Functional organization of glomerular maps in the mouse accessory olfactory bulb

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The mammalian accessory olfactory system extracts information about species, sex and individual identity from social odors, but its functional organization remains unclear. We imaged presynaptic Ca2+ signals in vomeronasal inputs to the accessory olfactory bulb (AOB) during peripheral stimulation using light sheet microscopy. Urine- and steroid-responsive glomeruli densely innervated the anterior AOB. Glomerular activity maps for sexually mature female mouse urine overlapped maps for juvenile and/or gonadectomized urine of both sexes, whereas maps for sexually mature male urine were highly distinct. Further spatial analysis revealed a complicated organization involving selective juxtaposition and dispersal of functionally grouped glomerular classes. Glomeruli that were similarly tuned to urines were often closely associated, whereas more disparately tuned glomeruli were selectively dispersed. Maps to a panel of sulfated steroid odorants identified tightly juxtaposed groups that were disparately tuned and dispersed groups that were similarly tuned. These results reveal a modular, nonchomotopic spatial organization in the AOB.

Neural circuits decode the sensory world through a highly refined series of synaptic connections. Because most neuronal circuitry in local, progress in dissecting functional interactions has long emphasized the nervous system’s spatial organization1–6. Indeed, discovering how sensory modalities map to regions of the brain was an essential step in deciphering their function. Discoveries in the visual and somatosensory cortices revealed links between sensory parameters and the location of neural responses, indicating that these sensory systems possessed topographic maps1,2. Whether topographic or nontopographic, sensory maps have served as a foundation for our understanding of the brain.

However, many regions of the brain do not have a natural correspondence to external space. There is considerable interest in understanding the principles of spatial organization that govern such circuits. In the chemical senses, one prominent form of spatial organization is observed in the olfactory bulb, in which olfactory sensory neurons expressing the same receptor gene project their axons to common regions of neuropil called glomeruli4,7. This organization pools the output of many individual sensory neurons with identical specificities for odorants. This glomerular organization serves as a form of sensory map because particular functional responses are reproducibly localized to particular regions8. In the main olfactory bulb (MOB) of rodents, numerous studies have defined the positions and tuning profiles of many of the glomeruli6,9–17. While this map is reproducible across animals, no overarching principle has been discovered that broadly describes its spatial organization18. The lack of a cohesive model for MOB topography might be a consequence of the huge diversity of odorous compounds and difficulties inherent in determining how ‘proximity’ in chemical space relates to odorant receptor structure and axon targeting.

To overcome these barriers, one promising approach is to study maps and topography in an olfactory system selective for a narrower range of stimuli. An attractive candidate is the accessory olfactory system (AOS), also called the vomeronasal system, which specializes in the detection of social odors19. Vomeronasal sensory neurons (VSNs), neuroepithelial cells in the vomeronasal organ (VNO), project axons to the first AOS circuit, the AOB. The AOB receives all of its synaptic inputs in a densely packed glomerular layer. In mice, this layer covers less than a square millimeter of the brain surface and is roughly 150 µm deep, in principle allowing optical observation of nearly all synaptic inputs into the AOB in a single experiment. An important difference between the AOB and the MOB is that VSNs expressing the same receptor gene (members of a given VSN type) target multiple AOB glomeruli, and do so with substantial variability across experimental subjects20,21. Until now, the only studies of the organization of the glomerular layer have been anatomical ones in which one20,21 or a few22 VSN types were tagged with a fluorescent label. Such studies provide a precise but narrow snapshot of the organization of AOB inputs. Moreover, because the molecules that activate VSN types are unknown, the relationships between glomerular receptive fields and physical positions have remained unexplored.

However, any attempt to determine the functional organization of the AOB must address a number of technical obstacles. AOB glomeruli are small (10–80 µm in diameter), variable in shape and stacked atop one another in depth, so obtaining an exhaustive functional map requires methods to quickly image activity in three dimensions through large volumes. We combined ex vivo methods that expose the AOB surface23 with calcium imaging via objective-coupled planar illumination microscopy24. By stimulating VSNs with AOS odorants, including urine from different developmental stages and sulfated steroids25, we obtained the first large-scale functional images of the AOB glomerular layer. Our results identify organizational features across spatial scales and reveal a modular organization in this circuit that physically juxtaposes certain inputs and selectively disperses others.

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RESULTS

We studied AOBs from adult male transgenic mice expressing the Ca\textsuperscript{2+}-sensitive fluorescent protein GCaMP2 in the cytoplasm of VSNs\textsuperscript{26}. We imaged GCaMP2 fluorescence in the glomerular layer of the AOB while stimulating peripheral VSNs with mouse urine and sulfated steroids in ex vivo preparations of the AOS\textsuperscript{27} (Fig. 1a). Image stack dimensions were approximately 700 µm along both the anterior-posterior and medial-lateral axes and nearly 200 µm deep into the tissue (Fig. 1b). These image stacks encompassed the entire anterior AOB (aAOB) and approximately 1/3 of the posterior AOB (pAOB; Fig. 1b and Supplementary Video 1) and were acquired every 5 s while stimuli were delivered to the VNO. Responses were recorded continuously for periods up to 1 h 40 min.

Sex-specific glomerular maps are activated by mouse urine

We hypothesized that male- or female-responsive glomeruli might occupy specific spatial domains in the aAOB. Previous electrophysiological recordings identified aAOB mitral cells responding to dilute male and female urine, without clear evidence of sex-dependent spatial organization\textsuperscript{28}. However, mitral cells possess extensive dendritic arbors with connections to many glomeruli spanning large distances\textsuperscript{22,29,30}, which might complicate understanding of local circuits. We observed fluorescence intensity changes in the glomerular layer when we stimulated the VNO with 100-fold dilute male, sexually naive BALB/c female and male mouse urine, but not with Ringer’s control saline (Fig. 1c). As in previous studies, we separately pooled the urine of many male and female mice from several consecutive days to produce an ‘average’ source of sex-specific urinary odors. We calculated a response reliability index (RRI, which reports signal-to-noise ratio) for each image voxel by normalizing the fluorescence changes to the across-trial noise (Fig. 1d). We used RRI values to identify regions of interest (ROIs) representing putative glomeruli. These glomerular ROIs showed stimulus-dependent increases in fluorescence, consistent with previous Ca\textsuperscript{2+} imaging from VSNs\textsuperscript{26,31} (Fig. 1e). In the AOB, glomerular boundaries are often poorly defined by anatomical criteria\textsuperscript{29}, but when visible these boundaries did align well with activated ROIs (Fig. 1f). Moreover, GCaMP2 activation highlighted regions that could not be distinguished on the basis of morphological cues alone (Fig. 1f).

To compare glomerular maps responsive to male and female urine, we aligned AOBs to visual boundaries in each image (Fig. 2; see Online Methods). In accord with previous reports\textsuperscript{24}, we observed strong aAOB responses to 1:100 BALB/c female urine (Fig. 2a). The sum volume of all glomeruli activated by 1:100 BALB/c female urine was four times that activated by 1:100 BALB/c male urine (4.4 ± 1.6 fold, \(n = 8\), \(P = 0.043\); Fig. 2a,b). A much larger fraction of male urine–responsive than female urine–responsive voxels were located in the visible regions of the pAOB (32 ± 8% male versus 2 ± 1% female, \(P = 0.007\), \(n = 8\); Fig. 2b). Female urine–responsive ROIs tended to be larger than male urine–responsive ROIs (Fig. 2c; \(P = 0.032\), Kolmogorov-Smirnoff test). Because ROI volumes increase with the normalized change in fluorescence intensity (\(\Delta F/F\)), this effect may be influenced by stronger maximal activation by BALB/c female urine than male urine, but might also suggest that female urine–responsive glomeruli possess a larger synaptic territory in the AOB.

We observed a small number of ROIs that responded to both male and female urine (Fig. 2a,d). To ensure that this effect was not due to inclusion of very weak responses to male urine, we evaluated glomerular ROI responses at several thresholds. The overlap persisted until very high thresholds, confirming that these glomerular ROIs were activated by cues found in both male and female urine (constituting 4 ± 3% of female and 9 ± 4% of male urine–responsive voxels at \(\Delta F/F > 2\%\), \(n = 8\); Fig. 2d). This population represented a larger fraction of the male urine–responsive ROIs, owing largely to the larger overall pool of female-responsive ROIs.

**Figure 1** Functional presynaptic Ca\textsuperscript{2+} imaging in the AOB. (a) Midline (top) and ventral (bottom) schematic of the objective-coupled planar illumination imaging setup for ex vivo preparations. VSNs and their axons are highlighted in green. LS, light sheet used to excite fluorescence; Obj., microscope objective; Stim., stimulus. (b) Top left, side view of AOB glomerular layer. Cyan lines illustrate three positions of the light sheet. Right, images at three light sheet positions. Bottom left, three-dimensional rendering of the AOB as seen from the surface. M, medial; A, anterior; P, posterior. Dotted line indicates the linea alba. (c) GCaMP2 fluorescence changes during VNO stimulation with dilute BALB/c female urine at the light sheet positions indicated in b. (d) Glomerular ROIs were defined based on \(\Delta F/F\) intensity and RRI. \(\Delta F/F\) responses to 1:100 BALB/c female (left) and male (right) mouse urine are shown for two different trials (top two rows). The RRI across five trials is shown in the bottom row. Color-coded arrowheads indicate the ROIs evaluated in e. (e) Fluorescence in three-dimensional glomerular ROIs was evaluated during peri-stimulus image stacks and compared to the response to Ringer’s control saline. Gray shading, stimulus timing; thin traces, individual trials; bold traces, across-trials mean. (f) Glomerular boundaries (arrowheads) were evident in the GCaMP2 baseline fluorescence in certain image frames (left). Stimulus-responsive voxels often followed visible anatomical boundaries (middle, 10 µM epipregnanolone sulfate). Nearby voxels activated by other stimuli (right, 10 µM 5-androsten-3\(\beta\)-diol disulfate) did not cross visible glomerular boundaries. Images represent mean \(\Delta F/F\) across 4 trials. Three adjacent optical sections are shown. Scale bars: b–d, 100 µm; f, 20 µm.
Glomerular maps encode both sex and maturity from urine

If the only function of the AOS were to distinguish sex, one might naturally wonder why so many glomeruli respond to female urinary cues. The fourfold difference in overall volume of the AOB glomerular layer activated by male and female urine mirrors observed differences in the number of VSNs demonstrating sex selectivity for urine\(^*\). Given that a small number of sex-selective VSNs and AOB neurons is theoretically sufficient for sex classification, we hypothesized that some of the abundant female urine–selective glomeruli may encode biologically relevant features other than sex—for example, sexual maturity. We therefore compared glomerular activity maps generated by VNO stimulation with juvenile (postnatal day 21), gonadectomized and sexually mature urine from the same mouse strain, BALB/c.

Each of these six urinary cues activated at least one glomerulus (Fig. 3). Glomerular maps generated by this stimulus set revealed the rich combinatorial nature of responses in the AOB (Fig. 3a–c). Many of the glomeruli demonstrated selectivity for certain urines, indicated by an increase the numbers of selective ROIs and a decrease in the number of nonselective ROIs at higher \(\Delta F/F\) thresholds (Fig. 3d). Most ROIs activated by sexually mature female urine also responded to juvenile and/or gonadectomized urine of one or both sexes (86 ± 3%...
at ΔF/F > 2%, n = 5; Fig. 3a,c,e). Thus, only a small fraction of the female urine–responsive aAOB glomeruli are exclusively tuned, or selective, for urine from adult intact females at this concentration (Fig. 3e). This was not the case for male urine–responsive glomeruli, which showed almost no overlap between intact, juvenile and castrated males (Fig. 3b). Including all stimuli, we found that most intact male urine–responsive ROIs were also selective for this stimulus (70 ± 14%, P = 3 × 10−3, one-way ANOVA, d.f. 24; max(P) = 5 × 10−5, unpaired t-tests with Bonferroni correction, n = 5; Fig. 3e). Intact adult female urine responses were nearly entirely abolished by sulfatase treatment (Supplementary Fig. 1), confirming that sulfated compounds dominate the AOS activity induced by female urine15.

Evaluation of all urine responses revealed specific, repeated tuning patterns among aAOB glomeruli (Fig. 3f). Clusters responded to anywhere from one to five of the six stimuli, with approximately half of the ROIs dominated by one or two stimuli. This classification of glomeruli into types serves as a foundation for analysis of the spatial organization of responses to natural stimuli (Fig. 3g and Supplementary Fig. 1).

Sets of functionally defined glomeruli form spatial modules

Because the AOBs evaluation and analysis of natural stimuli might be based on local circuitry, we wondered whether glomeruli exhibit patterns in the relationship between their receptive fields (Fig. 3f) and their spatial organization (Fig. 3g). To evaluate such questions statistically, we compared the observed positional patterns against those that arose from randomly shuffling the cluster label assigned to each ROI.

Several glomerular clusters were preferentially spatially distributed along the three orthogonal axes (Fig. 4a,b and Supplementary Fig. 1). Intact adult female urine–selective glomeruli (cluster 1) were preferentially located near the anterior tip of the AOB (P = 0.001 compared to shuffle test expectations, n = 5; Fig. 4b). In contrast, intact adult male urine–selective glomeruli (cluster 2) were preferentially located more posteriorly, very near the linea alba dividing the aAOB and pAOB (P = 0.012 compared to shuffle test expectations, n = 5; Fig. 4b). Cluster 3 glomeruli were preferentially located approximately midway between clusters 1 and 2 along the anterior–posterior axis (P = 0.001 compared to shuffle test expectations, n = 5; Fig. 4b). Cluster 1 glomeruli were frequently among the most medial of all activated glomeruli and had an unusually strong tendency to be superficial rather than deep (medial–lateral P = 0.002, depth P = 0.0001 compared to shuffle test expectations, n = 5; Fig. 4b). Cluster 3 glomeruli were biased toward deeper positions (P = 0.026 compared to shuffle test expectations, n = 5; Fig. 4b).
In addition to the regularities in absolute positioning exhibited by three of these clusters, we asked whether there might be reproducible patterns in the relative positioning between two or more clusters\(^3\). Again using comparisons against shuffled maps (Fig. 4c–f), we found that the largest effects consisted of dispersals: for example, cluster 1 ROIs (selective for intact adult female urine) and cluster 4 ROIs (selective for juvenile urine) were much farther apart than expected by chance (Fig. 4c, d; \( P < 10^{-7}, n = 5 \)). A similar result held for clusters 1 and 2 (\( P < 10^{-7} \)), clusters 4 and 5 (\( P < 10^{-3} \)) and clusters 4 and 6 (\( P < 10^{-8}, n = 5 \); Fig. 4d–f). We also found two clear instances of preferentially juxtaposed functional groups (Fig. 4d–f). Cluster 3 (responsive to intact adult female and juvenile urine) and cluster 4 (responsive to juvenile urine only) tended to be near one another (Fig. 4d-f; \( P < 0.01, n = 5 \)). Likewise, cluster 5 (responsive to all female urines and juvenile male urine) and cluster 6 (responsive to all urines except intact adult male urine) tended to be closer than expected by chance (\( P < 0.01; \) Fig. 4d–f). Clusters 1 and 2, selective for intact adult female urine and intact adult male urine, only showed preferential dispersal with other groups. Clusters 4, 5 and 6 showed preferential juxtaposition in some pairwise comparisons and preferential dispersal in others.

In both cases of selective juxtaposition, the glomeruli were similarly tuned, with the two members of each pair differing by only one response. Selectively dispersed groups, by contrast, were more dissimilarly tuned (that is, differing by at least two responses). At face value, this suggested that relative spacing among glomeruli may selectively group similarly tuned inputs and disperse dissimilarly tuned inputs. Such an organization would suggest that the AOB possesses ‘identity-based’ chemotopy based on relative glomerular position. However, because the blend of odorants in each urine source is unknown, it remains unclear from these data whether preferential juxtaposition and dispersal among glomeruli reflects sensitivity to similar molecules.

**Steroid-responsive glomeruli span the aAOB and pAOB**

To make explicit comparisons between glomerular spatial organization and odorant chemical structures, we generated maps to a panel of 11 synthetic sulfated steroids, a prominent class of natural ligands\(^2\). We delivered this panel of sulfated steroids, spanning the androgon, estrogen, pregnanolone and glucocorticoid families, at 10 \( \mu \)M, a concentration previously found to activate at least ten functionally separable VSN populations\(^27,31\). Across 10 adult experimental subjects, we identified 1,078 glomeruli that responded to at least one of the 11 synthetic sulfated steroids with \( \Delta F/F > 1\% \). We observed reliable, concentration-dependent glomerular activity across stimulus trials (Fig. 5a, b, Supplementary Fig. 2 and Supplementary Video 2).

In glomerular ROIs, fluorescence peaked within the first image stack (within 5 s) and then accommodated over subsequent image stacks (Fig. 5b). Single-plane imaging at 10 Hz indicated that fluorescence increases began within 1–2 s of stimulus onset and peaked within 3–5 s (Supplementary Fig. 2 and Supplementary Video 3). The vast majority (1,012 of 1,078, 94\%, \( \Delta F/F > 1\% \), \( n = 10 \)) of steroid-responsive ROIs were located in the aAOB, but they were widely distributed within this subregion (Fig. 5c–f, Supplementary Fig. 3 and Supplementary Video 4). Since VSNs expressing type 1 vomeronasal receptors (V1Rs) selectively innervate the aAOB\(^20,21\), these results confirm that many VSNs expressing V1Rs are sensitive to sulfated steroids\(^11,33\).

We observed a notable number of glomeruli responsive to sulfated pregnanolones in the posterior AOB (56 of 576 pregnanolone-responsive glomeruli, representing 85\% of all posterior responses). These glomeruli likely derive from a pregnanolone-responsive population of basal VSNs expressing type 2 V1Rs (V2Rs)\(^3\). We further investigated this pAOB activity by comparing activity maps at different sulfated steroid concentrations (Supplementary Fig. 2). We found reliable aAOB responses to most of the sulfated steroids in the panel starting at 1 \( \mu \)M (Supplementary Fig. 2), in accord with previous results\(^25,34\). Glomeruli in the pAOB responded to allo-pregnanolone sulfate (P3817; see Supplementary Table 1) at 10 \( \mu \)M (Supplementary Fig. 2). At 100 \( \mu \)M, activity in the pAOB increased markedly to another sulfated pregnanolone, epipregnanolone sulfate (P8200), but less so to other sulfated steroids (Supplementary Fig. 2). Since the pAOB receives input from V2R-expressing VSNs, these results suggest that sulfated pregnanolones elicit activity in both major vomeronasal receptor families.

Because sulfated steroid abundance is high in BALB/c female urine\(^25\), we investigated spatial relationships between synthetic steroid...
ROIs and female urine–responsive ROIs. We observed many glomerular ROIs that were activated by both 10 µM corticosterone-21-sulfate (Q1570) and 1:100 intact adult BALB/c female urine, consistent with previous results (Supplementary Fig. 4). We also observed many glomeruli that responded to both 10 µM epiteosterone sulfate (A6940) and 1:100 BALB/c female urine (Supplementary Fig. 4), suggesting that female urine contains odorants that activate the same inputs as certain sulfated androgens. Glucocorticoid overlap was also seen for juvenile male and female and ovariectomized female urine (Supplementary Fig. 4), suggesting these glucocorticoids are present at comparable concentrations in these urine sources. Also apparent in this comparison was the low incidence of overlap between these sulfated steroids and intact adult male urine (for example, Q1570 overlap was 7 ± 1% for female urine and 0 ± 0.1% for male urine, \( P = 6 \times 10^{-4} \), Wilcoxon rank-sum test, \( n = 7 \); Supplementary Fig. 4). Glomerular juxtaposition does not imply similar tuning

To determine whether individual glomeruli are organized by their tuning for molecular features, we first analyzed the glomerular activity patterns to synthetic sulfated steroids using clustering algorithms (Supplementary Fig. 6a–c). We found that glomerular responses to these steroids fell into at least ten classes of reproducible pattern (Fig. 6a–c). These classes match previous physiological recordings in VSNs (Supplementary Fig. 5), and nearly every glomerulus (1,071 of 1,078, 99%) could be assigned to one of these classes. Cluster features remained consistent across response thresholds (Supplementary Fig. 5). This suggests that glomerular responses to sulfated steroids reflect these functionally defined peripheral populations.

To compare receptive fields to molecular features of the ligands (Fig. 7a), we calculated 1,666 molecular descriptors for each sulfated steroid in the panel, then computed the average pairwise Euclidean distance between the normalized odorant descriptors sensed by each VSN class. This produced quantitative measurements of receptive field differences between all VSN classes (Fig. 7b). The distinctions among most VSN response classes had a clear corresponding structural basis—compounds with similar molecular features were more likely to activate the same glomerulus—but some classes showed selectivity for steroids differing in subtle aspects poorly captured by these descriptors (for example, the three sulfated pregnanolones).

The absolute glomerular positions for most VSN classes showed no evidence of selective positioning along the three orthogonal axes. The exceptions were a strong posterior bias among class 5 glomeruli (selective for allopregnanolone sulfate) and a bias among class 7 glomeruli (selective for 17β-estradiol disulfate) to lie laterally along the linea alba (Supplementary Fig. 6). Visual inspection of activity maps for similarly tuned VSN classes revealed no evidence of a positive link between physical closeness and receptive field similarity (Fig. 7c). For example, the three pregnanolone-responsive classes had highly similar receptive fields but were not similar in their projection patterns (Fig. 7b,c). At face value, these data indicate that, at fine spatial scales, VSNs sensing highly similar odorants do not necessarily project to physically adjacent locations.

We next sought to determine whether any of these functionally defined classes showed evidence of higher order spatial relationships.
We compared pairwise distances between the 45 combinations of VSN class pairs and identified two tightly juxtaposed pairs. The most tightly juxtaposed pair was classes 4 and 8 ($P = 4 \times 10^{-5}$; Fig. 8a and Supplementary Video 4). The second pair was classes 6 and 10 ($P = 0.026, n = 10$). As was the case for our investigation of spatial similarity among urine-responsive glomeruli (Fig. 4), we observed glomeruli for many VSN class pairs (15 total) that were preferentially dispersed in comparison to shuffle-test expectations ($P < 0.05, n = 10$; Fig. 8b). In all, 17 of the 45 VSN class pairs (38%) showed a symmetrical statistically significant spatial relationship (Fig. 8b). We also observed several asymmetrical significant spatial relationships (for example, classes 1 and 3; Fig. 8b), indicating tight juxtaposition between subsets of glomeruli in certain class pairs.

The juxtaposed glomeruli in the class 4–class 8 pair arose from VSN classes with the most distant receptive fields in our analysis ($P = 1.8 \times 10^{-5}$; Fig. 8c,d). The receptive fields for classes 6 and 10 were also dissimilar, but less strongly so ($P = 0.014$; Fig. 8c,d). Among the most spatially dispersed pairs was class 3–class 5, sensitive to pregnanolone stereoisomers, the most molecularly similar ligands in our stimulus set (Fig. 7a,c). To test whether there was a consistent relationship between glomerular receptive fields and relative spacing, we generated a spring embedding model in which receptive field similarity was used as the spring constant and spatial similarity was used for all VSN class receptive fields. (d) Isomap analysis of physical spacing with receptive field similarity. The closeness of each colored point to others indicates the tendency for that pair to be spatially juxtaposed. The darkness of the shaded connecting lines indicates the similarity of receptive fields, with black representing the most similar and white the most dissimilar. The unclustered group is omitted. The $P$ value reflects the probability that the observed relationships indicate preferential juxtaposition of similarly tuned inputs (spring embedding model, 100,000 simulations). A, anterior; L, lateral. Scale bars, 100 µm.

**DISCUSSION**

**A distributed glomerular code for sex and maturity**

Many individual VSNs and AOB mitral cells are selective for the sex, strain and species of urine sources. Many fewer ROIs responded to intact adult male urine, but most (70%) of these ROIs were exclusively activated by it. As such, the activity generated by the AOS in response to mature male urine appears to be more directly indicative of a sexually intact BALB/c male. This also indicates that sexually mature males, but not juvenile or gonadectomized males, cease producing urinary odors common to sexually mature female mice. This implies that changes in metabolic pathways in intact males result in halted production, metabolic shunting or accelerated degradation of many AOS odors.

Our combinatorial approach to studying AOB glomerular maps also revealed that juvenile female and male urine were the most potent sources of aAOB activators in our study, activating nearly twice as many glomerular ROIs than even intact BALB/c female urine. Nearly one-third
of these ROIs were exclusively activated by both juvenile male and female urine. Discovery of this population suggests that a large number of inputs to the aAOB possess the capacity to discriminate sexually immature animals at weaning age (postnatal day 21) from sexually mature adults. These inputs are prime candidates for guiding behaviors toward these young animals.

**Juxtaposed glomeruli have correlated urine tuning**

The clearest indication so far that the AOB glomerular layer possesses a systematic organization at fine scales came from anatomical studies labeling two or more members of the same clade\(^22\). In these studies, VSNs types in the same clade were found to closely appose their glomeruli\(^22\). Because clade definitions were based on amino acid homology for the entire receptor, this result suggested a modular glomerular organization that juxtaposes inputs with similar receptive properties. We measured the pairwise distances between glomeruli with different receptive fields for sexually mature and immature urines and found strong evidence for preferential juxtaposition among two of these glomerular populations. We also found evidence for strong preferential dispersal among other glomerular populations. These findings suggest that relative spacing between glomerular populations may be a prominent ‘axis’ on which biologically relevant spatial relationships might be built in the AOB.

The preferentially juxtaposed glomerular groups all responded to juvenile urine of both sexes but differed in their sensitivities to various adult urines. Each of these juxtaposed pairs differed only by sensitivity to just one of these stimuli, whereas each of the preferentially dispersed glomeruli differed by more than one. At face value, this might seem to suggest that preferential glomerular juxtaposition is associated with similar organizational status, which would constitute a form of chemotopy. However, the urinary odorants activating juxtaposed glomeruli may or may not be molecularly similar. To address this question, we performed similar experiments using a defined panel of sulfated steroid odorants known to activate distinguishable VSN populations\(^27,31\).

**Sulfated steroids activate aAOB and pAOB glomeruli**

At the macroscopic scale, the AOB has a tripartite organization determined by innervation of VSNs expressing receptors from different families\(^31\). However, how these broad regions correspond to receptivity to defined ligands has scarcely been explored. We chose the 11 synthetic sulfated steroids to match previously identified functional groups of similarly tuned VSNs\(^27,31\). The breadth of our stimuli allowed us to compare glomerular activation patterns to chemically similar and distinct odorants. AOB glomerular responses to sulfated steroids showed concentration dependence, with sensitivity to some sulfated steroids at 1 \(\mu\)M. AOB responses to steroids at 1 to 10 \(\mu\)M showed increased peak fluorescence within glomeruli but few changes in the macroscopic activity pattern. This was in stark contrast to the responses at 100 \(\mu\)M, which dramatically changed both the intensity and number of activated glomeruli. At 100 \(\mu\)M, activated glomeruli retained some stimulus specificity, in accord with previous reports\(^25,33\) and indicating that, even at 100 \(\mu\)M, sulfated steroids do not gate a nonspecific conductance in VSNs.

The physical location of steroid-responsive glomeruli was dispersed across the aAOB. This is consistent with previous observations that sulfated steroids activate apical\(^31\) and V1R-expressing\(^33\) VSNs. The small (6%) but consistent population of steroid-responsive glomeruli in the pAOB was dominated by glomeruli responding to certain sulfated pregnanolones. This posterior activity was strongly concentration dependent but was present in some cases even at 1 \(\mu\)M, suggesting either that a displaced V1R-expressing population projects to the pAOB or that some VSNs expressing V2Rs are sensitive to sulfated pregnanolones.

The glomerular activity patterns across the panel of 11 synthetic sulfated steroids matched those previously observed in VSNs using multielectrode array recordings\(^31\) and population calcium imaging\(^31\). This suggests that our experimental setup was capable of accurately producing topographical maps in the AOB downstream of functionally defined sets of VSNs. Some glomerular ROIs assigned to functional classes possessed lower intensity activity patterns that resembled those of other classes (see Fig. 6b). These particular ROIs may encompass parts of two anatomical glomeruli.

**Glomerular proximity is not based on odorant similarity**

In chemical senses, regional specificities are apparent, but scarce evidence of systematic positional relationships between these regions has been found\(^18\). However, this question has never been considered for the AOB, which is activated by a narrower range of stimuli. We explored the hypothesis that systematically juxtaposed glomeruli have similar receptive fields. Similarly tuned glomeruli were not always found in tightly packed groups. This was especially evident for classes 3 and 5, responsive to pregnanolone stereoisomers, which showed preferential dispersal rather than juxtaposition. Additionally, the most preferentially juxtaposed pair, classes 4 and 8, responded to the most disparate ligands in the study (sulfated pregnanolone and estrogen-selective inputs, respectively). In combination with evidence that receptors in the same clade, at least in some circumstances, target neighboring glomeruli\(^22\), one possible interpretation of this result is that receptor amino acid sequence is more directly related to axonal targeting than to ligand binding. It is worth noting that our functional clustering scheme may lump multiple, similarly tuned VSN types together. As such, it may be the case that some ROIs correspond to a clump of indistinguishably tuned glomeruli. Our results do not suggest that tight glomerular apposition cannot exist between similarly tuned VSN types, but rather that strong, reproducible juxtaposition does not require similar odorant tuning.

These observations comprise, to our knowledge, the first clear examples of functionally defined AOB glomerular modules and demonstrate that the individual glomeruli in these modules can be tuned to odorants with very different molecular features. The apparent discrepancy between juxtaposition and receptive field similarity when evaluating urine-stimulated maps and steroid-stimulated maps may indicate that the organizational principle in the AOB is based on phenotypic, and not molecular, similarity between odorants. This would perhaps indicate that only biologically significant odorant combinations are selectively juxtaposed in the AOB. The computational benefits of juxtaposing these particular glomeruli remain to be elucidated. Are these adjacent signals integrated by downstream mitral cells or specifically inhibited by local interneurons? Does physically juxtaposing these inputs enable the AOB to identify specific hormonal or behavioral states? Answering these questions will certainly improve our understanding of the logic of this important social and reproductive sensory pathway.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*
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AUTHOR CONTRIBUTIONS

J.P.M. and D.T. developed the AOB imaging infrastructure, J.P.M. and G.F.H. conducted the experiments, and J.P.M., G.F.H. and T.E.H. analyzed results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Driver mice with an OMP-ires-tTA (CS7B/6 background) transgene were mated with transgenic effector mice harboring a tetracycline transactivator response element and minimal promoter 5’ of the coding region for the Ca2+ protein GaCaMP2 (ref. 26). Ten adult (>60 day postnatal) male mice were used as experimental animals, with one AOB imaged per animal. Mice were housed in single-sex cages of no more than five animals from weaning until experimental use with ad libitum access to food and water. Animals were housed on a standard 12 h–12 h light/dark cycle. All BALB/c mice used for urine collection, including surgically altered mice, were purchased from The Jackson Laboratory. All animal procedures were approved by the Washington University Animal Studies Committee.

Ex vivo preparations. Ex vivo dissections were performed as described previously23,42. Briefly, mice were deeply anesthetized with isoflurane and rapidly decapitated into ice-cold, oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM) NaCl 125, KCl 2.5, CaCl2 2, MgCl2 10, NaHCO3 25, Na2HPO4 1.25, myo-inositol 3, sodium pyruvate 2, sodium ascorbate 0.4, glucose 25. A single hemisphere (typically the right) of the dorsal mouse snout and head, up to and including the olfactory bulb and accessory olfactory bulb, was dissected away from the head, glued with Veobond (3M, St. Paul, MN, USA) to a small plastic plank and then adhered with silicone vacuum grease (Dow Corning, Midland, MI) to a dissection chamber. In the dissection chamber, the preparation was superfused rapidly with room temperature (22–25 °C) aCSF. The vomeronasal nerves were exposed to the superfusate via a secondary microdissection to remove the septal cartilage. The preparation was moved to a customized tissue imaging chamber, and a 0.0056-inch inner diameter polyimide cannula was carefully placed inside the vomeronasal organ (VNO) lumen. We delivered a steady stream (0.2 ml/min) of Ringer’s saline, into which all stimuli were diluted, to the VNO. At this flow rate, the VNO lumen (approximately 2 µl in volume) was replaced approximately every 0.5–1 s. Ringer’s saline contained (in mM) NaCl 115, KCl 5, CaCl2 2, MgCl2 2, NaHCO3 25, HEPES 10, glucose 10.

Stimuli. On the day of the experiment, VNO stimuli were dissolved to their final concentrations (100 nM–100 µM) in Ringer’s solution. Sulfated steroids were purchased from Steraloids, Inc. (Newport, RI, USA) and stock solutions (20–100 µM) produced by dissolving steroids into methanol or into filtered distilled water. The sulfated steroids applied are listed in Supplementary Table 1.

Although VNO recordings have not demonstrated sensitivity of vomeronasal sensory neurons to methanol at 2000–10,000-fold dilutions, control Ringer’s stimuli (to which other responses were compared) always contained the maximum methanol concentration applied across all stimuli. BALB/c urine was collected using methods described previously31. For intact adult male and female urine, single-sex cages of intact adult (>P60) BALB/c male or female mice were suspended in wire-mesh bottom cages above liquid nitrogen. Frozen urine from several cages was pooled across several days’ collections, then centrifuged and filtered to remove particulates. Pooling female mouse urine likely prevented compound responses as compared to Ringer’s treated urine.

To prepare the treated samples, we incubated for 2 h at 37 °C. In order to prepare the treated samples for physiology and mass spectroscopy, we ran the reactions over a Phenomenex Torus120 C8 column (Sigma-Aldrich) was added to the first dilution. Both sulfatase and control reactions were incubated for 2 h at 37 °C. In order to prepare the treated samples for physiology and mass spectroscopy, we ran the reactions over a Phenomenex Torus120 C8 column. Columns were washed with one volume methanol and one volume water before the buffered reactions were applied. After applying the reactions to the column, the resin was washed with one volume water/methanol/acetic acid mixture at a 78:20:2 ratio. One column volume of methanol was then used to extract soluble compounds from the resin, and these were dried under nitrogen gas. Samples were subsequently dissolved in 1:2,000 methanol to their original urine volume in order to match the methanol volume in sulfated steroid stimuli. Mass spectrometry was used to measure the loss of sulfate precursor ions in the sulfated treated sample compared to the control sample.25. We observed that compounds in the range of m/z 300 to 350 were lost in the sulfatase-treated but not control-treated urine.

AOB GaCaMP2 Ca2+ imaging. Volumetric images of the mouse AOB were acquired from ex vivo preparations inside the custom tissue imaging chamber using an objective-coupled planar illumination (OCPi) microscope43. The microscope consists of a 20x water immersion objective (Olympus, Center Valley, PA, USA) mounted on a piezoelectric objective actuator (Piezosystem Jena, Hopedale, MA, USA). A custom-designed coupler linked an optical fiber, a light collimator and a light sheet–forming cylindrical lens oriented at 90° to the focal plane of the microscope objective. Images were taken using a 1,004 × 1,002 pixel EM-CCD camera (Andor, South Windsor, CT, USA) located after a 100-mm tube lens (Infinity Photo-Optical Company, Boulder, CO, USA). Volumetric images were produced by scanning the light sheet across the tissue. The total scan range along the z axis was 400 µm, and the z step size was typically 8 µm. Voxel dimensions were 0.71 µm × 0.71 µm × 8 µm, and thus the total dimensions of image stacks were 713 µm × 712 µm × 400 µm. The total time to acquire and write each image stack to disk was 5 s.

Before image acquisition, the superfusing aCSF was warmed to 33–35 °C. Eleven sulfated steroids (1–100 µM), 100-fold diluted BALB/c male and female urine and control Ringer’s stimuli were delivered to the VNO through the polyimide cannula using a pressurized, computer-controlled stimulus delivery system (AutoMate Scientific, Berkeley, CA, USA) or an UltiMate 3000 analytical autosampler ( Dionex, Sunnyvale, CA). Each stimulus lasted approximately five image stacks (25 s) and was followed by a recovery period of approximately ten image stacks (50 s). All stimuli were presented in 3–5 randomized, interleaved blocks. In a small subset of experiments, we acquired images from single frames at 10 Hz, stimulating the VNO with P8200 or Q1570 for 10 s with a recovery period of 50 s.

Ca2+ imaging analysis. Volumetric movies (each ~100 GB in raw form) were analyzed in MATLAB (MathWorks, Natick, MA, USA) using custom software described previously31. Each image stack was registered to a reference stack acquired between stimulus presentations near the middle of the experiment. Image registration typically reduced the mean squared difference between the voxels in the stack by ~100-fold. To measure resting-state fluorescence from GaCaMP2 signals, we averaged the fluorescence intensity (F) at each voxel in 3–5 consecutive stacks before stimulus onset (Frest). To measure stimulated activity, we averaged the fluorescence during the 3–5 stacks of stimulus presentation to the vomeronasal organ (Fstim).

From these, we computed the relative change in fluorescence intensity:

\[
\frac{\Delta F}{F} = \frac{F_{\text{stim}} - F_{\text{rest}}}{F_{\text{rest}}}
\]

To measure the reliability of responses across repeated stimulus presentations, we also computed a response reliability index (RRI):

\[
\text{RRI} = \frac{\text{mean}(\Delta F/F)}{\text{stdev}(\Delta F/F)}
\]

where stdev represents s.d. of the mean across all stimulus repetitions. This produced a five-dimensional (n×n×n×2 images × 2 metrics) activity matrix. We thresholded 3D activity matrices, then subjected 4D maximum projections across stimuli to image flow, wherein each voxel was associated with a nearby peak in ∆F/F by traversing an ‘uphill’ path of its nearest neighbor voxels. This process produced volumetric ROIs. Some adjacent ROIs shared identical response patterns and no obvious physical discontinuities (that is, a ‘split’ ROI). We manually merged such ROIs using a customized graphical user interface in MATLAB.

For each volumetric ROI, a scalar response value was produced by weighting the activity of each voxel by its dot product with the mean response, then summing all activity within the ROI. Thus, the Ca2+ activity for experiment was condensed to a matrix of dimension n stacks × n ROIs. Only ROIs that responded with ∆F/F exceeding the various thresholds and that showed statistically significant responses as compared to Ringer’s controls for at least one stimulus (P < 0.05, Wilcoxon rank sum test) were included in subsequent analyses.
For the concentration-response analysis in Supplementary Figure 2, we calculated a GCaMP2 signal strength, the sum of the ∆F/F signal for all voxels that crossed experiment-specific RRI thresholds. We then normalized these values for each experiment by the maximum value across stimuli and concentrations. Because the maximum value was not always elicited by 100 μM P8200, the average across experiments, even for 100 μM P8200, was less than 100%.

Clustering of glomerular response patterns. From each set of experiments, an n stimuli × n ROIs matrix of ∆F/F values was computed as described above. We used clustering algorithms (based on mean shift clustering) to identify common patterns of activity in the population (Figs. 3f and 6b). We evaluated sulfated steroids cluster results at multiple ROI thresholds (Supplementary Fig. 5). The resulting patterns strongly agreed with patterns identifying functional classes of VSNs from somatic recordings. For steroid clusters (VSN classes), labels were arranged to match a common numeric assignment (classes 1–10 plus an unclustered group; Fig. 6b).

Analysis of ROI positions. Several methods were used to compare the positions of ROIs. To measure absolute glomerulus positions (Fig. 4 and Supplementary Fig. 6), we first identified ROIs on the basis of their responses to one or more stimuli. For each experiment, we generated a set of three orthogonal vectors indicating the anterior–posterior, medial–lateral and depth axes using a planar fit to the surface of the tissue and the visible division between the aAOB and pAOB of ROIs. To measure absolute glomerulus positions, we computed the dot product between the ROI position and the orthogonal vectors. We then normalized the ROI positions along each axis. We simplified each ROI as a sphere (matching the centroid and volume of observed ROIs) and then measured the tendency for ROIs of each class to occupy positions along each axis. We did this by normalizing to the total occupancy across all classes and then comparing these relative occupancies to those expected from 100,000 shuffled glomerular maps. In the shuffle test, ROI positions remained static but cluster identities were scrambled. Shuffle tests were used to indicate the 95th percentile, corresponding to a 95% confidence interval for relative occupancy. Glomerular classes showing preferential occupancy along any axis show relative occupancy exceeding this 95th percentile.

For measurements of relative spacing between glomeruli (Figs. 4e and 8b), pairwise distances $d_{ROI(1,2)}$ were computed as

$$d_{ROI(1,2)} = d_{C(1,2)} - \sqrt{\frac{3}{4\pi}} V_{g(1,2)}$$

where $d_{C(1,2)}$ is the Euclidean distance between ROI centroids and $V_{g(1,2)}$ is the geometric mean of the ROI volumes. In this case, we used a shuffle test ($N = 10,000$) to establish the propensity for randomly chosen elements in the set to produce a given measured distance. The median minimum pairwise distance from each ROI in cluster or class 1 was calculated for members of all other clusters or classes, 2–10 (first columns of Figs. 4e and 8b). This value was then compared to mean values taken from 10,000 sample sets with the same number of ROIs per class but with class identities shuffled. We calculated the likelihood of encountering each value by computing a physical spacing index (PSI):

$$PSI = \frac{\text{mean}_{\text{observed}} - \text{mean}_{\text{shuffled}}}{\text{stdev}_{\text{shuffled}}}$$

The PSI thus resembles the statistical z-score. PSI values less than 0 indicate values closer together than expected in shuffled sets, and vice versa.

Chemical space and receptive field analysis. For each sulfated steroid, the chemical structure was entered into online DRAGON software, which produced a set of 1,666 of molecular descriptors. Of these descriptors, 1,319 varied across the 11 stimuli. We normalized the values using the formula

$$n_{i,j} = \frac{x_{i,j} - \text{min}(x_j)}{\text{max}(x_j) - \text{min}(x_j)}$$

where $x_{i,j}$ represents the raw value of the jth molecule for the ith descriptor and $n_{i,j}$ is the normalized value. Thus, the range of normalized values for each descriptor ranged from 0 (the minimum observed value for a particular descriptor) to 1 (the maximum observed value for a particular descriptor). We then computed the chemical dissimilarity for each VSN class receptive field pair by calculating the mean Euclidean distances between all components of each class pair. For example, the dissimilarity between class 1 (responsive to Q1570 and Q3910) and class 4 (responsive to P3817, P3865 and P8200) was the average Euclidean distance between the 1,319-dimensional representations of all steroid comparions. A pairwise dissimilarity matrix was made and a 2D visualization of this matrix calculated using nonclassical multidimensional scaling (MATLAB function “mdsfile”, Fig. 7a). This analysis summarizes the differences across all 1,319 chemical descriptors without weighting any particular descriptor as being more or less important for its binding properties. Because the features that relate structure to receptor binding are not clear, this remains one of the best objective ways to compare odorant structures.

We calculated a relative chemical space index (Fig. 8c) by comparing pairwise chemical dissimilarity values to 10,000 iterations of a shuffle test. In each instance of the shuffle test, the identity of the steroids giving rise to each receptive field was shuffled (for example, for class 4, each of the three steroids giving rise to the receptive field was randomly resampled, without replacement within the panel of 11 steroids). This process produced values assessing the relative spacing of these receptive fields, with closeness indicated by values < 0 and farness by values > 0. Note that this comparison is specific to the molecules and activity patterns found with this stimulus set.

Isomap and spring embedding analysis. We evaluated the tendency for glomerular juxtaposition and dispersal to be correlated (or anticorrelated) with receptive field similarity using a spring embedding model (Fig. 8d). In this model, normalized estimates of receptive field similarity $R_k$ were used as the spring constant $k_E = R_k^2$. The relative physical spacing between glomeruli was measured using two-dimensional isomap analysis ("Isomap.m" in MATLAB dimensionality reduction toolbox). To produce the 2D isomap, we identified the six nearest-neighbors for each ROI and measured the probability of members of each class being among these neighbors. This produced a matrix of relative juxtapositions $J$ between classes that was symmetrized and normalized ($\hat{h}_{norm}$), and then evaluated by isomap analysis. The isomap $\tau$ term was set to the median value in $\hat{h}_{norm}$. The spring embedding model was thus

$$E = \sum_{i,j} k_{E(i,j)} D_{ij}^2$$

where $E$ is the model energy, $k_{E(i,j)}$ is the receptive field difference between classes $i$ and $j$, and $D_{ij}$ is the pairwise Euclidean distance between the 2D isomap positions of classes $i$ and $j$. The observed values for $E$ were compared to 100,000 shuffle test computations of $E$ ($E_{sim}$) in which the $D_{ij}$ terms remained constant while $k_{E(i,j)}$ were scrambled. Values of $E$ significantly lower than $E_{sim}$ (that is, less than the 5th percentile of the shuffle test) would indicate that similarly tuned glomeruli are preferentially juxtaposed (that is, chemotopy based on glomerular juxtaposition or dispersal), and values of $E$ higher than $E_{sim}$ would indicate anti-chemotopy. The observed value of $E$ in our data set (7.1) was just slightly higher than $E_{sim}$ (6.8 ± 0.8).

A Supplementary Methods Checklist is available.