Fig. 8A–B) has a similar layer-dependent trend as the total blood volume (black line, Fig. 2F); while the CBVw activation peak is shifted to GL and EPL (red lines, Fig. 8A–B) where there is a local increase in micro-vasculature blood volume (Fig. 2D–F). In addition, the large BOLD

responses observed in superficial layers will attenuate the CBVw responses here. However, the decreased T_E for CBVw images will mitigate such BOLD contributions, which is evident in that large activation is observed in GL for both methods.

Alternatively, the biological explanation is that the highest CBV change indeed occurs at GL and EPL. Support for this conclusion can be drawn from previous MION studies in the cortex that showed that the spatial peak of activation does not change with increasing iron doses (Keilholz et al., 2006; Kim et al., 2013). In addition, the largest CBVw change was measured in layer 4 of the cortex, where the microvascular density is the highest, in gradient-echo and spin-echo measurements (Zhao et al., 2006). These previous findings indicate that the CBV peak in olfactory GL and EPL is biologically genuine. Our observations in the olfactory bulb are consistent with previous CBVw fMRI studies in the cortex and suggest that small blood vessels nearer to the source of metabolically active cells are the likely source of functional change.

4.5 Comparison of Laminar-Dependent 2-DG and fMRI Changes

Although the current study was not intended for odor mapping, distinct activation patterns were observed for two odor conditions and provide evidence that different odorants can be functionally differentiated with CBVw fMRI. CBVw activation is spatially consistent with 2-DG activation for the same or similar odorants (Johnson et al., 2005; Johnson et al., 1998), but differences are observed. For example, some of the odor activation maps have unilateral activation (Fig. 7A–B) in both BOLD and CBVw images, whereas 2-DG studies show symmetrical, bilateral activation. Such difference is most likely due to differences in experimental design, such as 1-min vs. 45-min lengths of odor exposure, anesthetized vs. awake physiological conditions, or directionally fixed introduction of the odorant to the nostrils vs. ambient odor diffusion for fMRI and 2-DG methods.

Laminar-dependent activation is compared between CBVw fMRI and 2-DG as follows. 1) Activation in ONL is observed in both measurements. Increased glucose metabolism in unmyelinated axons partially accounts for the 2-DG changes; however, the vascular composition of ONL is composed predominantly of larger blood vessels and contains little micro-vasculature. In addition, previous studies provide evidence that the energy demands of axons in ONL may be met by either passive diffusion of oxygen from arterial vessels or capillaries from the adjacent glomeruli, or by anaerobic respiration (Borowsky and Collins, 1989; Chaigneau et al., 2003; Lecoq et al., 2009; Tsai et al., 2003). This evidence brings into question whether fMRI activation in ONL is indicative of an active axon-specific hemodynamic regulation or is due to other vascular reasons mentioned in Section 4.2. 2) GL contains a large density of capillaries (Borowsky and Collins, 1989) and synapses, and corresponds to the peak vascular and metabolic responses. This provides evidence that CBVw fMRI indeed measures vascular changes near the site of metabolically active synapses. 3) EPL and MCL activity is similar for both methods in that they have decreased, but detectable, activation compared to GL. A measurable functional change in deeper layers is particularly important, since mitral cells are the primary output neurons of the bulb. 4) GCL activity is relatively low for CBVw fMRI as compared to 2-DG, which may be due to insignificant neural activity elicited by odor stimulation, a small blood volume fraction (Fig. 2E–F), insufficient sensitivity or a lack of hemodynamic coupling to inhibitory granule cells located here. Further fMRI study of GCL activation is necessary to understand the contribution of inhibitory neurons to the vascular response.

Consistency between 2-DG and fMRI methods provides a means to better understand how the brain processes odor using non-invasive methods. This is of particular importance because odor dysfunction is shown to predict many neurodegenerative and mental disorders before the onset of the more debilitating symptoms (Doty, 2012a; Doty, 2012b). For this

reason, “scratch and sniff” tests are becoming increasingly popular as a way to diagnose certain diseases at a time critical for early therapeutic intervention. However, the accuracy of such tests in discriminating different diseases cannot be fully realized without measuring whole brain function. Therefore, continued study of olfactory bulb fMRI is necessary to investigate *in vivo* olfactory processing that can one day benefit clinical applications.

4.6 Technical Disadvantages and Advantages of CBVw fMRI in the Olfactory Bulb

The olfactory bulb is positioned near air sinuses and, therefore, is a difficult structure to image due to large susceptibility artifacts and reduced T_2^* . These pitfalls cause conventional, fast fMRI imaging modalities, such as gradient-echo echo planar imaging, to have poor high-resolution image quality in the bulb; although odor activation maps have been reported with this method at a 1 s temporal resolution (Martin et al., 2007). Thus, FLASH technique is preferred in the bulb but at the cost of decreased temporal resolution: 8 s for this study. Compressed sensing techniques, in which k-space is partially collected to reconstruct an entire image, can be considered to improve the fMRI temporal resolution in the olfactory bulb, and to increase the CNR of the functional response by increasing the number of time points. This technical improvement is necessary to increase the sensitivity and reproducibility of the measured fMRI signal changes and will greatly improve the detection of small functional changes associated with neuroplasticity or disease in the bulb (Holland et al., 2013; Jung et al., 2009). Spin-echo echo planar imaging is another alternative, fast imaging method that will decrease susceptibility effects in the bulb but will have lowered sensitivity to hemodynamic changes caused by stimulation (Lee et al., 1999). Such technical improvements should be explored to reduce susceptibility artifacts and increase temporal resolution for olfactory bulb fMRI.

When the FLASH technique is used, CBVw fMRI has the advantages of reduced T_E and reduced in-flow effects compared to BOLD. First, T_E for BOLD and CBVw experiments was optimized to the baseline T_2^* values measured at GL before and after MION injection, respectively. The reduced T_2^* and, thus, T_E for CBVw experiments further reduces T_2^* susceptibility effects caused by sinus air and provides higher quality fMRI images in the layers near the air-brain interface, like ONL and GL. In addition, BOLD fMRI experiments with short T_R are confounded by in-flow effects, in which incoming intravascular protons have an increased baseline SNR compared to parenchyma protons. Increased baseline SNR attributed to in-flow effects was observed in the high-resolution T_2^* images in the current study as white hyper-intensities (Fig. 2A). However, for CBVw fMRI, the intravascular signal is completely eliminated by the increased susceptibility effects of MION, which prohibits SNR intravascular weighting. FLASH-based CBVw fMRI provides technical advantages in the olfactory bulb, but future improvements in temporal resolution are necessary for future applications.

CBVw fMRI commonly uses contrast agents for enhancing functional sensitivity and reducing large vessel signal contributions. Alternatively, CBVw fMRI can be obtained without contrast agents using the vascular-space-occupancy (VASO) technique, which separates blood and tissue signals with differential T_1 values (Lu et al., 2004). Since arterial CBV change is dominant during stimulation (Kim et al., 2007; Kim and Kim, 2011), arterial CBV MRI techniques can be used for CBVw fMRI, including arterial spin labeling with magnetization transfer (Kim and Kim, 2005) or bipolar gradient (Kim and Kim, 2006), and BOLD with magnetization transfer (Kim et al., 2008). However, these non-invasive CBV approaches are less sensitive compared to contrast agent CBVw and BOLD fMRI.

5. Conclusions

We found the blood volume content in the bulb to be layer-dependent in that total blood volume progressively decreases from the bulb surface to core, while a significant microvascular enhancement is measured in GL, EPL and MCL. Compared to BOLD, CBVw fMRI provides greater sensitivity and reproducibility in the bulb. In addition, CBVw better represents the layer-dependent metabolic changes reported by previous 2-DG studies and, thus, is more accurate to the location of neurophysiological activity in the bulb at high spatial resolutions. Finally, CBVw is able to differentiate activation patterns associated with two odor conditions. Our study indicates that CBVw fMRI is a viable tool to investigate *in vivo* olfactory bulb function and can be applied to basic neuroscience study.

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Abbreviations

fMRI	functional magnetic resonance imaging
BOLD	blood oxygenation level-dependent
CBVw	cerebral blood volume weighted
MION	monocrystalline iron oxide nanoparticles
ONL	olfactory nerve layer
GL	glomerular layer
EPL	external plexiform layer
MCL	mitral cell layer
GCL	granule cell layer
2-DG	[¹⁴ C]-2-deoxy-D-glucose
FLASH	fast low-angle shot
ROI	region of interest

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Highlights

- We compared BOLD and cerebral blood volume-weighted (CBVw) fMRI in the olfactory bulb
- Baseline total blood and microvessel volumes were layer-dependent
- CBVw had greater sensitivity and reproducibility than BOLD
- Layer-dependent CBVw activation better represented previous metabolic 2-DG changes
- CBVw activation patterns of two odor conditions were differentiated

Fig. 1. fMRI experimental overview

A: A custom-built odor delivery system was used to present the different odor stimuli for fMRI activation studies. Compressed air continuously blew through the system at 1 L/min while the MRI acquisition computer controlled the opening and closing of solenoid valves that diverted air flow through one of three flasks: 100% mineral oil (control), 5% amyl acetate or 1% pyridine. Each odor had dedicated tubing that converged at the rat nose cone for quick transitions. One-way check valves (►) prevented odor mixing and a vacuum line removed odors from the rat nose. **B:** The olfactory bulb is located posterior to the nasal sinus, is anterior to the cortex in rats (red square) and receives direct input from olfactory sensory neurons. To increase experimental sensitivity, a custom 1-cm diameter radio frequency (RF) surface coil was placed dorsal to the bulb and whole bulb spin-echo navigator images were acquired to determine the image position of five experimental slices ($7 \times 7 \text{ mm}^2$ FOV, 0.5 mm slice thickness). High-resolution fMRI slices were acquired every 8 s and each slice had an in-plane resolution of $110 \times 110 \mu\text{m}^2$. **C:** Anatomical images ($55 \times 55 \mu\text{m}^2$ in-plane resolution) were acquired and the individual bulb layers were clearly identified using endogenous T_2 contrast. The anatomical images were used to draw seven region-of-interest (ROI) masks and include the major layer subdivisions of the bulb: 1) Surface, 2) ONL, 3) GL, 4) EPL, 5) MCL, 6) GCL and 7) Core ROIs. The same color ROIs were shown in following figures. These ROI masks were then used to analyze layer-dependent baseline blood volumes and activation.

Fig. 2. Layer-dependent baseline total blood and micro-vasculature volumes

All images (AD) have an in-plane resolution of $55 \times 55 \mu\text{m}^2$. Color overlay in the left bulb shows laminar ROIs. **A:** T_2^* -weighted image before MION injection shows large signal hyper- (due to large vessel in-flow effects) and hypo-intensities (due to large vein induced magnetic susceptibility effects) that are primarily localized to the bulb surface and midline (red arrows). **B:** T_2 -weighted image before MION injection shows hypo-intensities at large vessels (red arrows). **C:** $\Delta R_2^*_{\text{MION}}$ maps measure the total baseline blood volume. Consistent to the location of large blood vessels in A and B (red arrows), large total blood volumes ($\uparrow \Delta R_2^*_{\text{MION}}$) are observed. In addition, vessels are clearly observed to penetrate perpendicular to the bulb surface that progressively reduce in volume toward the deeper bulb layers. **D:** ΔR_2_{MION} has an enhanced sensitivity to detecting micro-vessel blood volume and provides a measure for the location of capillary beds. A small, hyper-intense band is observed to follow the contour of layers GL (green overlay), EPL, and MCL (orange overlay). A Gaussian spatial smoothing filter ($40 \mu\text{m}$ width) was applied to this image for display purposes only. **E:** $\Delta R_2^*_{\text{MION}}$ (black line) and ΔR_2_{MION} (red line) line profiles at the location indicated by the red box overlays in C and D. Seven voxels in the dorsal-ventral axis were averaged (mean $\pm$ s.e.m., $n=7$, no spatial smoothing) for each point reported in the line profile along the left-right axis. Local maxima are observed in GL for both total and micro-vascular-enhanced CBV, with sustained increases in ΔR_2_{MION} through EPL and MCL. **F:** ROI analysis includes five slices from four animals (mean $\pm$ s.e.m., $n=4$) and shows the laminar profile of baseline total CBV (black line, $\Delta R_2^*_{\text{MION}}$) and capillary-sensitive CBV (red line, ΔR_2_{MION}). Each animal was normalized to GL and, therefore, reports relative (Rel.) changes in R_2^* and R_2 due to MION. Surface ROI was removed from the plot due to large vessel intravascular R_2 changes that are not indicative of microvasculature blood volume. In addition, Surface and ONL ROIs were removed from statistical analyses because a previous study shows few capillaries are present in these regions of the dorsal bulb (Chaigneau et al., 2003) and is supported by the ΔR_2_{MION} line profile in E. One-way ANOVA and post-hoc t-tests indicate ROIs that are significantly different from GL (* $p < 0.013$, $\alpha = 0.05/4$ ROI comparisons, $n=4$ animals) for each ΔR_2_{MION} and $\Delta R_2^*_{\text{MION}}$.

Fig. 3. Sensitivity of BOLD and CBVw fMRI responding to 5% amyl acetate

Statistical t-value maps with the t-value threshold of 1.5 (A) and 4 (B) laid over the anatomical images are shown in the central three slices from three representative rats. Note that since an increase in CBV decreases fMRI signal due to increased susceptibility effect, the sign of all CBVw t-values was reversed. Decreases in BOLD signals during odor exposure (blue and cyan voxels) co-localize with hypo-intensities in the T_2 -weighted anatomical images and are associated with large blood vessels. Generally, BOLD fMRI activation is more spurious and contains less hot pixels compared to CBVw.

Fig. 4. BOLD and CBVw time courses at each laminar ROI

One hundred pixels with the most significant t-values common to BOLD and CBVw images were chosen across five slices for the time course analysis of each ROI. **A–B:** The anatomical locations of these pixels are shown in three slices. **C–D:** BOLD and CBVw fMRI average time courses of ONL, EPL, and GCL ROIs (C) and Surface, GL, MCL, and Core ROIs (D) are shown for better visualization (mean $\pm$ s.e.m., $n = 100$ pixels). The black horizontal bar indicates the period of the 5% amyl acetate odor stimulation. Generally, BOLD signals increase, while CBVw signals decrease during odor stimulation. The sign of the signal changes was preserved during the time course analysis; thus, positive and negative changes cancel. This effect is greatest in Surface ROI for BOLD.

Fig. 5. Reproducibility of BOLD and CBVw activation maps to 5% amyl acetate by comparing even and odd fMRI runs

Three center slices from a single representative animal are shown. **A–B:** Statistical t-value maps of even and odd runs are qualitatively similar for both BOLD and CBVw fMRI. However, across techniques, the sensitivity increase of CBVw is apparent by the increased number of hot pixels. **C–D:** Even and odd maps with thresholds of $t > 4$ and $t > 2$ were overlapped, where activation from only even runs is in green, only odd runs in yellow and common activation in red. The SCC values for these particular images are displayed in the lower right corners. **E:** SCC values were calculated across 5 animals to quantitatively evaluate reproducibility (even $\cap$ odd). All SCC values are above 0.2, indicating significant overlap ($p < 0.01$) and good reproducibility for both BOLD and CBVw maps. In addition, CBVw fMRI had significantly greater reproducibility compared to BOLD in GL at the higher threshold ($* p < 0.05$, paired Student's t-test, $n = 5$).

Fig. 6. Activation foci of BOLD vs. CBVw fMRI maps

Due to sensitivity differences, top 3%, 6% and 12% most active voxels were chosen so that an equal number of voxels were activated for both methods (A–B). Blue arrows indicate the co-localization of the negative BOLD responses to large blood vessels (hypo-intensities in the T₂-weighted anatomical image). Corresponding absolute t-value thresholds are displayed in the bottom right corner of each slice. Activated voxels were laid over the anatomical image and ROI mask to show laminar location. A single representative slice was chosen and voxel clusters less than two were removed. To directly compare activation foci of BOLD (green) and CBV (yellow), the overlap maps were generated (C). Common active voxels are shown as red and the SCC values ($\text{BOLD} \cap \text{CBVw}$) for the specific slice and threshold are shown in the bottom right corners. Generally, BOLD activation is primarily located at superficial layers including ONL, while CBVw activation shifts to deeper layers including GL, EPL and MCL. **D:** SCC values averaged across all the six animals for the whole bulb and for the individual ROIs. Negative SCC values indicate segregation of the BOLD and CBVw maps at higher thresholds. In addition, no mean SCC value is above 0.2, indicating that there is low overlap of the two types of fMRI contrast. Animals that did not have active pixels at 3% and 6% thresholds for certain ROIs were removed from analysis for that ROI only. The number of animals, if less than six, is displayed above each ROI. Asterisks (*) indicate significant SCC values ($p < 0.01$). One-way ANOVA shows no significant difference between Surface through GCL ROIs for all three thresholds chosen ($p > 0.05$).

Fig. 7. Single line profile across the bulb layers through a hot focus

Location of activation is shown with single line profiles. **A-B:** BOLD (**A**) and CBVw (**B**) t-value maps showing the line paths through BOLD (L_{BOLD}) and CBVw (L_{CBV}) foci. **C-D:** T-value profiles of BOLD (black line) and CBVw (red line) along L_{BOLD} (**C**) and L_{CBV} (**D**). **E:** 2-DG line profile from Sharp et al. (1977) through a 2-DG focus following odor exposure. Cellular glucose metabolism ($\uparrow$ radioactivity) peaks in GL but remains detectable through MCL and superficial parts of GCL. The CBVw profile in **D** is more similar to the 2-DG profile.

Fig. 8. Layer-dependent ROI analysis of BOLD and CBVw activation

ROI analysis included all five slices (mean $\pm$ s.e.m., $n = 6$ animals). The top 3%, 6% and 12% most active voxels were selected for A and B, and all pixels were used for C. **A:** Distribution of active voxels (# of active voxels in each ROI/total # of active voxels) normalized by the ROI volume fraction (# of voxels in each ROI/total # of voxels). **B:** The mean absolute t-value for the three thresholds. Animals that did not have active pixels at 3% and 6% thresholds for certain ROIs were removed from analysis for that ROI only. The number of animals, if less than six, is displayed adjacent to each point. Generally, the magnitude of BOLD activation is similar in shape to the layer-dependent total blood volume measurements (Fig. 2F, black line, $\Delta R_2^* \text{ MION}$). However, the peak of CBVw activation magnitude is slightly shifted toward layers containing a microvascular enhancement (Fig. 2D–F). **C:** Mean absolute t-values without threshold (top 100% t-values) were calculated for each layer ROI. Generally, BOLD t-values are greatest in the superficial bulb ROIs (Surface, ONL and GL) ROIs, while CBVw t-values peak in GL and EPL but have comparable activation in MCL. One-way ANOVA and post-hoc t-tests indicate ROIs that are significantly different from GL reference (A–C, * $p < 0.008$, $\alpha = 0.05/6$ ROI comparisons).

Fig. 9. CBVw fMRI maps responding to 5% amyl acetate vs. 1% pyridine

Three center slices from one representative animal are shown. **A–B:** Statistical t-maps with t-value threshold of 1.5 for 5% amyl acetate (**A**, 5% AA) and 1% pyridine (**B**, 1% Pyr) exposure. Locations of 5% AA focal activations (cyan arrows) differ from 1% Pyr foci (white arrows); while global activation in the ventral-medial bulb (white contour) is absent in 1% Pyr condition. **C–D:** Top 3% and 12% threshold activation maps for 5% AA (green voxels) and 1% Pyr (yellow voxels) were overlaid to measure the extent of overlap (red voxels). The SCC values for the specific images shown are displayed in the right corners. Strict 3% threshold (**C**) isolates activation foci that differ in location, as is indicated by the lower SCC values. Liberal 12% threshold (**D**) better shows global activation differences (white contour). **E:** SCC values for reproducibility (red, even $\cap$ odd for AA only) and two different odors maps (black, AA $\cap$ Pyr). In all cases, the mean SCC value is less for the different odor maps compared to the single odor reproducibility maps. For the higher threshold only, there is a significant difference between the SCC values (* $p < 0.05$, paired Student's t-test, $n = 4$), indicating that CBVw is able to detect differences in the functional maps of the two odors.