Neuroscience

Representation of male features in the female mouse Accessory Olfactory Bulb, and their stability during the estrus cycle

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eLife Assessment

In this detailed study, Cohen and Ben-Shaul characterized Accessory Olfactory Bulb (AOB) cell responses to various conspecific urine samples in female mice across the estrous cycle. The authors found that AOB cell responses varied depending on the strain and sex of the sample, but no clear differences were observed between estrous and non-estrous females. These findings provide **convincing** evidence that the AOB functions as a stable sensory relay, without directly modulating responses based on reproductive state, which supports the role of downstream brain regions in integrating reproductive state. Overall, this study provides **valuable** insights for researchers in the fields of olfaction and social neuroscience.

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Abstract

Most behaviors result from integration of external and internal inputs. For example, social behavior requires information about conspecifics and internal physiological states. Like many other mammals, female mice undergo a reproductive cycle during which their physiology and behavioral responses to males change dramatically: during estrus, they are more receptive to male mating attempts. A critical element in reproductive behavior is the investigative stage, which in mice, and many other species, strongly relies on chemosensation. While the initial approach mostly involves the main olfactory system (MOS), once physical contact is established, the vomeronasal system (VNS) is engaged to provide information about potential partners' characteristics. Given the estrus-stage dependent behavioral response, we asked whether representations of male features in the first brain relay of the VNS, namely, the accessory olfactory bulb (AOB), change during the cycle. To this end, we used a stimulus set comprising urine samples from males from different strains and virility levels, and from estrus and non-estrus females. The stimulus set was designed to reveal if response patterns of AOB neurons conform to ethologically relevant dimensions such as sex, strain, and particularly, male virility state. Using extracellular recordings in anesthetized female mice, we find that most ethological categories contained in our data set

are not over-represented by AOB neurons, suggesting that early stages of VNS processing encode conspecific information efficiently. Then, comparing neuronal activity in estrus and non-estrus females, we found that overall, response characteristics at the single neuron and population levels remain stable during the reproductive cycle. The few changes that do occur, are not consistent with a systematic modulation of responses to male features. Our findings imply that the AOB presents a stable account of conspecific features to more advanced processing stages.

Introduction

To efficiently navigate the environment, social animals must derive information about conspecifics, and evaluate it in light of their own state. One notable example is reproduction, which requires coordination of sexual behavior with internal physiology [1, 2]. In mice, females undergo a 4-to-5-day cycle, characterized by hormonal and physiological changes that effect the reproductive system, and the females' responses to male stimuli [3, 4]. The cycle includes a fertile estrus/proestrus phase accompanied by receptive behavior towards males, and a non-estrus phase, during which males are avoided [5]. Sexual behavior is often divided into appetitive, consummatory and refractory stages [2, 5], and the appetitive stage can be further divided into detection, approach, and investigation.

In many species, chemosensation is a central sensory modality which plays a key role in social interactions, and is critical during the appetitive stage. In rodents and most vertebrates, information about conspecifics is obtained via two major chemosensory systems. The main olfactory system (MOS) plays many roles, and among other functions is required for social behaviors [6, 7]. The vomeronasal system (VNS) in contrast, appears dedicated to cues from other organisms, and conspecifics in particular [8]. An important functional distinction between the systems involves stimulus uptake. The MOS detects volatile cues, and can thus sense distant objects. During social interactions, it is likely required to decide whether to approach or withdraw. In contrast, the VNS requires physical contact for efficient stimulus uptake [8–10]. Notably, once contact is made, the VNS can provide further information that is required for more fateful behavioral decisions. Thus, the VNS is particularly important during the investigative part of the appetitive stage [11–13].

The first brain stage of the VNS is the accessory olfactory bulb (AOB), and its principal neurons are known as mitral-tufted cells (AOB-MCs). AOB-MCs represent a key node, as they receive information from vomeronasal sensory neurons (VSNs) and project to downstream limbic stages [8]. Previous studies have shown that AOB-MCs respond to various bodily secretions including feces [14], saliva [15], vaginal secretions [15], and most prominently, urine [16, 17]. These studies also revealed that AOB neurons show differential responses to urine from males versus females [16–18], from mice of different strains [16, 18], and from females in different physiological states [15]. Thus, AOB provides critical information to guide social decisions, such as required during reproductive behavior.

Previous studies have shown that female mice prefer male over female stimuli [19–21], dominant over non-dominant males [22], and intact vs. castrated males [23]. Here, to reveal how sensory representations about mating partners is represented in the AOB, we applied a stimulus set that includes urine from castrated, sexually naïve, and proven-breeder dominant males (**Fig. 1**). These stimuli correspond, respectively, to males that *cannot* breed, *may* be able to breed, and males that have reproduced successfully. Our stimulus set therefore represents varying levels of virility, allowing us to reveal if AOB-MCs represent male reproductive status.

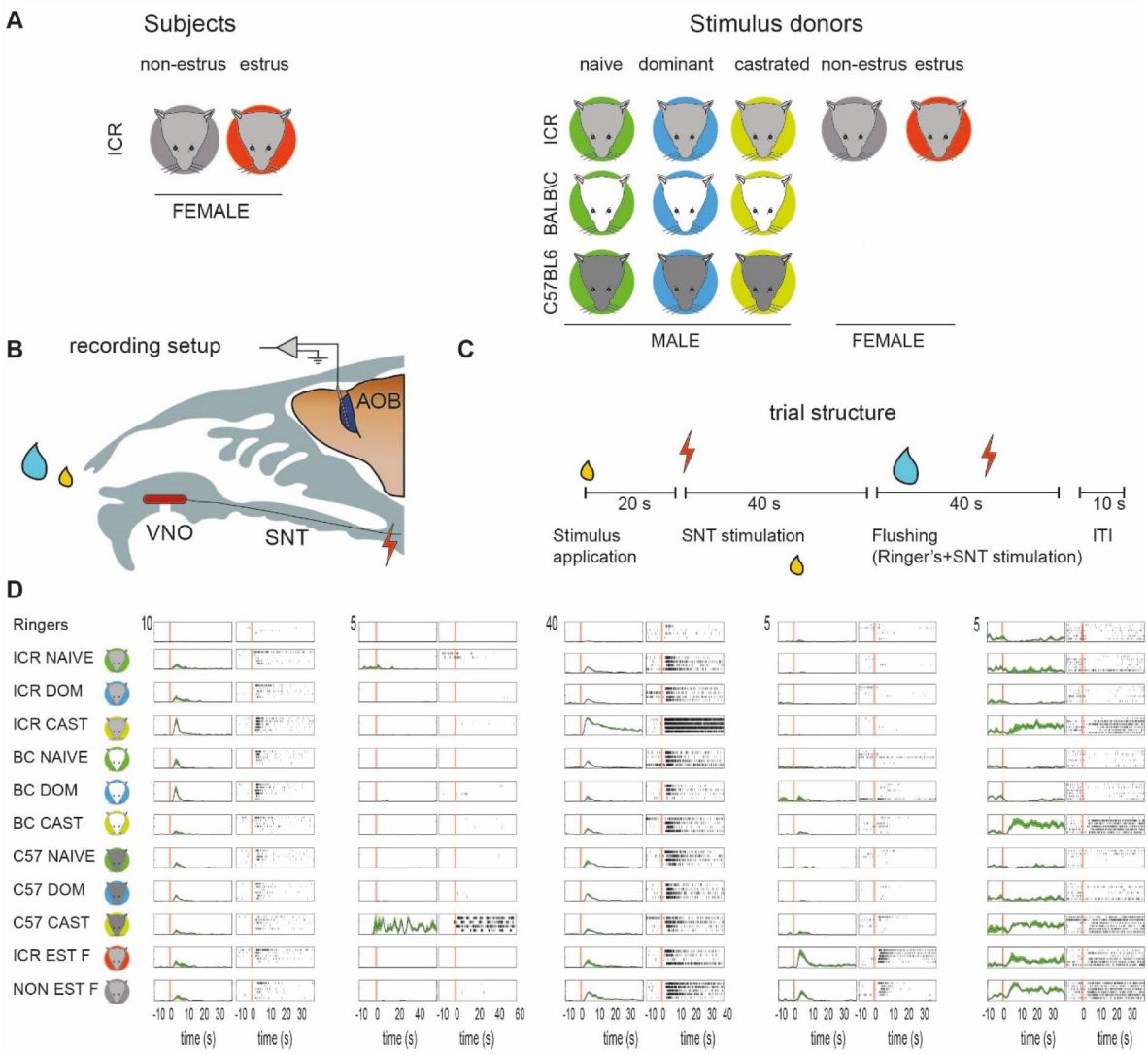

Figure 1.

Experimental design and examples of single unit recordings.

A. Icons describing the different subject and stimulus donors. In these icons, mouse colors denote the strain, while the background circles denote the status (estrus, non-estrus for females, naïve, dominant, castrated for males). **B.** Schematic of the recording setup. A multisite electrode probe is advanced to the external cell layer of the AOB. Stimuli (yellow drop) are applied to the nostril and, after a delay, the sympathetic nerve trunk (SNT) is stimulated. Between stimulus applications, the nasal cavity and VNO are flushed with Ringer's solution. VNO: Vomeronasal organ. **C.** Timeline of an individual trial. After a 20 second delay, the SNT is activated to induce VNO activation. Following another 40 seconds, the flushing procedure is initiated. An inter-trial interval (ITI) of 10 seconds is applied between consecutive stimulus applications. During the experiment, stimuli are delivered repeatedly in blocks. Within each block, each of the individual stimuli are presented in a random order. **D.** Examples of single unit responses to all stimuli. For each unit, responses to each of 11 stimuli are shown. Left panels show the mean firing rate (PSTH, peri-stimulus time histogram), while the right panels shown raster displays for each of the individual trials. The red vertical line indicates stimulus application. For unit 2, time 0 indicate stimulus application, as in this case, responses began after stimulus application, prior to stimulation.

Notably, observation of differential neuronal responses as a function of some property, is often interpreted as "tuning" to it. However, this is unwarranted unless selectivity is demonstrated across a diverse stimulus set, representing multiple sources of variability [24]. For example, to demonstrate that a neuron is tuned say, to 'dominant males', selectivity should be shown across a wide range of stimuli, including different instances of dominant male samples. Since the range of possible stimuli is unlimited, this goal can never be fully attained. Nevertheless, any expansion of the stimulus set allows a better characterization of response properties. Guided by these considerations, for each male virility state, we applied male urine stimuli from three distinct strains. This not only reduces the risk for misleading strain-specific conclusions, but also allows testing if AOB neurons are tuned to specific ethologically relevant features embedded in our stimulus set. Response properties of AOB MCs are particularly intriguing given their dendritic fields that can sample multiple glomeruli [25, 26]. This organization that can give rise to response profiles corresponding to combinatorial molecular patterns, and hence to ethologically relevant features.

As mentioned above, reproductive behavior requires evaluation of information about potential mating partners in light of the subject's own physiological states. Some studies [27, 28] (but see also [29, 30]) indicate that not only the probability of mating, but even the initial preference towards male stimuli is modulated by the estrus stage. Thus, in the context of female sexual behavior, it is expected that at some stage of processing, neuronal representations of male stimuli would depend on reproductive state. Consistent with this idea, it was shown that neuronal activity and/or connectivity in hypothalamic brain regions change during the female mouse estrus cycle [30–38]. Furthermore, response properties of vomeronasal sensory neurons (VSNs) are also altered during the estrus cycle [39–42]. However, it was not known if and how AOB sensory representations are modulated during the reproductive cycle. To this end, we recorded neuronal activity from naturally cycling female mice and examined how response properties of AOB-MCs change during the cycle. We hypothesized that responses to "virile stimuli" will be somehow amplified during estrus, reflecting heightened sensitivity, or perhaps increased discriminatory ability.

Thus, this study addresses two interrelated goals. First, to reveal how male reproductive status is reflected by AOB neuronal activity. Second, to determine if representation of the males' reproductive state is altered during the course of the estrus cycle.

Results

We recorded stimulus induced responses from the AOB of estrus and non-estrus anesthetized adult ICR female mice (**Fig. 1A, B**). Our stimulus presentation protocol includes sample application to the nostril, followed by electric stimulation of the sympathetic nerve trunk (**Fig. 1C**). During each session, we repeatedly present one of several stimuli in a pseudorandom order, with each presented at least four times. The VNO and nasal cavity were washed with Ringer's solution between consecutive stimulus applications. The stimulus set includes urine from sexually naïve, castrated, and sexually experienced dominant male mice, from each of three strains: BALB/C (BC), C57BL6 (C57), or ICR (**Fig. 1A**). These stimuli span two independent dimensions: male reproductive state, and strain. For brevity, we designate the sexually-experienced dominant mice as dominant. In addition, we presented urine from estrus and non-estrus ICR females (**Fig 1A**). Response magnitudes were quantified as the baseline-subtracted average firing rate, within a temporal window starting at stimulus application, and ending 40 seconds after electrical stimulation of the sympathetic nerve trunk. Examples of single unit responses are shown in **Fig. 1D**. Our dataset includes 241 single units, from 21 recording sites, from 17 estrus females, and 305 single units, from 28 recording sites, from 20 non-estrus females.

Population level representations and general response features

Responses from all recording sessions (for both estrus and non-estrus females, n = 546 single units) to each of the stimuli are shown in **Fig. 2A**. Responses are shown as a normalized response matrix, with non-significant responses set to 0. As a population, AOB neurons are responsive to each of the stimuli that were presented. To compare population activity patterns across stimuli, we applied the correlation distance metric and classical multi-dimensional scaling. This representation shows that the two female stimuli elicit similar patterns, distinct from all male stimuli (**Fig. 2B**). As for intact male stimuli, the strongest determinant of response similarity appears to be strain rather than physiological status: naïve and dominant C57 stimuli form one cluster, while another contains naïve and dominant BC and ICR males. Castrated urine response patterns are distinct from male or female induced responses, but here too, ICR and BC castrated urine are more similar to each other than to C57 urine.

The distribution of the number of stimuli that elicit significant responses per neuron is shown in **Fig. 2C**. The most common response profile is the most selective (responding to only one stimulus), although a significant fraction shows minimal selectivity (responding to all stimuli). The number of neurons with significant rate increases and decreases to each of the stimuli is shown in **Fig. 2D** . Consistent with previous studies in the AOB, rate increases are far more frequent than rate decreases [43, 44]. Also consistent with other studies, female stimuli activate more neurons than male stimuli [17, 44, 45]. Somewhat surprisingly, yet also consistent with previous work [43], castrated stimuli are at least as effective as intact male stimuli.

Mean response strengths for each of the stimuli are shown in **Fig. 2E**. This value effectively combines the number of responsive neurons with their response magnitudes, and provides an estimate of the summed activity across the AOB elicited by each stimulus. This representation shows that within each strain, female and castrated male stimuli elicit the strongest activity.

Defining response patterns of AOB neurons

Here next examine the frequency of response patterns of AOB neurons. Using stimuli from three strains and three male reproductive states, we can define patterns corresponding to strain, and to male virility status. While there are only two female stimuli (estrus and non-estrus ICR urine), they are useful for defining response patterns corresponding to sex or strain. Response patterns are defined by response strength (**Fig. 3A**). For illustration, a *dominant* pattern (DOM in **Fig. 3**) means that responses to all of the *dominant* male stimuli are stronger than to each of the other stimuli. The complementary response pattern (~DOM) is equally informative. Intuitively, by setting a threshold, neurons with these particular response patterns suffice to determine if the stimulus source is a dominant male. Note that any determination of the stimulus source is only valid *given* that the stimulus is a member in our stimulus set. We also stress that we use this approach to define neuronal receptive fields, and are not implying that decoding is actually achieved in this manner.

We also considered another type of response pattern (**Fig. 3B**), which we designate as “strain adjusted” or “virility state adjusted” patterns. The rationale is that some chemical feature may be consistently modulated by reproductive state, although its baseline will also depend on the strain. Alternatively, a feature could be consistently modulated by strain, with its baseline determined by reproductive state. For example, a *dominant strain adjusted* pattern implies that for each strain, the response to the dominant stimulus is larger than the responses to the other states. Here too, complementary patterns are relevant as well. Adjusted patterns are more permissive than basic patterns. For example, a neuron that satisfies a dominant male pattern necessarily complies with a strain adjusted dominant pattern as well.

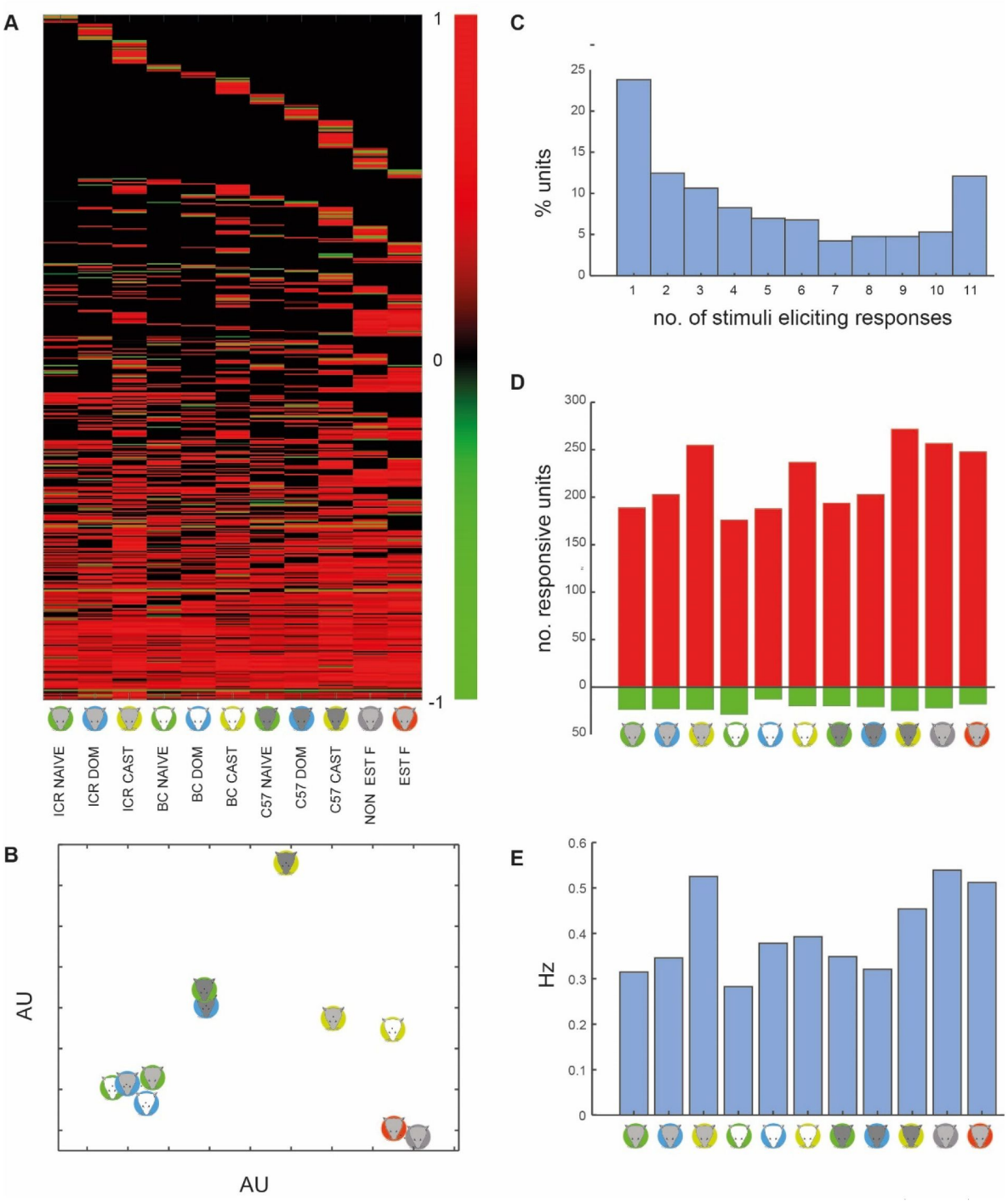

Figure 2.

Basic response characteristics.

A. Colormap of responses. Each neuron (n=546) is represented by one row, and each stimulus by one column, as indicated at the bottom. Responses are normalized between −1 and 1. Non-significant responses are assigned a value of 0. **B.** Classical multidimensional scaling of population level responses. The mean error due to the reduced 2-D representation is 0.29 (arbitrary units). **C.** Distribution of number of significant responses across all neurons in the dataset. **D.** Number of neurons responding to each of the stimuli, separately for rate increases (upper bars, red), and decreases (lower bars, green). **E.** Mean response magnitude, across all recorded neurons to each of the stimuli.

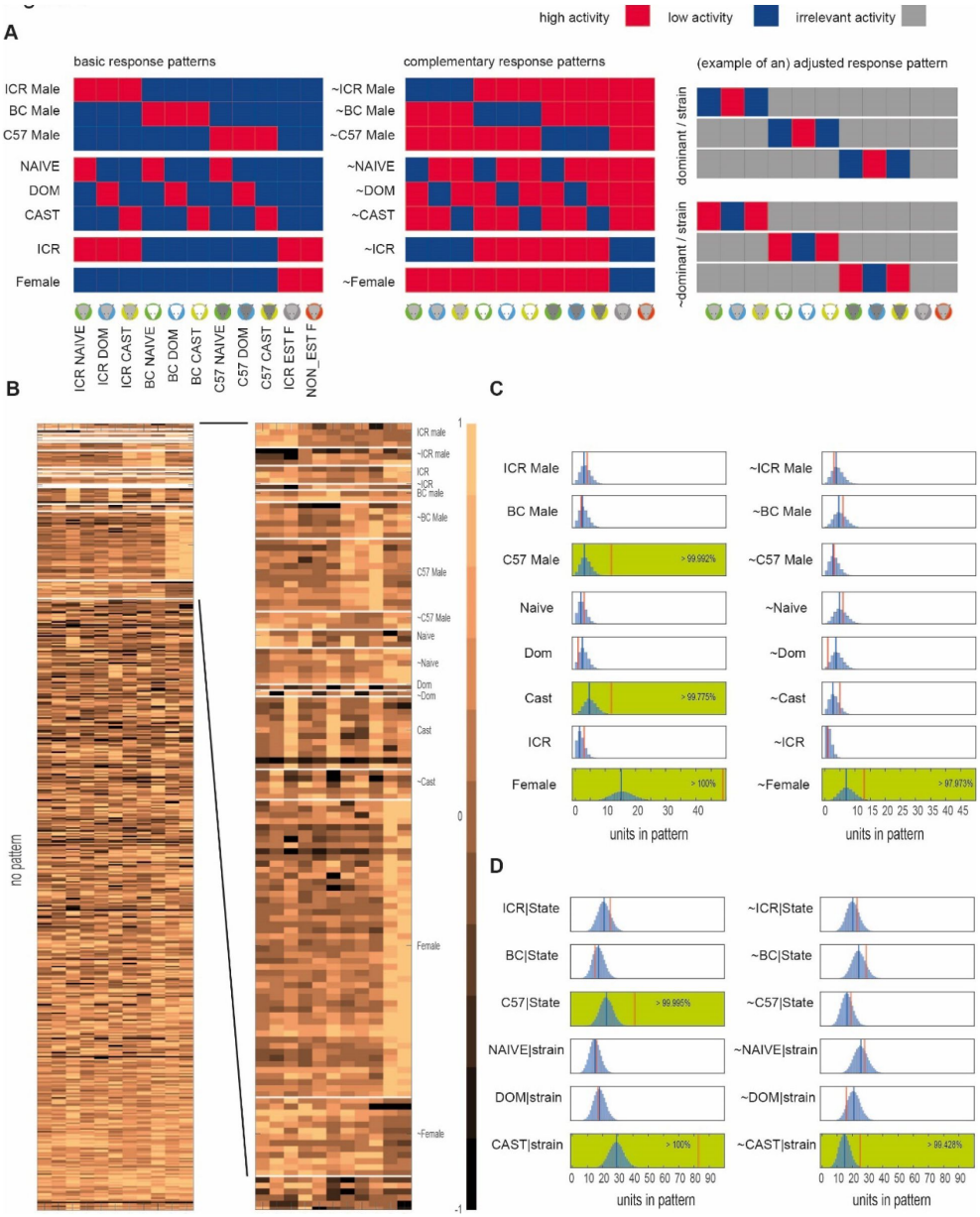

Figure 3.

Analysis of response patterns.

A. Graphical representation of response pattern definitions. In these representations, each of the stimuli indicated in red should elicit a stronger response than each of the stimuli in blue. The left and center panels show the basic response patterns and their complementary patterns, respectively. The panels on the right provide two examples of adjusted response patterns. For example, a dominant/strain pattern requires fulfilment of three conditions, so that within each strain, responses will be strongest to the dominant stimulus. Within a given condition, gray squares indicate stimuli that are irrelevant. The complementary adjusted responses patterns are not shown here. **B.** All response patterns across neurons. A neuron may fulfill more than one pattern and thus may appear in more than one row. The right panel is an expanded view of predefined response patterns. The adjusted categories are not shown in this representation. **C.** Frequency analysis of each of the basic and complementary response patterns. The response pattern name is indicated next to each panel. Within each panel, the blue histogram shows the shuffled distribution of pattern frequency (n = 100,000). The vertical blue lines show the mean value of the shuffled distribution. Red vertical lines indicate the actual number of observed patterns. Green panels indicate significant cases (i.e., the estimated probability to obtain the observed number of cases by chance is less than 5%). In most cases, the probability is considerably less (as indicated by the numbers within each panel).

Most response patterns are not over-represented

Responses of single neurons, sorted according to response types (**Fig. 3A**, only the basic and complementary response patterns are shown) are shown in **Fig. 3B** . Clearly, most neurons do not conform to any of the response patterns (78%, 433 of 557 total patterns; the number of patterns exceeds the number of neurons since a neuron may conform to more than one pattern). While some patterns (e.g., *female*) are frequent, others are associated with only a few neurons (e.g., *naïve* and *dominant* patterns). However, to gain insight about the sampling properties of AOB neurons, it is important to know if patterns are represented more than represented by chance. We define chance under the assumption that each neuron's responses to each of the stimuli are independent. Practically, we apply bootstrapping (n = 100,000, see Methods) to derive chance probabilities to observe each of the patterns. Bootstrapped distributions and the number of observed neurons for each of the patterns are shown in **Fig 3B**. Considering basic and reverse patterns, we find four that are overrepresented ($p < 0.05$): ~female, C57 male, and castrated (**Fig. 3C**). F or strain or state adjusted patterns (**Fig. 3D**), we find over presentation of *C57/state*, *cast/strain* and *~cast/Strain*. The frequencies of the observed patterns (and the deviations from the expected values) are affected by the chemical nature of the stimuli and the sampling properties of individual neurons. This idea and its implications are further developed in the Discussion. Presently, we note that most of the male strain specific patterns, and the male-virility-state patterns are not more frequent than expected by chance.

Representational space is broadly stable over the estrus cycle

We begin the comparison of response properties of AOB neurons under estrus and non-estrus states by examining responses at the population level. **Fig. 4A** shows responses from **Fig. 2A** partitioned according to the estrus state. Each pair of stimuli (i.e., each pair of columns in **Fig. 4A**) is associated with a distance, which we quantify using two common metrics: correlation and Euclidean distances. Our 11-stimulus set yields 55 pairwise distances, which together provide a measure of representational space. We then compare these measures under both states, using each of the distance metrics. As shown in **Fig. 4B, C**, using both metrics, there is a positive and highly significant correlation between the two states' representations. This indicates that overall, the structure of representational space remains similar across the estrus cycle. Yet, several points in **Fig. 4B, C** diverge from the diagonal, indicating that representations are not identical under the two states. We next consider various hypotheses concerning these differences.

Response intensity of most stimuli is stable across the estrus cycle

We next ask if the intensity of specific stimuli varies across the cycle, and particularly, whether stimuli associated with virile males elicit stronger responses during estrus. Specifically, we examine response magnitudes, as expressed by the fraction of responsive neurons, and the mean response strength across the population, to each of the stimuli. The fraction of responsive neurons and mean response magnitude in estrus and non-estrus females are shown in **Fig. 5A, B**. After correction for multiple comparisons, the only significant difference is that during estrus, the *fraction* of responsive neurons to castrated BC male urine increases. There are no significant differences in response *magnitude* to any of the stimuli. Relaxing the requirement for significance, and searching for estrus state changes related to virility which appear across all strains, the only consistent patterns are a mild increase in response frequency to naïve males in estrus (icons with a green background in **Fig. 5A**), and a mild decrease in the magnitude of responses to dominant stimuli (icons with a blue background in **Fig. 5B**). Neither of these changes reflects increased responsiveness to more virile stimuli during estrus. Thus, we conclude that there are no consistent changes in response strength across the estrus cycle, and particularly, no increase in response efficacy of stimuli associated with higher virility.

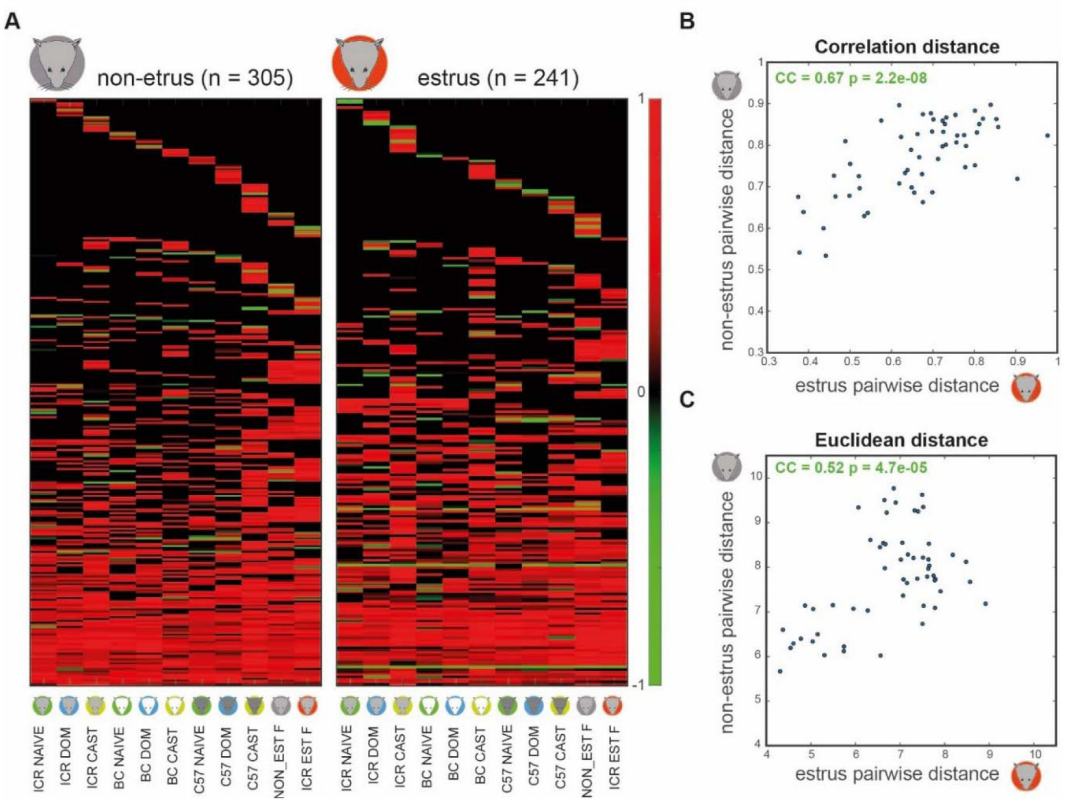

Figure 4.

Comparison of population level representations in estrus and non-estrus females.

A. Responses of individual neurons. Same data as in **Fig. 2A**, divided according to reproductive state. **B.** Correlation of population level pairwise distances across the two reproductive states, using the correlation distance measure. **C.** Same as in B using the Euclidean distance. In both B and C, the correlation coefficient (CC) and the probability to obtain it by chance (p-value) are indicated within each panel.

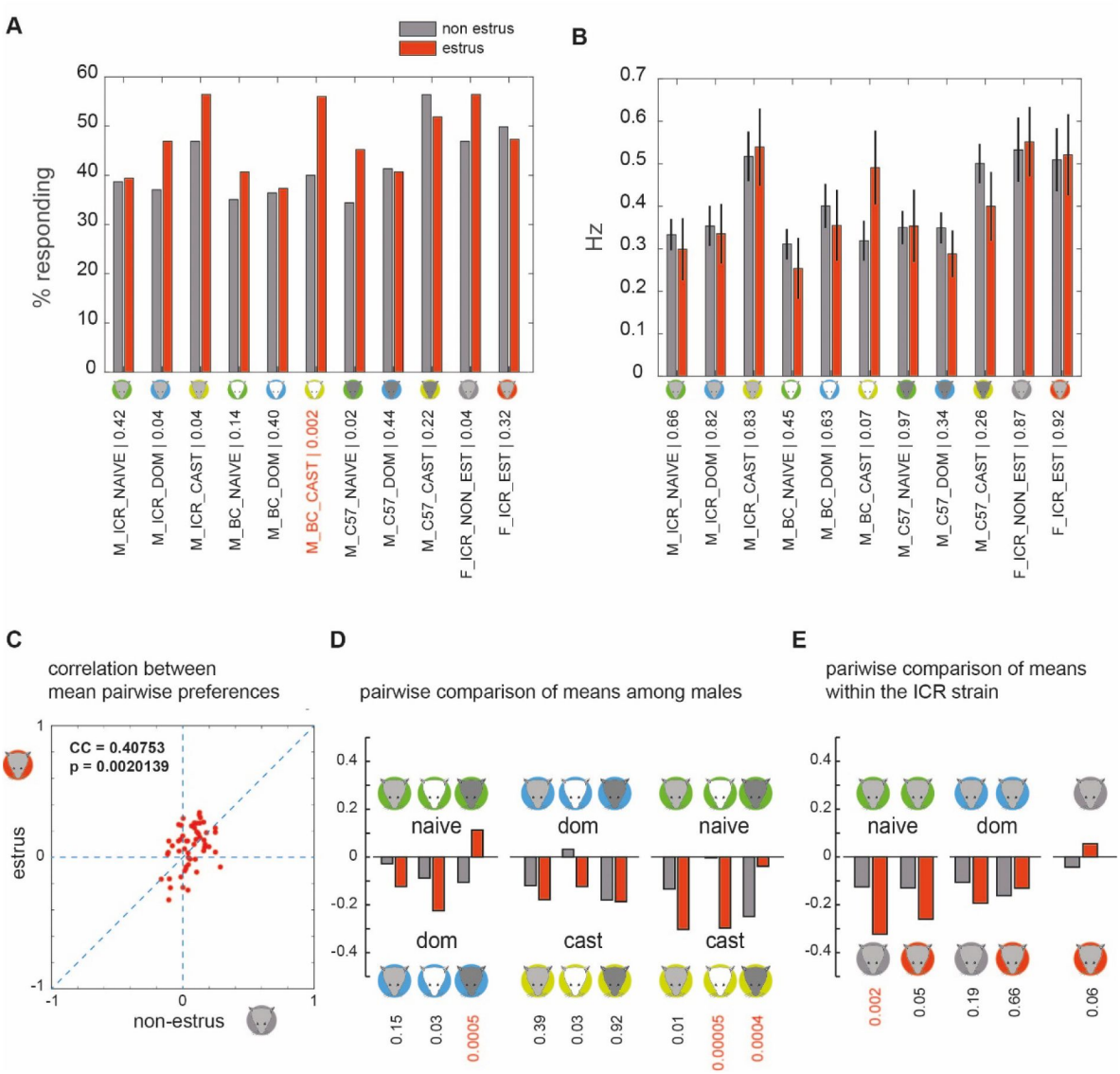

Figure 5.

Comparison of responses in estrus and non-estrus females.

A. Percent responding neurons to each of the stimuli in non-estrus (gray) and estrus (red) neurons. **B.** Mean response magnitude to each stimulus under the two states. In A and B, p-values correspond to the binominal exact test. Bonferroni adjusted p-value given 11 comparisons for the 0.05 level is 0.00454. The one significant difference is indicated by red text. In A and B, n = 305 non-estrus, and n = 241 estrus. **C.** Correlation between mean pairwise preference indices under the two reproductive states. The correlation coefficient and the probability to obtain it by chance are indicated. **D.** Comparison of pairwise preference indices under the two states. Each pair of bars corresponds to one comparison. The stimuli are indicated by the icons and the text. For example, the first pair of bars on the left corresponds to pairwise comparison of dominant and naive ICR male urine. In both estrus states, there is a stronger response to the dominant stimulus as indicated by the downward pointing bars. However, the difference is not significant. **E.** Comparison of pairwise preference indices between stimulus pairs comprising male and female stimuli. In D and E, significance is determined using a shuffling test (n = 100,000). Bonferroni adjusted p-values at the 0.05 level are 0.0056 and 0.01 for D, and E, respectively. Significant differences among the states are indicated in red. Note that in most cases, significant differences correspond to cases where responses during estrus are stronger to the *less* virile male stimulus.

The relative magnitude of response strengths remains stable across the estrus cycle

Although our previous analyses did not reveal changes in absolute response magnitudes, they do not rule out finer changes in *relative* response magnitude. For example, the ratio of response strengths to dominant stimuli relative to naïve stimuli could shift during estrus. To explore this and other scenarios, we define a preference index $PI_{AB} = (R_A\text{-}R_B) / (R_A + R_B)$, where R_A and R_B are the responses to stimulus A and B, respectively. PI_{AB} ranges between −1 (exclusive response to stimulus B) and 1 (exclusive response to stimulus A). After calculating these values for each neuron, we derive their means across the population. Finally, we compare each of the 55 preference indices (defined by our 11 stimuli dataset) for estrus and non-estrus females. The comparison (**Fig. 5CB**) reveals a positive (0.41) and significant (p = 0.002) correlation between the two states.

Despite this similarity, in some cases pairwise indices change and even reverse signs across the states (points located in the upper left and lower right squares in **Fig. 5C**). To reveal if these reflect estrus-dependent changes in responsiveness to *male* features, we first tested pairwise indices associated with virility levels. To account for strain related effects of response strength, we conducted this analysis separately for each strain. As shown in **Fig 5D**, for comparisons of naïve vs. dominant urine, there is a significant shift for C57 stimuli, with responses to naïve urine becoming stronger during estrus (p = 0.0005, non-parametric ANOVA, Bonferroni corrected p-value: 0.0056). For comparisons of castrated vs. dominant urine, there are no significant differences. Finally, for comparisons between castrated and naïve stimuli, there are two significant shifts, but in opposite directions. Specifically, for BC derived stimuli, responses to castrated stimuli are increased during estrus (p=0.00005), while the opposite is true for C57 stimuli (p = 0.00036). Summarizing these comparisons, there are no consistent trends across the three strains, and specifically, no evidence for stronger responses to more virile stimuli, as we had initially hypothesized.

Another possibility is that during estrus, responses to male stimuli will become stronger relative to female stimuli. To test this, we focused on comparisons involving female and intact male stimuli from the same strain (ICR). In all four comparisons (**Fig. 5E**), we find that female urine elicits a stronger response than male urine. The only significant difference (p=0.0024, Bonferroni corrected p-value: 0.01) is that during estrus, responses to non-estrus female stimuli are strengthened compared to naïve male stimuli, a change that does not reflect enhanced responsiveness to male stimuli during estrus. We also compared responses to estrus and non-estrus female urine (**Fig. 5E**), and observe reduced sensitivity to urine associated with the subject female's own state. However, although the sign is reversed, the change is mild, and non-significant.

In conclusion, relative response magnitudes are also broadly conserved across estrus states. The changes that do take place do not reflect systematic modulation of sensitivity to particular virility states.

Increased selectivity during estrus, but no consistent changes associated with virility state

Thus far, we examined hypotheses related to response magnitudes, following the rationale that changes in physiological state can enhance the mean responses to selected stimuli. Another possibility is that individual neurons will become more *selective* to particular stimuli, without changes in mean response magnitudes across the population. We begin this analysis by examining lifetime sparseness, a normalized measure of selectivity based on response strength which ranges between 0 (uniform response magnitude), and 1 (exclusive response to one stimulus). As shown in

Fig. 6A, the sparseness of the population is larger for estrus neurons (p = 0.0079, Kruskal-Wallis non parametric ANOVA). To determine if these changes reflect selectivity to particular virility states, we next examine them directly.

To account for strain related effects of response strength, we conduct this analysis for each strain separately. In the triangle plots [46] of **Fig. 6B**, vertices correspond to virility states, and each dot represents the *relative* response magnitude of one neuron to each virility state. Thus, dots near the center represent non-selective neurons, while selective neurons are represented by dots near the vertices. Across all strains, dots corresponding to estrus and non-estrus neurons widely overlap, particularly in the centers. Consistent with our previous observations, (**Fig. 5B**), the region near the naïve vertex (lower left) is relatively sparse, whereas the vertex representing castrated stimuli is denser. To determine if selectivity changes with reproductive state, we calculated a response selectivity score [46], for each neuron for each strain (i.e., each panel in **Fig. 5B**). Like lifetime sparseness, the selectivity score ranges between 0 (uniform response strength) and 1 (exclusive response to one stimulus). We hypothesized that during estrus, selectivity among male stimuli will be enhanced. This hypothesis appears true for ICR male stimuli, with a small yet significant increase in selectivity during estrus (p = 0.0017, Kruskal-Wallis test). However, this is not the case for BC and C57 derived stimuli (**Fig. 6C**), indicating that it is not a general feature of the estrus state.

Finally, while the triangle plots compare responses to the three virility states, they are not convenient for assessing selectivity for pairs of stimuli. Therefore, we also analyzed pairwise response selectivity, by considering, for each neuron, the *absolute* value of the preference index (see Methods), which ranges between 0 (equal responses to both stimuli), and 1 (exclusive response to one of the stimuli). We then compared distributions of these indices across the two estrus states. The analysis (**Table 1**) does not support the presence of increased acuity during estrus for reproductively relevant cues. For example, of all pairwise comparisons involving a dominant and non-dominant stimulus donor from the same strain, the only significant change involves a decrease in selectivity during estrus between dominant and naïve C57 male urine. The pairwise comparisons (**Table 1**) also suggest that the observed selectivity changes for the three-way comparisons to ICR stimuli mostly result from increased selectivity to castrated urine. Summarizing these analyses, while as a population, AOB-MCs from estrus females show sparser responses, there are no consistent shifts in selectivity associated with male virility state.

Neuronal receptive field distributions across the estrus cycle

Finally, we return to examine response patterns, and compare their frequencies across the estrus cycle. Such changes, if exist, could reflect shifts in tuning properties that would not necessarily be revealed by our previous analyses. Response pattern frequencies for units recorded in estrus and non-estrus females, for the basic and adjusted patterns are shown in **Fig. 7A, B**. Applying a binominal exact test (see Methods), and correcting for multiple comparisons, none of the patterns show a significant state-dependent difference in frequency. After relaxing significance criteria (by removing the multiple comparisons correction), three differences are apparent, two of which involve castrated males. Specifically, the *cast* and the *cast*|*strain* patterns are more prominent in estrus females. The *~ICR*|*strain* category is more frequent in estrus female neurons. Again, none of these changes suggests an obvious ethological function, nor do they reflect a different representation of dominance categories. In summary, response pattern distributions remain stable during the cycle, and those changes that do take place, are not compatible with increased tuning to reproductively relevant stimuli.

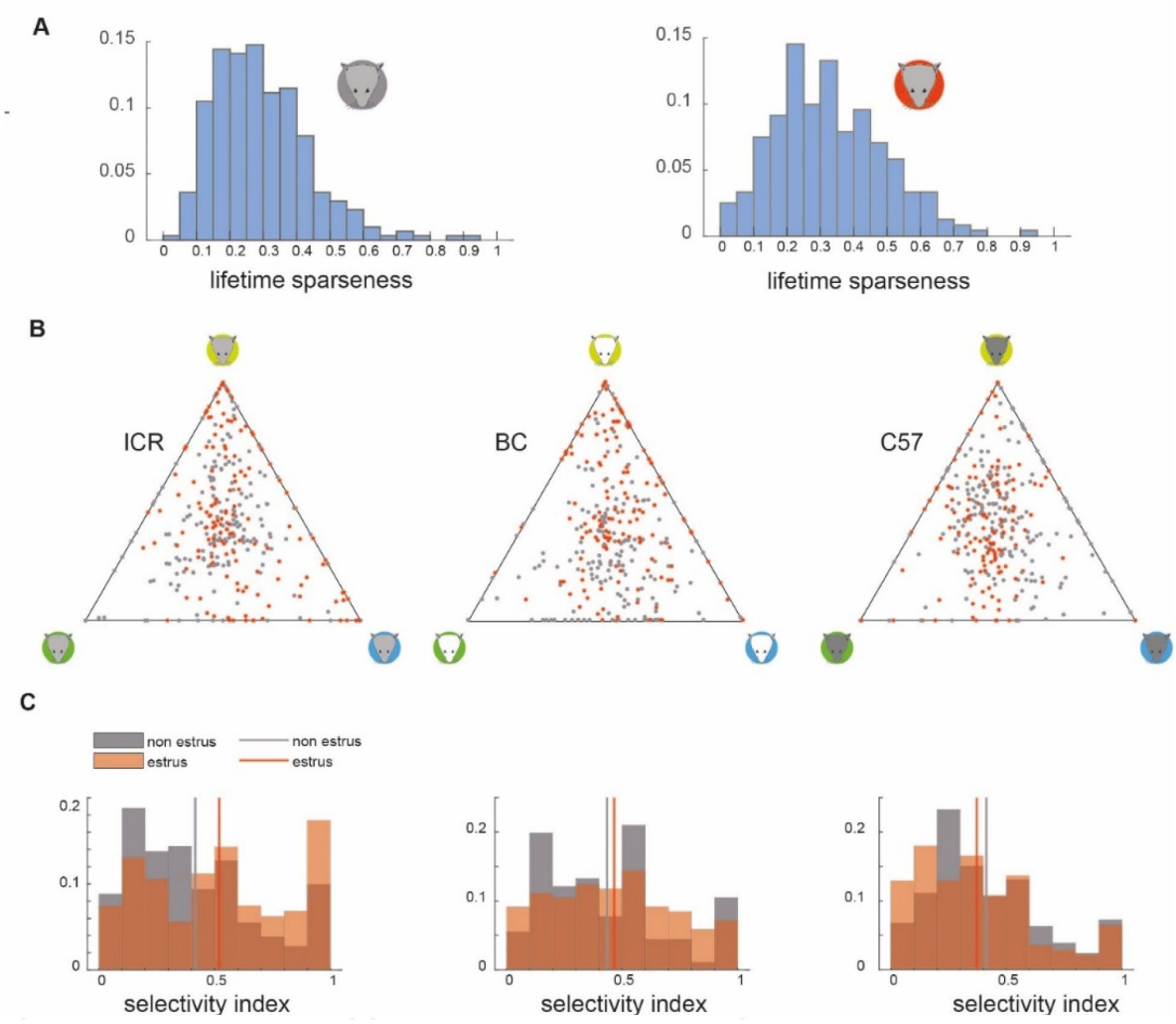

Figure 6.

Response selectivity under the two reproductive states.

A. Lifetime sparseness distributions for non-estrus (left, n = 305, mean: 0.29 median: 0.27), and estrus (right, n = 241, mean: 0.33, median: 0.31), neurons. Non-parametric ANOVA p-value: 0.0078, reflecting higher selectivity during estrus. **B.** Triangle plots showing relative response magnitude to stimuli from each of the three male states, separated according to strains. Neurons recorded in non-estrus and estrus females are indicated in gray and red, respectively. **C.** Distributions of the male state selectivity index (calculated from the data shown in B) under the two reproductive states, calculated for each strain separately. p- values corresponding to non-parametric analysis of variance (Kruskal Wallis test): (ICR: KW p: 0.0017, BC: KW p: 0.24, C57: KW p: 0.08). Higher selectivity during estrus is observed for the stimuli from ICR male mice (p = 0.0017). Mean selectivity scores: ICR: estrus: 0.52, n = 161, non-estrus: 0.42, n = 181. BC: estrus: 0.47, n = 153, non-estrus: 0.43, n = 181. C57: estrus: 0.37, n = 139, non-estrus: 0.41, n = 206.

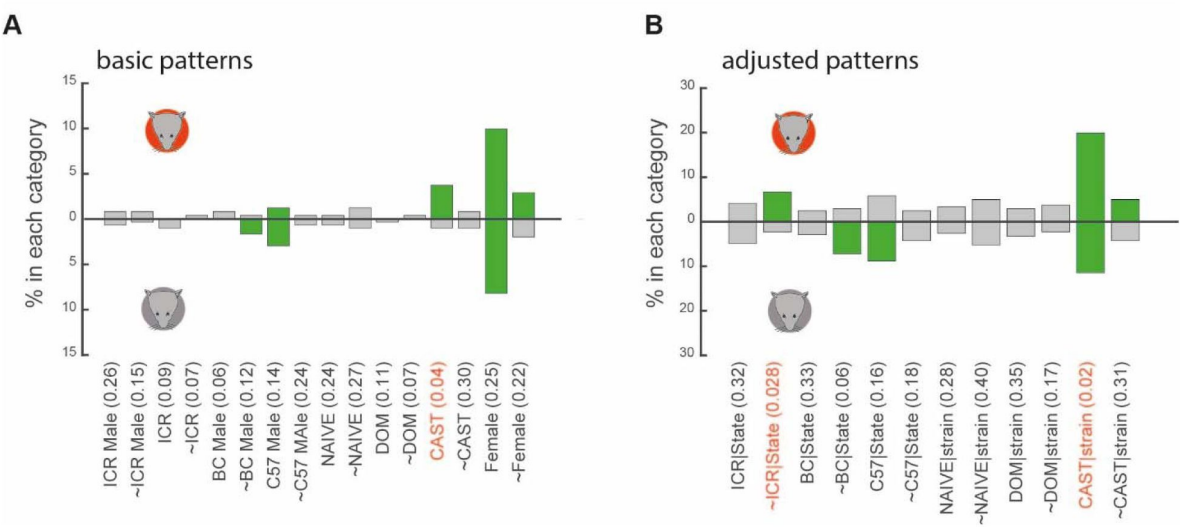

Figure 7.

Comparison of response frequency under the two reproductive states.

A. Frequencies of basic patterns (including complementary patterns). **B.** Frequencies of adjusted patterns (including complementary patterns). In both panels, frequencies in estrus and non-estrus females are shown above and below the horizontal axis, respectively. Significantly represented categories (using a shuffling test as described in **Fig. 3**) are indicated in green. P-values for differences for each pattern under the two states, using the binomial exact test, are listed. The Bonferroni adjusted p-values at the 0.05 level are 0.0031 (0.05/16) and 0.0042 (0.05/12), for A and B, respectively, and thus none of the differences are significant. Relaxing the correction, three categories fulfil the 0.05 criterion. None of them correspond to increased representation of dominant male stimuli during estrus. Note that in some cases, a category is overrepresented only during one of the states, but this is not necessarily reflected by a significant difference among the two states (e.g., ~female).

Discussion

The first stage in sexual behavior is the appetitive stage, which is further divided into detection, approach, and investigation [2]. In rodents, the VNS is probably most important during investigation, which takes place after contact is established, allowing a thorough analysis of a potential partner's state [11, 12]. We had originally hypothesized that during estrus, responses to "virile stimuli" will be accentuated, reflecting heightened sensitivity or discriminatory ability. Instead, we found that overall, AOB representations remain stable. This conclusion is based on analysis of response magnitudes to individual stimuli and relationships between response magnitudes (**Fig. 5**), selectivity to specific stimuli (**Fig. 6**), population level representations (**Fig. 4**) and the frequency of response categories (**Fig. 7**). It is consistent with our recent observations showing representational stability in virgin and in pregnant female mice, at least under temporal scales reflecting brief investigation bouts [43]. Combined with analysis of responses in male and female subjects [16, 46] and in males from different strains [44], we propose that the AOB conveys detailed information, but does not "interpret" it as a function of an organism's current state. Thus, the AOB provides an unbiased account of a potential partner's features, leaving evaluation of the appropriate response to downstream processing stages. Below, we first discuss what our study can reveal about response patterns of AOB neurons, and then we proceed to discuss the implications for state dependent processing.

Response patterns of AOB-MCs

While the primary focus of this study was state-dependent processing, we first considered the broader topic of response properties of AOB-MCs. Chemosensory space cannot be easily parameterized like visual or auditory space, and presentation of all potential ligands (and their combinations) is clearly unfeasible. Thus, receptive fields properties of central chemosensory neurons, including AOB-MCs, are poorly characterized. The selective responses to mice from different sex and strain combinations that we observed here are consistent with previous electrophysiological [44], and chemical analyses [47]. Selectivity to castrated vs. intact males has also been shown before [43], while selectivity to male dominance is consistent with chemical analyses [48] and AOB immediate-early-gene expression profiles [49]. Here, we asked if AOB-MC response patterns reflect behaviorally relevant conspecific features. We found that most response patterns (**Fig. 3**) are not more frequent than expected by chance.

What can we learn from our analysis of response pattern frequency? Generally, pattern frequency is determined by the fraction of molecules (or their combinations) common to stimuli that share a feature, and, the degree to which AOB-MCs sample these shared molecules (or their combinations). This idea is illustrated in **Fig. 8**, where for simplicity, we ignore the fact that that molecule levels are graduated rather than binary, that multiple AOB-MCs can sample the same molecule, that the same molecules can be sampled by multiple AOB-MCs, and that trait selective information is often conveyed by combinations rather than single molecules. While these are critical facts, ignoring them does not alter the essence of our explanation. **Fig. 8A** represents three individuals that share a common trait (e.g., dominance), each of which emits a certain number of molecules, chosen randomly from a "world" of molecules (depicted in a 30 x 30 grid). Some of the molecules are unique to each individual (shown in in green), while others are related to the common trait and thus shared by all individuals (red). The individual-selective molecules may convey information related to individuality or to some other unrelated trait. The three rows in **Fig. 8A** represent scenarios with varying ratios between trait-selective and individual-selective molecules. Each row shows the molecules associated with each individual, as well as their union (right column).

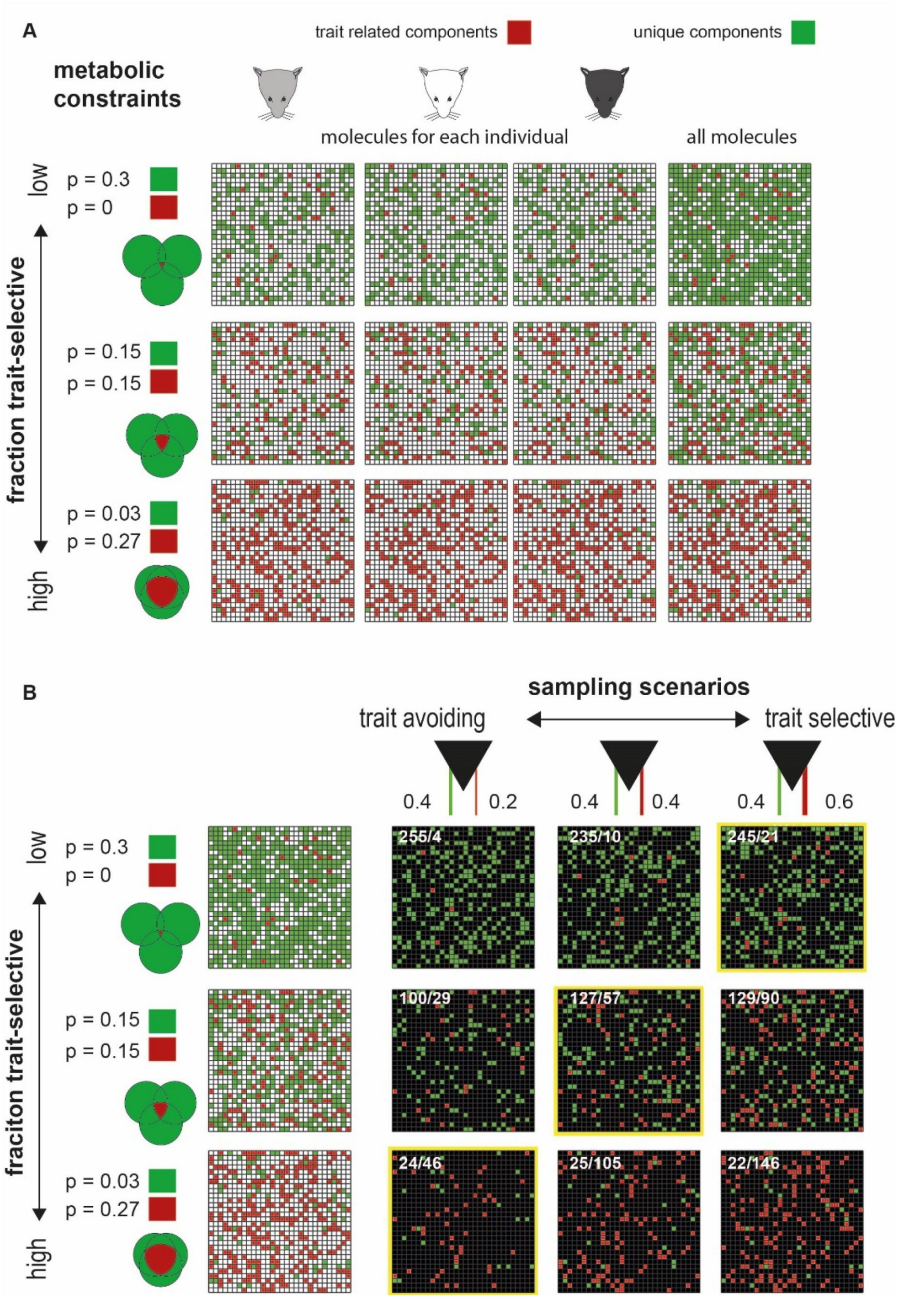

Figure 8.

Schematic illustration of sampling of trait selective molecules.

A. Molecular profiles associated with 3 different individuals sharing a common trait. Trait selective molecules are shown in red and all others (unique molecules) are shown in green, within a 30 x 30 grid representing all (900) molecules in the world. The three individuals are indicated on the three columns on the left. The rightmost column shows their union. Rows correspond to different proportions of individual specific and trait selective molecules. **B.** Different sampling scenarios by AOB neurons. The three rows correspond to the scenarios shown in A. The left column is the union of all molecules under each scenario, as shown in A. The three other columns represent sampling schemes that differ in the proportion of trait selective and other molecules sampled, moving from trait avoiding, to balanced, to trait selective sampling. The two values above each column indicate the probability of sampling unique (left), or common (trait-selective) molecules (right). We speculate that sampling properties are matched to component frequencies, thereby balancing robustness of trait detection with redundancy. While only AOB-MCs are depicted here, sampling actually involves VNSs and the manner by which they are sampled by AOB-MCs.

For other organisms to identify this trait, they must detect trait-selective molecules, and this involves the second element that dictates response frequency, namely the effective sampling properties of neurons (of course, AOB-MCs do not directly sample molecules, but rather VSNs, which do). Assuming some noise in production and detection of these molecules, robust trait detection should involve sampling of multiple molecules. Given a tradeoff between robustness of detection, and redundant sampling (since resources are limited), there is likely an optimal range of molecules/neurons required for identification of any given trait. We hypothesize that tuning properties of the AOB-MC population are adapted to efficiently sample trait-selective molecules. Generally, three scenarios are possible (with respect to each set of trait-selective molecules): non-biased sampling, over-sampling, and undersampling of trait-selective molecules (**Fig. 8B**). Combined with the proportions of trait-selective and other molecules, these sampling statistics determine the frequency of particular response patterns across the population.

Taking these considerations into account, we speculate that optimal sampling by the VNS (reflecting both VNS tuning properties and AOB-MCs connectivity) involves selective sampling of rarely represented trait-related molecules, and selective avoidance of common redundant molecules. Such an organization may optimize information retrieval with limited resources, and can account for our observation that most patterns, which correspond to what we consider as meaningful traits, are not over presented.

However, if sampling is optimal, why then are some patterns in our data more frequent than expected by chance? In the case of male vs. female stimuli, there may be numerous molecules that are more prevalent in female that in males. We speculate that under-sampling can reduce, but not overcome this excess. While recent analyses indicate that only a small number of urinary molecules are unique to all males or females from different strains [47], our pattern types do not depend on unique, but rather on increased or decreased concentrations associated with specific traits. Similar considerations may apply to the C57 male category which was also over-represented in our data. Notably, analysis of chemical levels indicates that VSN selectivity for both sex and strain is less prominent than expected based on urinary contents [47]. This supports our ideas that the VNS acts to reduce the redundancy present in molecular levels. Our present results suggest that despite this reduction, some patterns are still over-represented.

The over-representation of castrated stimuli is puzzling at first, since castrated males are not natural stimuli. Yet, this may in fact explain the over-representation. Specifically, if castration gives rise to sweeping changes in molecular composition, then the (hypothetical) evolutionary process of under-sampling could not happen. This would account for the puzzling fraction of castrated urine selective neurons and the strong responses to these stimuli (**Fig. 2D, E**), observed here, and elsewhere [43].

Along the same lines, in contrast to castrated stimuli, the dominant pattern was very infrequent (in fact, only one single unit in our dataset fulfilled it, **Fig. 3C**). While this is not significantly less than expected by chance, it is still somewhat surprising. We note that this is not because dominant stimuli are less potent than naïve stimuli; they are generally stronger (see **Fig. 2 D, E**). Nor is it likely to be due to our modest sample size, as similar pattern distributions are observed when multi-unit activity is also included in the analysis (not shown). Following our line of reasoning, this scarcity may represent an optimal allocation of resources to separate dominant from naïve males. Note also that population activity levels elicited by dominant and naïve stimuli are similar (see **Fig. 2B**), suggesting that unlike castration, dominance does not give rise to sweeping changes that outweigh those associated with strain. Indeed, the number of strain-adjusted dominant patterns is not negligible (**Fig. 3D**), implying that evaluation of male state might be done in the context of that male's strain. Ultimately, lacking knowledge of the chemical space associated with each of the stimuli, this and all the other ideas developed here remain speculative. A

comprehensive analysis of tuning properties of AOB-MCs must take into account all the features that we ignored in our simple explanation, and consider the balance between encoding multiple traits in parallel, some of which must involve overlapping molecules.

Implications for female reproductive state dependent processing

Considering the second theme of this research, namely state dependence, our results are not at odds with multiple reports of estrus-state dependent behavioral changes in female mice. They are also easily reconcilable with state-dependent representations in downstream regions that directly, or indirectly integrate fluctuating hormonal states. However, our findings are unexpected in light of estrus-state dependent changes in the earlier processing stage, namely at the level of VSNs [39–41]. One possible explanation is that our approach can detect global changes rather than finer adaptations within dedicated processing streams [50]. This scenario applies in [39], which showed changes in a subset of VSNs sensitive to male stimuli. Two other studies revealed more global sex hormone dependent changes in VSNs [40, 41]. Lacking knowledge of the functional implications of such changes at the network level in the AOB, it is difficult to explain why we did not observe broad changes. One simple possibility is that such effects will generally increase excitability. However, we did not observe such changes, perhaps due to compensatory mechanisms within the AOB. Finally, we note that despite the global stability, some of our comparisons yielded significant differences among the two states. While these differences do not represent increased (or decreased) responsiveness to virile stimuli, we cannot rule out their connection with the changes reported in VSNs.

Our experimental approach has inherent advantages and disadvantages for detecting state dependent effects. Since mice are anesthetized, changes involving active exploration or arousal cannot be detected. This includes the possible effects of feedback that may themselves be state-dependent, such as those from estrogen receptor expressing neurons in the medial amygdala [51]. On the other hand, in awake mice, there is no simple way to monitor the dynamics of stimulus uptake, which may vary with state, and thus confound state dependent neuronal responses with state dependent sampling. The anesthetized preparation ensures that stimulus uptake is consistent under both states and allows repeated delivery of multiple stimuli, regardless of the subject's engagement or motivation. In this context, we note that MOS activation using our preparation is likely minimal, thereby reducing its influence on sampling behavior, or in more direct forms of cross talk between the two systems.

In conclusion, the two themes of this study inspire two future research directions. The first calls for continued investigation of state dependent representations, in both the AOB under the awake state, and in deeper brain regions, in both anesthetized and ultimately, awake animals. The second direction involves a broader effort, to understand how behaviorally relevant features are encoded in chemical space, in early sensory processing, and eventually, decoded by downstream neurons that receive sensory inputs.

Materials and Methods

Subjects

For recordings, we used adult sexually unexperienced female mice from the ICR strain (Envigo Laboratories, Israel). All experiments complied with the Hebrew University Animal Care and Use Committee.

Determination of the estrus stage

Female estrus stage was determine using a vaginal lavage of 15 ul of saline. The saline sample was then deposited on a Superfrost plus slide (Thermo Scientific), and dried. Slides were stained with Wright Stain Solution (Sigma), and photographed under a light microscope. The estrus stage was determined using the cytological composition of the smear, as described in [52, 53].

Male status

Our stimulus set includes urine samples from castrated, naïve, and dominant proven breeder mice. Proven breeder and naïve male mice were purchased (Envigo Laboratories, Israel) and then assigned to cages containing 2 proven breeders and 2-3 naïve mice. One to two days after mice were placed in these cages, we identified candidate dominant male (always one of the two proven breeders) by observation of the mice in their home cages. Then, to confirm that these mice were indeed dominant, we conducted dominance tests involving each proven breeder mouse with each of the other cage mates (including all naïve cage mates and the other proven breeder male). The test included a "tube test", conducted in a new cage with clean bedding. The two mice were placed at opposite ends of a cylindrical tube and the mouse that forced the other to exit was designated as dominant. In addition, immediately following this tube test, the same pair of mice was monitored for an additional 10 minutes, and the mouse displaying aggressive behavior (biting, chasing, tail rattling) towards the other was identified as dominant. The outcomes of these tests were unambiguous in most cases. In some cases, we repeated the dominance test to confirm our initial evaluation. We also confirmed that the established hierarchy was maintained throughout the entire period of urine collection, over the course of ~4 weeks, by monitoring the behavior of these mice in their home cages. Dominant male mice consistently showed aggressive behaviors, whereas non-dominant mice engaged in self grooming behaviors more frequently. Castrated male urine was collected from 8 weeks old males that were has their testes removed under Isoflurane anesthesia at the age of 4 weeks (before puberty).

Stimuli and dataset

Stimuli were collected from castrated, naïve, and sexually experienced male mice from the BALB/C, C57BL/6 and ICR strains and adult estrus and non-estrus sexually-naïve female mice from the ICR strain (Envigo Laboratories, Israel). For urine collection, mice were gently held over a plastic sheet until they urinated. The urine was collected in plastic tubes, flash-frozen in liquid nitrogen, and then stored at −80°C. Each of the samples presented here comprised urine from 3 different individuals, diluted 1:10 in Ringer's solution. Urine from each individual was collected over a period of ~ 4 weeks and pooled before combining it with urine from the males of the same strain and status.

Surgical procedures and electrode positioning

Experimental procedures were detailed in [54]. Briefly, mice were anesthetized with 100mg/kg ketamine and 10mg/kg xylazine, tracheotomized, and a cuff electrode was implanted around the sympathetic nerve trunk. Incisions were closed with Vetbond (3M) glue, and the mouse was placed in a stereotaxic apparatus where anesthesia was maintained throughout the experiment (0.5-1% isoflurane in oxygen).

A craniotomy was made immediately rostral to the rhinal sinus, the dura was removed, and electrodes were advanced into the AOB at an angle of ~30° with an electronic micromanipulator (MP-285; Sutter Instruments, Novato, CA). We used 32 channel probes (NeuroNexus Technologies, Ann Arbor, Michigan) with 8 channels on each of 4 shanks. Before recordings, electrodes were dipped in fluorescent dye (DiI, Invitrogen, Carlsbad, CA) to allow subsequent confirmation of placement within the AOB external cellular layer (ECL).

Stimulus presentation procedure

Each session contains several distinct stimuli. Typically, each stimulus was presented repeatedly in a pseudorandom order, at least 4 times, and typically 5 times within each session. Stimuli were presented in blocks, and within each block all stimuli were presented in a random order. During each presentation, 2 μl of stimulus were applied directly into the nostril. After a delay of 20 s, a square-wave stimulation train (duration: 1.6 s, current: ±120 μA, frequency: 30 Hz), was delivered through the sympathetic nerve cuff electrode to induce vomeronasal organ suction. Following another 40 s delay, the nasal cavity was flushed with 1-2ml of ringer's solution, and drained with suction from the nasopalatine duct. The cleansing procedure lasted 50 s and included sympathetic trunk stimulation to facilitate stimulus elimination from the vomeronasal organ lumen.

Electrophysiology

Neuronal data was recorded using an RZ2 processor, PZ2 preamplifier, and two RA16CH head-stage amplifiers (TDT, Alachua, FL). Single-unit and multi-unit activity were sampled at 24414 Hz and band-pass filtered at 300-5000 Hz. Custom MATLAB codes were used to extract spike waveforms. Spikes were sorted automatically according to their projections on two principal components on 8 channels of each shank using KlustaKwik [55, 56] and then manually verified and adjusted using Klusters [56]. Spike clusters were evaluated by their waveforms, projections on principal component space (calculated for each session individually) and autocorrelation functions. A cluster was defined as a single-unit if it displayed a distinct spike shape, was fully separable from both the origin (noise) and other clusters, and if its autocorrelation function demonstrated a clear trough around time 0 of at least 10 ms. Clusters apparently comprising more than one single-unit were designated as multi-units. Under these definitions, multi-units could represent the activity of as few as two units. Such units are not included in our dataset. As a further criterion for single-unit classification, we examined the "shape symmetry" of all spike shapes. Shape symmetry was defined as the correlation coefficient of the two sides (margins) of the spike waveform around its peak (in absolute value, with one of the margins reversed). For example, if the waveform contains 9 samples with a peak at sample 5, the correlation will be calculated between the vectors defined by samples 1-4 and a reversed version of the vector defined by samples 6-9. If the shape is symmetric, the symmetry will be 1. When the peak is not in the middle, as is usually the case, the correlation is calculated for vectors determined by the smaller margin. A high symmetry (>0.95) is helpful for identifying noise accidentally misclassified as spikes. Using a graphical representation of all spike shapes, we examined the entire data set for such noise-like spikes and removed them from further analyses.

Data analysis

All data analyses and visualizations were performed using custom and built-in MATLAB code. The response of a unit to a given stimulus was defined as the average firing rate change over a 60 s window following sympathetic nerve stimulation (change measured compared to the 17 s period preceding VNO activation). This broad window covers both the post application and post stimulation periods, as in [44]. This window was chosen because in some cases, responses began after stimulus application, and prior to electric stimulation of the nerve. Response significance of a given unit to a given stimulus was determined by comparing its spiking rate distribution following all repeats to the baseline firing frequency distribution during the 17 s period prior to stimulus application. Response significance (of a particular unit to a given stimulus) was determined by a non-parametric analysis of variance *kruskalwallis* test) comparing the set of post-stimulation rates to the set of preceding baseline rates (preceding rates were pooled across all stimuli). In the image in **Fig. 2A**, the response magnitude of each unit was normalized by the absolute value of the maximal response (i.e., the response to the stimulus that elicited the strongest response, in absolute value). Thus, all response values range between −1 and 1. In addition, in this image and in some analyses, all non-significant responses were set to 0. For **Fig.**

2B, we applied classical multi-dimensional scaling, using the data in each of the rows in **Fig. 2A**, using the MATLAB *cmdscale* function, and the *correlation* distance metric (defined as one minus the sample correlation).

For analysis of response pattern frequencies, we initially defined several response patterns, as shown in **Fig. 3A** . For example, an ICR male pattern (upper row in left matrix in **Fig. 3A**) is fulfilled by neurons whose response to each of the three ICR male stimuli is larger than all of the other stimuli. In the complementary pattern (upper row in central matrix), the response to each of these stimuli is smaller than all the other stimuli. To qualify for a dominant/strain pattern, a neuron's response, within each strain, must be strongest to the dominant stimulus. We note that in initial analyses we defined response patterns according to presence or absence of responses. However, because response designation is inevitably arbitrary, and because this procedure results in a very small number of neurons conforming to each pattern, we applied more permissive criteria involving response magnitude.

In our analyses, we first found the number of neurons that conform to each of the patterns. Then, we calculated the probability to observe that number of cases by "chance". To analyze if response frequencies occur more often than expected by chance, we constructed a null distribution by shuffling the response data matrix. In this matrix, (as in **Fig. 2A**), each row corresponds to one neuron, and each column corresponds to one stimulus. In each of 100,000 iterations, we randomly shuffled the order of all elements within a column, and calculated the frequency of each response pattern. This frequency for each pattern was calculated across all iterations, allowing us to evaluate the probability to obtain the observed frequencies given only the marginal probabilities to each stimulus.

To correlate global response similarity between neurons recorded in estrus and non-estrus female, we used the MATLAB *pdist* function, which calculates pairwise distances along arrays. The arrays in our cases were the population responses (after normalization, as shown in the matrices in **Fig. 4A**). We used the *correlation* and *Euclidean* distance measures for **Fig. 4B** and **Fig. 4C**, respectively. To calculate the correlation coefficient and the p-value (the probability to observe such a large correlation under the hypothesis of no correlation), we used the MATLAB *corrcoef* function.

To calculate significance of differences in the number of neurons responding under each of the two states (**Fig. 5A**), we tested the null hypothesis that response probability is identical under both states. This was done by calculating the observed probability (based on the total number of responding neurons, per stimulus, across both states), and then calculating four different probabilities under the binomial distribution: the probability to obtain the observed number or more responding neurons in estrus, the probability to obtain the observed number or less of responding neurons in estrus, and the corresponding probabilities for neurons recorded in non-estrus females. The smallest (i.e., most unlikely) value of these four probabilities was then considered as the p-value. Since the smallest p-value is taken, this yields a sensitive test of differences. To calculate significance of differences between mean response magnitudes for each stimulus (**Fig. 5B**), we used a two-tailed non-parametric analysis of variance (*kruskalwallis* test), comparing the distributions of response strengths across all neurons recorded in estrus and in non-estrus females.

The pairwise preference index PI_{AB} for two stimuli A and B, (as shown in **Fig. 5C**), is defined as $(R_A-R_B) / (R_A + R_B)$, where R_A and R_B are the responses to stimulus A and B after rectification (i.e., after setting negative responses to zeros). Rectification was applied to keep the range of the preference indices between −1 and 1. For each pair of stimuli, we only considered neurons that showed a significant response to at least of the stimuli. Then, the mean preference indices were

calculated for each stimulus pair, as shown in **Fig. 5B**, for neurons recorded in estrus and non-estrus females. The correlation coefficient and p-value were derived using the *corrcoef* MATLAB function, as described above.

To compare pairwise preference indices across states (**Fig. 5D, E**), we also used a bootstrapping approach. Specifically, we first found the absolute difference between the mean preference indices for a given stimulus pair under the two states. Then, we repeatedly shuffled the data set (effectively, neurons were randomly assigned to one of the two reproductive states) 100,000 times, and calculated the absolute difference across the two (shuffled) states. Then, we derived the p-values as the fraction of cases in the shuffled distribution which were larger than the actual observed distribution. In other words, these p-value reflect the probability to obtain such a state-dependent difference in preference indices by chance.

To calculate lifetime sparseness (S), we used the definition given in [57]:

$$S = \left[1 - \frac{(\sum_i |r_i|/N)^2}{\sum_i (r_i^2)/N}\right] \Big/ \left[1 - \frac{1}{N}\right]$$

Where r_i is the response of neuron to the i^{th} stimulus (averaged across all trials) and N is the number of stimuli (N=11 in our case). S varies between 0 and 1, with 0 indicating uniform responses to all stimuli and 1 indicating a response to only one stimulus. Lifetime sparseness distributions across states were compared using a non-parametric analysis of variance (*kruskalwallis*).

Triangles plots in **Fig. 6B** were generated by considering the response of each neuron to each of three stimuli. Negative responses were truncated to 0 in this analysis. The position of each point is determined by the relative weights of three unit vectors pointing from the triangle center to each of the three vertices. The selectivity index (**Fig. 6C**) was calculated for each neuron (and each stimulus triplet) as in [46]. Specifically, it is the normalized distance from the center of the triangle, with values ranging between 0 (lowest selectivity, center of triangle), and 1 (highest selectivity, vertices of the triangles).

$$Si = \frac{\sqrt{(a-m)^2 + (b-m)^2 + (c-m)^2}}{d}$$

where a, b, and c are the responses to each of three stimuli. The value corresponding to equal responses to all stimuli is m, which is equal to 1/3. d is the maximum possible value of the numerator of the equation, and is equal to $\sqrt{2/3}$. Selectivity index distributions under the two states were compared using a non-parametric analysis of variance (*kruskalwallis*).

For comparison of category frequency distributions under the two states (**Fig. 7A, B).** We applied a binomial exact text under the hypothesis of equal distributions under two states. Essentially, we followed the same procedure described above for comparing the number of responsive neurons, with the difference that here we calculated the probability to obtain a certain number of neurons corresponding to each pattern. Bars in **Fig. 7** are shaded in green if the probability to obtain the observed pattern frequency (calculated separately for each state), is smaller than 0.05, employing the same shuffling approach described for **Fig. 3C**.

All the raw data, processed data, and codes will be provided by the authors upon request.

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Additional files

Table 1

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Reviewer #1 (Public review):

Summary:

In this detailed study, Cohen and Ben-Shaul characterized the AOB cell responses to various conspecific urine samples in female mice across the estrous cycle. The authors found that AOB cell responses vary with the strains and sexes of the samples. Between estrous and non-estrous females, no clear or consistent difference in responses was found. The cell response patterns, as measured by the distance between pairs of stimuli, are largely stable. When some changes do occur, they are not consistent across strains or male status. The authors concluded that AOB detects the signals without interpreting them. Overall, this study will provide useful information for scientists in the field of olfaction.

Strengths:

The study uses electrophysiological recording to characterize the responses of AOB cells to various urines in female mice. AOB recording is not trivial as it requires activation of VNO pump. The team uses a unique preparation to activate the VNO pump with electric stimulation, allowing them to record AOB cell responses to urines in anesthetized animals. The study comprehensively described the AOB cell responses to social stimuli and how the responses vary (or not) with features of the urine source and the reproductive state of the recording females. The dataset could be a valuable resource for scientists in the field of olfaction.

Weaknesses:

(1) The figures could be better labeled.

(2) For Figure 2E, please plot the error bar. Are there any statistics performed to compare the mean responses?

(3) For Figure 2D, it will be more informative to plot the percentage of responsive units.

(4) Could the similarity in response be explained by the similarity in urine composition? The study will be significantly strengthened by understanding the "distance" of chemical composition in different urine.

(5) If it is not possible for the authors to obtain these data first-hand, published data on MUPs and chemicals found in these urines may provide some clues.

(6) It is not very clear to me whether the female overrepresentation is because there are truly more AOB cells that respond to females than males or because there are only two female samples but 9 male samples.

(7) If the authors only select two male samples, let's say ICR Naïve and ICR DOM, combine them with responses to two female samples, and do the same analysis as in Figure 3, will the female response still be overrepresented?

(8) In Figure 4B and 4C, the pairwise distance during non-estrus is generally higher than that during estrus, although they are highly correlated. Does it mean that the cells respond to different urines more distinctively during diestrus than in estrus?

(9) The correlation analysis is not entirely intuitive when just looking at the figures. Some sample heatmaps showing the response differences between estrous states will be helpful.

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Reviewer #2 (Public review):

Summary:

Many aspects of the study are carefully done, and in the grand scheme this is a solid contribution. I have no "big-picture" concerns about the approach or methodology. However, in numerous places the manuscript is unnecessarily vague, ambiguous, or confusing. Tightening up the presentation will magnify their impact.

Strengths:

(1) The study includes urine donors from males of three strains each with three social states, as well as females in two states. This diversity significantly enhances their ability to interpret their results.

(2) Several distinct analyses are used to explore the question of whether AOB MCs are biased towards specific states or different between estrus and non-estrus females. The results of these different analyses are self-reinforcing about the main conclusions of the study.

(3) The presentation maintains a neutral perspective throughout while touching on topics of widespread interest.

Weaknesses:

(1) Introduction:
The discussion of the role of the VNS and preferences for different male stimuli should perhaps include Wysocki and Lepri 1991

(2) Results:
a) Given the 20s gap between them, the distinction between sample application and sympathetic nerve trunk stimulation needs to be made crystal clear; in many places, "stimulus application" is used in places where this reviewer suspects they actually mean sympathetic nerve trunk stimulation.
b) There appears to be a mismatch between the discussion of Figure 3 and its contents. Specifically, there is an example of an "adjusted" pattern in 3A, not 3B.
c) The discussion of patterns neglects to mention whether it's possible for a neuron to belong to more than one pattern. For example, it would seem possible for a neuron to simultaneously fit the "ICR pattern" and the "dominant adjusted pattern" if, e.g., all ICR responses are stronger than all others, but if simultaneously within each strain the dominant male causes the largest response.

(3) Discussion:
a) The discussion of chemical specificity in urine focuses on volatiles and MUPs (citation #47), but many important molecules for the VNS are small, nonvolatile ligands. For such molecules, the corresponding study is Fu et al 2015.
b) "Following our line of reasoning, this scarcity may represent an optimal allocation of resources to separate dominant from naïve males": 1 unit out of 215 is roughly consistent with a single receptor. Surely little would be lost if there could be more computational capacity devoted to this important axis than that? It seems more likely that dominance is computed from multiple neuronal types with mixed encoding.

(4) Methods:
a) Male status, "were unambiguous in most cases": is it possible to put numerical estimates on this? 55% and 99% are both "most," yet they differ substantially in interpretive uncertainty.
b) Surgical procedures and electrode positioning: important details of probes are missing (electrode recording area, spacing, etc).
c) Stimulus presentation procedure: Are stimuli manually pipetted or delivered by apparatus with precise timing?
d) Data analysis, "we applied more permissive criteria involving response magnitude": it's not clear whether this is what's spelled out in the next paragraph, or whether that's left

unspecified. In either case, the next paragraph appears to be about establishing a noise floor on pattern membership, not a "permissive criterion."
e) Data analysis, method for assessing significance: there's a lot to like about the use of pooling to estimate the baseline and the use of an ANOVA-like test to assess unit responsiveness.
But:
i) for a specific stimulus, at 4 trials (the minimum specified in "Stimulus presentation procedure") kruskalwallis is questionable. They state that most trials use 5, however, and that should be okay.
ii) the methods statement suggests they are running kruskalwallis individually for each neuron/stimulus, rather than once per neuron across all stimuli. With 11 stimuli, there is a substantial chance of a false-positive if they used $p < 0.05$ to assess significance. (The actual threshold was unstated.) Were there any multiple comparison corrections performed? Or did they run kruskalwallis on the neuron, and then if significant assess individual stimuli? (Which is a form of multiple-comparisons correction.)

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Author response:

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
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> *In this detailed study, Cohen and Ben-Shaul characterized the AOB cell responses to various conspecific urine samples in female mice across the estrous cycle. The authors found that AOB cell responses vary with the strains and sexes of the samples. Between estrous and non-estrous females, no clear or consistent difference in responses was found. The cell response patterns, as measured by the distance between pairs of stimuli, are largely stable. When some changes do occur, they are not consistent across strains or male status. The authors concluded that AOB detects the signals without interpreting them. Overall, this study will provide useful information for scientists in the field of olfaction.*
>
> *Strengths:*
>
> *The study uses electrophysiological recording to characterize the responses of AOB cells to various urines in female mice. AOB recording is not trivial as it requires activation of VNO pump. The team uses a unique preparation to activate the VNO pump with electric stimulation, allowing them to record AOB cell responses to urines in anesthetized animals. The study comprehensively described the AOB cell responses to social stimuli and how the responses vary (or not) with features of the urine source and the reproductive state of the recording females. The dataset could be a valuable resource for scientists in the field of olfaction.*
>
> *Weaknesses:*
>
> *(1) The figures could be better labeled.*

Figures will be revised to provide more detailed labeling.

> *(2) For Figure 2E, please plot the error bar. Are there any statistics performed to compare the mean responses?*

We did not perform statistical comparisons (between the mean rates across the population). We will add this analysis and the corresponding error bars.

> *(3) For Figure 2D, it will be more informative to plot the percentage of responsive units.*

We will do it.

> *(4) Could the similarity in response be explained by the similarity in urine composition? The study will be significantly strengthened by understanding the "distance" of chemical composition in different urine.*

We agree. As we wrote in the Discussion: “Ultimately, lacking knowledge of the chemical space associated with each of the stimuli, this and all the other ideas developed here remain speculative.”

A better understanding of the chemical distance is an important aspect that we aim to include in our future studies. However, this is far from trivial, as it is not chemical distance per se (which in itself is hard to define), but rather the “projection” of chemical space on the vomeronasal receptor neurons array. That is, knowledge of the chemical composition of the stimuli, lacking full knowledge of which molecules are vomeronasal system ligands, will only provide a partial picture. Despite these limitations, this is an important analysis which we would have done had we access to this data.

> *(5) If it is not possible for the authors to obtain these data first-hand, published data on MUPs and chemicals found in these urines may provide some clues.*

Measurements about some classes of molecules may be found for some of the stimuli that we used here, but not for all. We are not aware of any single dataset that contains this information for any type of molecules (e.g., MUPs) across the entire stimulus set that we have used. More generally, pooling results from different studies has limited validity because of the biological and technical variability across studies. In order to reliably interpret our current recordings, it would be necessary to measure the urinary content of the very same samples that were used for stimulation. Unfortunately, we are not able to conduct this analysis at this stage.

> *(6) It is not very clear to me whether the female overrepresentation is because there are truly more AOB cells that respond to females than males or because there are only two female samples but 9 male samples.*

It is true that the number of neurons fulfilling each of the patterns depends on the number of individual stimuli that define it. However, our measure of “over-representation” aims to overcome this bias, by using bootstrapping to reveal if the observed number of patterns is larger than expected by chance. We also note that more generally, the higher frequency of responses to female, as compared to male stimuli, is obtained in other studies by others and by us, also when the number of male and female stimuli is matched (e.g., Bansal et al BMC Biol 2021, Ben-Shaul et al, PNAS 2010, Hendrickson et al, JNS, 2008).

> *(7) If the authors only select two male samples, let's say ICR Naïve and ICR DOM, combine them with responses to two female samples, and do the same analysis as in Figure 3, will the female response still be overrepresented?*

We believe that the answer is positive, but we can, and will perform this analysis to check.

> *(8) In Figure 4B and 4C, the pairwise distance during non-estrus is generally higher than that during estrus, although they are highly correlated. Does it mean that the cells respond to different urines more distinctively during diestrus than in estrus?*

This is an important observation. For the Euclidean distance there might be a simple explanation as the distance depends on the number of units (and there are more units recorded in non-estrus females). However, this simple explanation does not hold for the correlation distance. A higher distance implies higher discrimination during the non-estrus stage, but our other analyses of sparseness and the selectivity indices do not support this idea. We note that absolute values of distance measures should generally be interpreted cautiously, as they may depend on multiple factors including sample size. Also, a small number of non-selective units could increase the correlation in responses among stimuli, and thus globally shift the distances. For these reasons, we focus on comparisons, rather than the absolute values of the correlation distances. In the revised manuscript, we will note and discuss this important observation.

> *(9) The correlation analysis is not entirely intuitive when just looking at the figures. Some sample heatmaps showing the response differences between estrous states will be helpful.*

If we understand correctly, the idea is to show the correlation matrices from which the values in 4B and 4C are taken. We can and will do this, probably as a supplementary figure.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Many aspects of the study are carefully done, and in the grand scheme this is a solid contribution. I have no "big-picture" concerns about the approach or methodology. However, in numerous places the manuscript is unnecessarily vague, ambiguous, or confusing. Tightening up the presentation will magnify their impact.*

We will revise the text with the aim of tightening the presentation.

> *Strengths:*
>
> *(1) The study includes urine donors from males of three strains each with three social states, as well as females in two states. This diversity significantly enhances their ability to interpret their results.*
>
> *(2) Several distinct analyses are used to explore the question of whether AOB MCs are biased towards specific states or different between estrus and non-estrus females. The results of these different analyses are self-reinforcing about the main conclusions of the study.*
>
> *(3) The presentation maintains a neutral perspective throughout while touching on topics of widespread interest.*
>
> *Weaknesses:*
>
> *(1) Introduction:*
>
> *The discussion of the role of the VNS and preferences for different male stimuli should perhaps include Wysocki and Lepri 1991*

Agreed. we will refer to this work in our discussion.

> *(2) Results:*
>
> *a) Given the 20s gap between them, the distinction between sample application and sympathetic nerve trunk stimulation needs to be made crystal clear; in many places, "stimulus application" is used in places where this reviewer suspects they actually mean sympathetic nerve trunk stimulation.*

In this study, we have considered both responses that are triggered by sympathetic trunk activation, and those that occur (as happens in some preparations) immediately following stimulus application (and prior to nerve trunk stimulation). An example of the latter Is provided in the second unit shown in Figure 1D (and this is indicated also in the figure legend). In our revision, we will further clarify this confusing point.

> *b) There appears to be a mismatch between the discussion of Figure 3 and its contents. Specifically, there is an example of an "adjusted" pattern in 3A, not 3B.*

True. Thanks for catching this error. We will correct this.

> *c) The discussion of patterns neglects to mention whether it's possible for a neuron to belong to more than one pattern. For example, it would seem possible for a neuron to simultaneously fit the "ICR pattern" and the "dominant adjusted pattern" if, e.g., all ICR responses are stronger than all others, but if simultaneously within each strain the dominant male causes the largest response.*

This is true. In the legend to Figure 3B, we actually write: “A neuron may fulfill more than one pattern and thus may appear in more than one row.”, but we will discuss this point in the main text as well.

> *(3) Discussion:*
>
> *a) The discussion of chemical specificity in urine focuses on volatiles and MUPs (citation #47), but many important molecules for the VNS are small, nonvolatile ligands. For such molecules, the corresponding study is Fu et al 2015.*

We fully agree. We will expand our discussion and refer to Fu et al.

> *b) "Following our line of reasoning, this scarcity may represent an optimal allocation of resources to separate dominant from naïve males": 1 unit out of 215 is roughly consistent with a single receptor. Surely little would be lost if there could be more computational capacity devoted to this important axis than that? It seems more likely that dominance is computed from multiple neuronal types with mixed encoding.*

We agree, and we are not claiming that dominance, nor any other feature, is derived using dedicated feature selective neurons. Our discussion of resource allocation is inevitably speculative. Our main point in this context is that a lack of overrepresentation does not imply that a feature is not important. We will revise our discussion to better clarify our view of this issue.

> *(4) Methods:*
>
> *a) Male status, "were unambiguous in most cases": is it possible to put numerical estimates on this? 55% and 99% are both "most," yet they differ substantially in interpretive uncertainty.*

This sentence is actually misleading and irrelevant. Ambiguous cases were not considered as dominant for urine collection. We only classified mice as dominant if they were “won” in the tube test and exhibited dominant behavior in the subsequent observation period in the cage. We will correct the wording in the revised manuscript.

> *b) Surgical procedures and electrode positioning: important details of probes are missing (electrode recording area, spacing, etc).*

True. We will add these details.

> *c) Stimulus presentation procedure: Are stimuli manually pipetted or delivered by apparatus with precise timing?*

They are delivered manually. We will clarify this as well.

> *d) Data analysis, "we applied more permissive criteria involving response magnitude": it's not clear whether this is what's spelled out in the next paragraph, or whether that's left unspecified. In either case, the next paragraph appears to be about establishing a noise floor on pattern membership, not a "permissive criterion."*

True, the next paragraph is not the explanation for the more permissive criteria. The more permissive criteria involving response magnitude are actually those described in Figure 3A and 3B. The sentence that was quoted above merely states that before applying those criteria, we had also searched for patterns defined by binary designation of neurons as responsive, or not responsive, to each of the stimuli (this is directly related to the next comment below). Using those binary definitions, we obtained a very small number of neurons for each pattern and thus decided to apply the approach actually used and described in the manuscript.

> *e) Data analysis, method for assessing significance: there's a lot to like about the use of pooling to estimate the baseline and the use of an ANOVA-like test to assess unit responsiveness.*
>
> *But:*
>
> *i) for a specific stimulus, at 4 trials (the minimum specified in "Stimulus presentation procedure") kruskalwallis is questionable. They state that most trials use 5, however, and that should be okay.*

The number of cases with 4 trials is truly a minority, and we will provide the exact numbers in our revision.

> *ii) the methods statement suggests they are running kruskalwallis individually for each neuron/stimulus, rather than once per neuron across all stimuli. With 11 stimuli, there is a substantial chance of a false-positive if they used $p < 0.05$ to assess significance. (The actual threshold was unstated.) Were there any multiple comparison corrections performed? Or did they run kruskalwallis on the neuron, and then if significant assess individual stimuli? (Which is a form of multiple-comparisons correction.)*

First, we indeed failed to mention that our criterion was 0.05. We will correct that in our revision. We did not apply any multiple comparison measures. We consider each neuron-stimulus pair as an independent entity, and we are aware that this leads to a higher false positive rate. On the other hand, applying multiple comparisons would be problematic, as we do not always use the same number of stimuli in different studies. Applying multiple comparison corrections would lead to different response criteria across different studies.

Notably, most, if not all, of our conclusions involve comparisons across conditions, and for this purpose we think that our procedure is valid. We do not attach any special meaning to the significance threshold, but rather think of it as a basic criterion that allows us to exclude non-responsive neurons, and to compare frequencies of neurons that fulfill this criterion.

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