The Bruce effect: Representational stability and memory formation in the accessory olfactory bulb of the female mouse

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SUMMARY

In the Bruce effect, a mated female mouse becomes resistant to the pregnancy-blocking effect of the stud. Various lines of evidence suggest that this form of behavioral imprinting results from reduced sensitivity of the female's accessory olfactory bulb (AOB) to the stud's chemosignals. However, the AOB's combinatorial code implies that diminishing responses to one individual will distort representations of other stimuli. Here, we record extracellular responses of AOB neurons in mated and unmated female mice while presenting urine stimuli from the stud and from other sources. We find that, while initial sensory responses in the AOB (within a timescale required to guide social interactions) remain stable, responses to extended stimulation (as required for eliciting the pregnancy block) display selective attenuation of stud-responsive neurons. Such temporal disassociation could allow attenuation of slow-acting endocrine processes in a stimulus-specific manner without compromising ongoing representations that guide behavior.

Graphical Abstract

The Bruce effect

Stranger

Father

Initial AOB representations are not altered by mating

accessory olfactory bulb (AOB)

brain

main olfactory bulb

AOB

Yet, during prolonged exposures, responses to the father are attenuated

Father

Stranger

brief interaction   prolonged interaction

In brief

Studying the neuronal correlates of the Bruce effect, Yoles-Frenkel et al. record neuronal responses to chemosensory cues in the accessory olfactory bulb (AOB) of female mice. They find that, while immediate neuronal representations of multiple stimuli remain stable following mating, extended presentation of the stud stimulus leads to attenuated responses.

INTRODUCTION

A key function of learning is to associate stimuli with appropriate behavioral responses. Often, learning involves incremental changes integrated over multiple exposures to a stimulus, but sometimes it can be much more rapid. A dramatic example of the latter is behavioral imprinting. This unique form of learning usually coincides with a crucial life event that stimulates acquisition of a memory during a single exposure (Hudson, 1993). Typically, imprinting involves linking a stereotyped and robust response to a complex stimulus that is *a priori* unknown. Among the best-known and most widely replicated examples of imprinting in mammals is the Bruce effect (BE), first described and repeatedly shown in various forms in mice (Bruce, 1959; Brennan and Keverne, 1997, Brennan, 2009) and later demonstrated or implicated in other species (Roberts et al., 2012; Eleftheriou et al., 1962; Clulow and Langford, 1971; Mallory and Brooks, 1980; Packer and Pusey, 1983; Rozenkrantz et al., 2020).

In mice, the BE involves two related components. The first is the innate sensory-mediated pregnancy block, in which exposure of a pregnant female mouse to an unfamiliar male mouse, or to his urine (Parkes and Bruce, 1962), during the first 72 h post mating (Bruce, 1959), leads to pregnancy failure. The second, and the focus of this work, involves learning. Specifically, during mating, the female forms a memory of the stud male, lasting several weeks, so that his odors will no longer elicit pregnancy failure. In the context of the BE, imprinting involves an interaction between individual recognition and the hypothalamic circuits that mediate basic physiological and endocrine responses. A series of studies has shown that both pregnancy block and the learning that selectively circumvents it involve sensory detection by the vomeronasal system (VNS), a chemosensory system devoted to processing cues from other organisms (Kaba et al., 1989; Kaba and Keverne, 1988; Lloyd-Thomas and Keverne, 1982). More specifically, it was suggested that activation of the VNS by unfamiliar male chemosignals would trigger a cascade of events involving the amygdala and hypothalamic dopamine (Li et al., 1989,1990) neurons that block secretion of prolactin, which in turn is required for maintenance of pregnancy during the initial stages (Li et al., 1994). Importantly, and consistent with a key role of the VNS in the BE, several studies have demonstrated the capacity of the VNS to represent information about conspecific individuals (Hurst, 2009; Hurst et al., 2001; Luo and Katz, 2004; Ben-Shaul et al., 2010).

What changes are triggered by mating that prevent the stud male from inducing a pregnancy block of his own litter? The prevailing theory is that mating leads to selective weakening or even silencing of stud-responsive neurons in the first brain relay of the VNS, the accessory olfactory bulb (AOB) (Brennan and Binns, 2005; Brennan and Keverne, 1997; Brennan and Zufall, 2006; Brennan et al., 1990). According to this idea, known as the negative template hypothesis (NTH), odors associated with the stud male will no longer trigger the hormonal cascade that leadsw to pregnancy block. While elegant, this solution faces two fundamental difficulties. First, complete silencing or graded attenuation of activity in response to the stud would render the female less sensitive to her mate, at least via the vomeronasal system, a scenario that appears maladaptive. Second, crosstalk between representations of different stimuli is likely to cause interference between the learned odors and other biologically relevant cues. AOB representations are combinatorial (Kaur et al., 2014; Xu et al., 2016; Fu et al., 2015; Arnson and Holy, 2013; Kahan and Ben-Shaul, 2016; Ben-Shaul, 2015; Bergan et al., 2014; Ben-Shaul et al., 2010), so single AOB neurons often respond to a range of stimuli. As a consequence, AOB neuronal ensembles associated with distinct stimuli, sometimes with highly divergent ethological implications, may display extensive overlap. This implies that silencing, or mere attenuation of studactivated neurons, will alter representations of multiple stimuli in a complex manner. These considerations address a broader issue, namely the balance between plasticity and stability in neuronal representations. While plasticity is an essential feature of sensory processing, when it alters sensory representations at an early processing stage such as the AOB, and thus compromises the stability of representations of multiple stimuli, it can complicate and even confound the readout of neuronal activity. How then can plasticity in the context of the BE take place without sacrificing representational stability?

One potential solution to this puzzle is suggested by a recent *in vitro* study investigating the effects of mating on AOB neuronal activity (Gao et al., 2017), which showed that

mating-activated projection neurons (mitral tufted cells [MTCs]) show reduced excitability. Critically, the reduced excitability was not apparent during a single current injection stimulus, but appeared only following an extended stimulation sequence. This raises the possibility that mating-induced plasticity is not expressed as a simple and direct weakening of activity, but rather as impaired sustainment of robust activation for extended periods. Under this scenario, mating would change the stud's ability to affect the slow endocrine processes required for induction of pregnancy failure (Brennan and Keverne, 1997), without altering the initial responses to sensory stimuli.

The goal of the present work was to test the effects of mating on sensory representations at the level of single units in the AOB *in vivo*. Most of this study is devoted to demonstrating that representations of stud male stimuli, and of various other stimuli, remain highly stable despite mating. Next, in attempt to better model prolonged bouts of sensory investigation, we test responses to an extended stimulation. Consistent with the prior *in vitro* work (Gao et al., 2017), we find that, in mated females, stud male urine evokes responses that decay substantially over repeated trials.

Taken together, our experiments suggest a scenario that reconciles plasticity (i.e., mating-induced protection of the stud's pregnancy-blocking ability) with the essential requirement to maintain stable sensory representations that are required for guiding social and defensive behaviors. We propose that these two apparently contradictory requirements can be met because of the distinct time scales required for sensory discrimination on the one hand, and considerably slower modulation of endocrine responses on the other.

RESULTS

To evaluate how mating affects representations of conspecific chemosignals, including those from the stud male, we performed extracellular ensemble recordings of AOB projection neurons while testing a panel of sensory cues derived from males and females from two strains. Our stimulus set included urine from two individual BALB/c (BC) males, two individual C57BL/6 (C57) males, as well as mixed urine samples from female mice of each strain, castrated mice from each strain, and a mix of urine from different predators. We note that multiple studies have shown that exposure to an unfamiliar male urine is sufficient to induce pregnancy block, whereas urine from castrated males or female mice does not have this effect (Bruce, 1965, Spironello Vella and Decatanzaro, 2001) (but see Kaba et al., 2020). All recordings were made from BC females, which were either sexually naive or mated with a male from the BC or the C57 strain (Figure 1A). Importantly, BC females are known to exhibit the BE with the particular male strains that we used. That is, BC females that mated with C57 males were susceptible to block following exposure to a BC male, and vice versa (Leinders-Zufall et al., 2004; Peele et al., 2003). In our experiments, females spent at least 48 h with the stud male, and we verified mating with a vaginal plug. When a plug could not be confidently identified, mating (i.e., pregnancy) was confirmed *post mortem*. Recordings were made in anesthetized female mice, in a period of 3–17 days following mating, a time during which memory formation is complete and strong (Kaba et al., 1988; Brennan and Keverne, 1997). Recording electrodes were targeted to the external cellular layer of the AOB, which contains the cell bodies of the MTCs (Figure 1B). Thus, our recordings reflect

the outputs sent by AOB neurons to downstream regions. To achieve controlled and repeated stimulus delivery, we applied urine stimuli to the nostril, followed by electric stimulation of the sympathetic nerve trunk via a cuff electrode. This stimulation serves to activate the VNO and induce stimulus uptake to the vomeronasal sensory neurons that are located within the VNO (see STAR Methods and Yoles-Frenkel et al., 2017). Our stimulation paradigm includes pseudo-random interleaved presentations of different stimuli separated by flushing of the nasal cavity (Figure 1C). Examples of neuronal responses showing selectivity across sexes, strains, and even individuals within a strain are shown in Figure 1D.

Stud-induced responses in naive females

We begin our analysis by examining selectivity of responses of MTCs in naive females to male stimuli (Figure 2A) (N = 104 single units and 169 multi-units, from 20 recording sites in 12 naive females). Overall, male urine activated a substantial portion of AOB neurons, with 39.5% and 37.5% of single units in naive female mice showing a significant response to BC and C57 urine stimuli, respectively, and 25% of the units responding to stimuli from both strains. Similar values are obtained when multi-unit data are also included (38% and 38% for the BC and C57 strains, and 23% to both strains). Given the four different male stimuli in our panel, and a binary response to each (as determined by response significance), there are 15 potential response patterns. These patterns are shown in Figure 2B along with the expected and observed number of neurons matching each pattern. Expected values were calculated using the estimated marginal response probabilities to each of the stimuli (shown in Figure 2C), and the assumption of independence in responses to them (see STAR Methods for details).

A significant difference between the observed number of neurons responding to a specific pattern (indicated by the blue bars), and the expected value as calculated above (indicated by the orange bars), implies that responses to each of the individual stimuli are not independent. In this analysis, we only include neurons that responded to at least one of the four male urine stimuli (see Table S1). Given the simple model of the NTH, and the observation that mating with an individual protects against pregnancy failure by another individual from the same strain (Bruce, 1968; Brennan, 2009), one expects to find neurons that show similar responses to two individual males from the same strain. In our dataset, this includes two response patterns (indicated by the horizontal yellow bars in Figure 2B): BC-selective neurons, and C57-selective neurons. Indeed, the observed number of neurons for both of these patterns is more common than expected by chance (although only significantly for BC-selective neurons). When including multi-unit data (Figure S1), both patterns are significantly over-represented. Another class of response patterns (indicated by the green bars in Figure 2B), also easily reconcilable with the coding scheme implicated by the NTH, are individual-selective neurons. Such neurons are useful for distinguishing among individuals from the same strain. In our data, we find that some, but not all, of these patterns are more frequent than expected by chance. The third class of patterns includes neurons that respond to stimuli from both strains (black horizontal bars). Although these patterns generally occur less than expected by chance, they still represent a substantial fraction of AOB neurons. These latter patterns are not easily reconciled with the NTH, as changes in the activity of such neurons would affect representations of individuals from distinct strains,

and this is inconsistent with selective modulation of responses in a strain-selective manner. These conclusions are also supported when multi-unit data are included (Figure S1). Overall, we find evidence for a diverse range of response properties, including broad and relatively nonselective tuning, as well as neurons that distinguish among strains and individuals within a strain.

Representations of the stud strain or stud individual are not attenuated or silenced following mating

Having characterized responses to males in naive females, we next explore how mating alters responses to male stimuli (BC mated females, n = 73 single units and 120 multi-units, 12 recording sites from seven females; C57 mated females, n = 49 single units and 85 multi-units, 18 recording sites from 11 females). According to the NTH, responses of stud-responsive neurons should be weakened following mating. However, this prediction may be interpreted in more than one way. Under one scenario, suggested by the observation that mating with an individual provides protection from another male of the same strain (Bruce, 1968), one would expect a weaker response to all males of the stud male strain. Alternatively, weaker responses could apply to the specific stud individual, and not to other individuals from the same strain. Furthermore, response weakening could manifest as complete silencing of responses to stud male stimuli (an extreme scenario), or, alternatively, as a subtler attenuation relative to other stimuli.

We begin our analysis with a rather extreme interpretation of the NTH, according to which the fraction of stud-responsive neurons should be reduced following mating. Specifically, we tested whether the proportion of stud-responsive single units (out of the total number of neurons that responded to any of the stimuli) was altered by mating. The results of this analysis are shown and described in detail in Figure S2, and indicate that mating does not lead to a reduction in the proportion of stud-strain- or stud-individual-responsive neurons.

Next, we test the more permissive interpretation of the NTH, according to which mating leads to a graded attenuation of response to the stud stimuli. In our analyses, we first consider responses to the stud male strain, and then to the specific stud individual. Responses of mated female neurons to male stimuli are shown in Figure 3A. To quantify the responses of neurons to male stimuli from each strain, we initially characterize each neuron's response as the larger (in absolute value) of the two responses to individuals from that strain. Then, we compare each neuron's response to male stimuli from the two strains. The scatterplots in Figure 3B suggest that relative response strengths are similar for neurons from the naive, BC mated, and C57 mated female groups. For a statistical comparison of the differences, we defined, for each neuron, a strain preference index. The index ranges between −1 and 1, with −1 corresponding to an exclusive response to C57 urine and 1 corresponding to an exclusive response to BC urine (see STAR Methods). Thus, absolute firing rates of individual neurons are normalized, yielding relative response magnitudes to the two stimuli. Analysis of the naive, BC mated, and C57 mated female groups indicates that the median of the strain preference index is not different from 0 for any of the groups, and, furthermore, is not different between any pair of groups (Figure 3C). Next, we combined data from both mated groups, and defined a stud strain preference

index, also ranging between –1 and 1, with 1 corresponding to an exclusive response to the stud male strain. The median of the stud strain preference index is also not different from 0 (Figure 3D). Taken together, these analyses indicate that mating does not lead to a general attenuation of responses to stimuli from the stud strain.

Having shown no general decrease in response strength to the stud *strain*, we next ask if there is a selective attenuation to the stud *individual*. To that end, we compare the responses to the stud individual and to the non-stud individual from the same strain by defining a stud-selective index that ranges between –1 (reflecting an exclusive response to the non-stud individual from the stud strain) and 1 (exclusive response to the stud individual himself). We find that, overall, responses to the stud individual are in fact stronger than responses to the non-stud individual Figure 3E). Thus, response magnitudes appear to be unaltered in both absolute Figure 3B) and relative (Figures 3C-3E), magnitudes.

The analyses shown in Figure 3 were also repeated with inclusion of multi-unit data, and also using the average response (rather than the maximum, as used above) across two individuals from the same strain, with both single unit and single and multi-units. Taken together, the results of these extended analyses further support the conclusions made above, namely, that stud stranger preference ratios are not modified following mating (Figure S3). Summarizing this series of analyses, we conclude that mating does not silence or attenuate AOB MTC responses to stimuli from either the stud strain or the stud individual.

Mating does not modify baseline rates, lifetime sparseness, relative response magnitude, or sex selectivity

While responses to the stud male are not modified following mating, mating may also induce changes in other features of AOB responses that might affect downstream processing. To address this possibility, we first compare baseline firing rates between naive and mated females (with both mated female groups combined, irrespective of the stud strain). Specifically, we calculated the mean baseline firing rate for neurons measured in each female, and compared those means across groups. This analysis indicates that mating does not induce a consistent change in baseline firing rates across females (Figure 4A). Next, we test the selectivity of neurons in mated and naive females, using the lifetime sparseness metric (see STAR Methods). As shown in Figure 4B, the distribution of sparseness values does not differ for the two groups, implying that mating does not lead to a general change in response selectivity.

We next examined neuronal responses using our entire stimulus set, which includes urine mixes from two female mouse strains, two strains of castrated males, and predator urine. Comparing the number of significant responses to each of the stimuli in naive and in mated females reveals a positive, although not significant, association (Figure 4C, correlation coeffecient [CC] = 0.6064, p = 0.0834). The correlation is higher and statistically significant when multi-units are also included (CC = 0.8, p = 0.0095).

A similar analysis of the population-mean response magnitudes to each of the stimuli (Figure 4D), also reveals similar patterns in the mated and naive female groups, with a correlation of 0.68 (p = 0.04). With multi-units included, the correlation is higher (0.93, p

= 0.0002). We note that, for both groups, female stimuli elicit stronger responses than male stimuli. This is a recurring observation in AOB recordings (Hendrickson et al., 2008; Bergan et al., 2014; Bansal et al., 2021) but, in the present experiments, may be due to female stimuli being mixes rather than individual mouse samples. Although responses appear to be overall stronger in mated females, particularly for female stimuli, statistical comparison (two-sided two sample Wilcoxon rank-sum test, with multiple comparisons) reveals that the only significant difference is for the female BC stimulus (Figure 4D).

Next, we asked if mating alters the *relative* response strength to male versus female stimuli. We defined a sex selectivity index (SSI) for each neuron and compared the distribution of the index in mated and unmated females. The index ranges between −1 and 1, with positive values indicating a stronger response to female stimuli (see STAR Methods). Comparison of the SSI distributions reveals no differences between the two groups (Figure 4E). Specifically, the mean SSI of naive female neurons is not significantly different from 0. Although neurons recorded in mated females had a mean SSI of 0.135, representing a tendency to respond more strongly to females (two-sided Wilcoxon signed rank-sum test p = 0.0074), the difference between the SSI distributions in mated and naive females is not significant (Figure 4E). When including multi-units, the SSI distributions assume more positive values (mean SSI naive, 0.1777; mated, 0.189) and are significantly larger than 0 in both groups (two-sided Wilcoxon signed rank test, naive, p = 3.9×10^{-7}; mated, p = 1.7×10^{-10}). However, here too, the difference between the mated and naive groups is not significant (two-sided Wilcoxon rank-sum test, p = 0.9704). Taken together, the analyses in this section indicate that mating does not induce a global change in response strength to conspecific and predator stimuli.

Stable population responses before and after mating

Finally, we examine whether mating alters population-level representation of stimulus responses. We use the Euclidean distance metric based on the firing rates elicited by each of the stimuli across all neurons, which defines a mapping between the stimuli and neuronal space. Smaller distances imply greater similarity (see STAR Methods). We use this metric to ask if mated and naive females share a similar representation of stimuli at the level of the AOB. Pairwise distance matrices for single-unit data are shown in Figures 5A and 5B (n = 90 and 111 single units for naive and mated females, respectively). For these analyses, we excluded responses to castrated males (and neurons that responded exclusively to castrated stimuli) as they did not show a consistent relationship with other stimuli, or with each other. As expected, the distance matrices show that pairs of male stimuli elicit responses that are generally similar to other male stimuli (rather than to female or predator stimuli). Comparing distances across pairs of stimuli between the two female groups, we find that the correlation is positive and significant (Figure 5C), indicating that mating does not induce global changes in representations of socially relevant stimuli by AOB neurons. A more specific, direct comparison of representations between BC and C57 mated females shows an even higher correlation (Figure 5D). The foregoing conclusions are also supported with a different distance metric (correlation distance), when including responses to castrated males, and after the addition of multi-units to the analysis (Figure S4).

Overall, we conclude that, when the AOB is presented with intermingled, time-varying sensory input from multiple individuals, stimulus representations at the level of the AOB are not altered by mating.

Selective response attenuation following extended stimulation

Thus far, we based our analysis on our standard stimulation paradigm in which a panel of stimuli are presented in an intermingled order. Under this mode of presentation, we demonstrated that AOB stimulus representations remain stable following mating. This stability fulfills the ongoing requirement to decode the environment in a consistent manner, but it provides no explanation of protection from the stud's pregnancy-blocking cues. Motivated by recent *in vitro* observations that mating-activated MTCs in the AOB show reduced excitability following an extended stimulation sequence (Gao et al., 2017), we sought to better model the effects of prolonged investigation in our experimental preparation.

Specifically, in a subset of our experiments at the end of our regular recording sessions, we presented two stimuli: urine from the stud male, and a urine mix comprising male, female, and predator urine (denoted as MFP mix, see STAR Methods for further details and rationale). The MFP mix includes stimuli that were presented during the regular stimulation trials (Figure 1). The extended stimulation paradigm (~3–4 min per stimulus; see Figure 6A and STAR Methods for details) comprised four consecutive presentations without washing of the nasal cavity between them. Following the four presentations, the nasal cavity and VNO were flushed with Ringer's solution and the second stimulus was presented using the same protocol. The order of stimulus presentation (stud urine or MFP mix) was randomly assigned in each experiment. In our analysis, we distinguish between stud-responsive units and MFP-responsive neurons. Responsiveness was assessed using responses to the conventional (interleaved) stimulus sequences that preceded the extended stimulation protocol. Neurons that responded to the stud urine (and potentially, also to the MFP mix), were designated as stud-responsive neurons. Units that responded to one or more components of the MFP mix, but not to the stud stimulus, were designated as MFP-responsive neurons. In naive females, the stud stimulus was arbitrarily designated by randomly selecting one of the four male stimuli. Examples of responses of individual units are shown Figure 6B.

This experimental design allowed us to evaluate the effects of learning on AOB representations by comparing sensory-driven activity in four groups of neurons: stud responses in mated and in unmated females, and MFP responses in mated and in unmated females. Although our conclusions are fully supported by analysis of single-unit data only, to increase sample size, we here show results using both single and multi-unit data (see Figure S6 for analysis only with single-unit data). To quantify changes in response strength, we defined an attenuation index (AI; see STAR Methods). The AI, defined for each neuron and stimulus, quantifies the reduction in response strength in the fourth presentation in comparison with the first, ranging from −1 to 1, with 1 corresponding to complete silencing and 0 corresponding to no change in response strength (see Figure S5 for further details). We find that the AI median is the largest for stud responses in mated females, indicating that attenuation is most prominent in this group (Figure 6C) and that it is the only group

for which the median of the AI distribution is significantly larger than 0. Furthermore, it is significantly different from the AI distributions in the three other groups (Figure 6C). As noted above, the same conclusions are obtained with a smaller dataset including only single-unit responses (Figure S6).

Finally, we wanted to confirm that reduced response strength is due to the continuous extended stimulation paradigm, and not merely to the repeated presentation of the stud stimulus. To that end, we revisited our standard stimulation data, to examine attenuation during the course of the experiment (~90 min). Here, we compared response strengths of the first and last (fifth) presentation of each stimulus, again using the AI. As above, for mated females, we analyzed responses to the stud male, while, in sexually naive females, we randomly designated one of the males as the stud. For both mated and naive females, we consider only neurons that showed a significant response to the stud stimulus. The analysis indicates that there is no consistent response attenuation to the stud stimulus in either group (Figure S7). Specifically, the median value of the AI is −0.18 and 0.004 for the mated and naive groups, and not significantly larger than 0 (p = 0.446 and 0.308 for the mated and naive groups, respectively; one-tailed signed rank test; n = 38 and n = 54 for the two groups). These conclusions are further supported after inclusion of multi-unit data, which substantially increases the sample sizes (Figure S7).

Taken together, the analyses in this section support the notion that mating leads to a decrease in the responses of stud-selective neurons upon prolonged continuous stimulation, resembling that involved in actual social investigation. These analyses provide key experimental evidence of altered sensory responses after mating, and, together with previous *in vitro* work showing altered membrane excitability (Gao et al., 2017), provide a potential explanation for the failure of stud odors to elicit the pregnancy block. Specifically, responses to isolated stimulations remain stable on faster time scales, allowing consistent decoding of stimulus information without requiring updating of readout rules by downstream processing stages. However, *extended* sampling of stud male chemosignals in pregnant females may result in decreased response magnitude, a smaller impact on the hormonal cascade that leads to pregnancy block, and, thus, a diminished pregnancy-blocking ability by the stud male.

DISCUSSION

In this work, we sought to identify the neuronal correlates of the BE in female mice. The BE is an intriguing example of behavioral imprinting of a conspecific individual, and various lines of study have assigned learning to the AOB. Since its characterization, the dominant framework for studying the BE was the NTH (Brennan et al., 1995; Matsuoka et al., 1997, 2004; Binns and Brennan, 2005; Brennan and Binns, 2005), which posits that AOB responses to stud stimuli following mating would be attenuated (silenced, or at least weakened). Such response attenuation can readily explain the acquired immunity (with respect to pregnancy failure) of the mated female to the stud male. When the NTH was first proposed, there were no direct recordings of AOB activity, and while the first single-neuron recordings from behaving mice supported the idea that individuals are sparsely represented by the activity of AOB projection neurons (Luo and Katz, 2004; Luo et al., 2003), subsequent studies painted a more complex picture, involving a distributed code

(Kaur et al., 2014; Xu et al., 2016; Fu et al., 2015; Arnson and Holy, 2013; Kahan and Ben-Shaul, 2016; Ben-Shaul, 2015). Furthermore, it is now clear that various compounds, namely low-molecular-weight urine constituents (Peele et al., 2003), MHC peptides (Leinders-Zufall et al., 2004), ESP peptides (Hattori et al., 2017), and mitochondrial encoded peptides (Kaba et al., 2020), can induce the block, implying that a substantial proportion of AOB neurons are involved in individual recognition. This argues against a narrow labeled line code in which individuals are represented by the activity of a highly selective, dedicated group of neurons. These observations and other recent data regarding the combinatorial coding schemes of social information in the AOB (Kahan and Ben-Shaul, 2016; Bergan et al., 2014; Ben-Shaul et al., 2010; Tolokh et al., 2013) highlight some of the potential difficulties with the NTH. Specifically, response attenuation would not only render the female less sensitive to her partner (via the vomeronasal system) but will also distort representations of other stimuli, thus confounding the animal's ability to respond appropriately to socially relevant stimuli.

Here, we make a distinction between responses to brief, exploratory encounters versus prolonged exposure as required to block pregnancy. This distinction can reconcile memory formation of the stud male without disrupting stable encoding of chemical cues. The disassociation is based on the distinct time scales of behavioral interactions and endocrine processes. While the VNS is inherently a slow system, behavioral interactions such as mating and fighting that require identification of features such as sex, genetic relatedness, and familiarity can be initiated by relatively rapid sensory sampling occurring within seconds. In contrast, endocrine processes are generally slower, lasting minutes to hours. Here, we show that, while initial stimulus presentations remain highly stable following mating, prolonged repetitive stimulation with stud stimuli in mated females leads to response attenuation.

Mechanistically, a key tenet of the NTH is that learning involves the coincidence of stud-responsive neurons with noradrenaline (NA), in turn leading to selective increases in inhibition of MTCs by granule cells. Various groups studied the effects of NA on AOB physiology. One study examined the effects of NA in the main olfactory bulb (MOB) (Shea et al., 2008) and showed selective response weakening following concomitant presentation with NA. These results are similar to those expected in the AOB following mating, but involve a shorter timescale than is relevant for the BE. Studies of the effects of NA on AOB responses in brain slices, or *ex vivo* AOB presentation, are limited to a shorter time range, and thus likely better reflect the conditions that facilitate memory formation rather than the long-term storage of information. Overall, these studies yield a complex picture. Some studies have shown evidence for disinhibition of MTC activity (Dong et al., 2009), while others suggest the opposite, namely that increased GABAergic signaling gives rise to silencing of AOB MTC activity (Smith et al., 2009; Araneda and Firestein, 2006). A more recent study using an *ex vivo* preparation showed an overall mild decrease in MTC responses, but the effect was heterogeneous, both across neurons and across stimuli for a given neuron (Doyle and Meeks, 2017). It may well be that experience-induced changes in response to extended stimulation could involve modification of MTC or granule cell excitability, as well as changes in the synaptic interactions between them (Cansler et al., 2017). Another possibility that cannot be entirely ruled out is that peripheral adaptation,

namely of vomeronasal sensory neurons (VSNs), plays a role, including in our extended stimulation paradigm (Spehr et al., 2009; Arnson and Holy, 2011; Holy et al., 2000). This is particularly relevant as such adaptation may be stimulus and subject dependent (Wong et al., 2018). However, while this may explain the differences between the MFP mix and the stud urine, it is harder to reconcile with the differences between mated and naive females. Furthermore, the same study (Wong et al., 2018) found little evidence for adaptation as expressed by spiking activity of VSNs, under intervals as short as 20 s. Thus, it is not likely that the selective attenuation observed here is due to processes at the level of VSNs.

To summarize, in the context of learning in the BE, our present work can reconcile memory for the stud male with maintaining constancy of stimulus representations in the AOB. These apparently contrasting ends can be achieved via the different temporal scales required for sensory processing to guide behavior (corresponding to initial representations) on the one hand, and those required for affecting downstream endocrine processing on the other. Thus, our results are in fact consistent with the NTH, but in a manner that is distinct from its original formulation. Future studies should directly test how extended AOB stimulation in response to learned and non-learned stimuli affects responses in downstream processing stages of the VNS, and the mechanisms that underlie this attenuation. More generally, our study suggests a form of multiplexing, a scenario in which the same channels convey different dimensions of information. Indeed, virtually all sensory systems detect and convey signals at various temporal scales, which may be subject to distinct modes of processing. It remains to be seen to what extent the phenomenon observed here applies in other contexts and sensory systems.

Limitations of the study

Although our observations help reconcile memory formation with maintained sensory range and acuity, we cannot confirm that the observed attenuation is sufficient to confer resistance to the stud male, nor can we exclude mating-induced representational changes under other conditions. For example, our experiments were conducted under anesthesia. The proposed mechanism according to the NTH is increased inhibition upon stud-selective MTCs (Brennan and Keverne, 1997), and one may argue that, under anesthesia, the effects of inhibition are diminished (Rinberg et al., 2006; Kato et al., 2012; Cazakoff et al., 2014). However, it has been shown that inhibition substantially shapes AOB responses in anesthetized mice (Hendrickson et al., 2008), and unlike the MOB, no prominent differences in baseline activity and response properties were reported between the awake and anesthetized AOB (Ben-Shaul et al., 2010; Luo et al., 2003; Hendrickson et al., 2008). However, even if inhibition is reduced in the anesthetized preparation, one would expect to see at least some degree of attenuation, and this is inconsistent with our observations. In this context, responses to the MFP mix may involve a larger contribution from inhibitory neurons than for the male stimuli (Hendrickson et al., 2008). This might introduce a confounding element into the comparison, especially if repeated stimulation influences excitatory and inhibitory interactions differently. We note, however, that, irrespective, of the potential limitations of the MFP stimulus, the key comparison regarding the extended paradigm concerns the difference between responses to male stimuli in mated and unmated females.

Finally, it is not clear how well our prolonged stimulation paradigm provides an adequate model of an actual ongoing sampling bout during social interaction.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yoram Ben-Shaul (yoramb@ekmd.huji.ac.il).

Materials availability—This study did not generate new unique reagents.

Data and code availability—Data reported in this paper will be shared by the lead contact upon request.

This paper does not report code that is conceptually original. The standard MATLAB code used to analyze data in this paper will be shared by the lead contact upon request.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Animals—All procedures were approved by the ethical committee of the Hebrew University Medical School. The dataset includes recording from 12 naive (unmated) and 18 mated BALB/c females. Stimuli were collected from adult male and female mice of the BALB/C and C57BL/6 strains. All mice were purchased from Envigo Laboratories (Israel). The age of mated mice ranged between 9 and 16 weeks (mean of 11.8 weeks). Ages of the naive group ranged between 9 and 14 weeks with (mean: 11.5 weeks). Mice were maintained on a 12:12 hour light dark cycle with free access to food and water.

METHOD DETAILS

Mating procedures—For mating, females were cohoused with individual male mice and mating were confirmed by the presence of vaginal plugs. Because, in some cases pregnancies were not associated with visible vaginal plugs, and only confirmed post mortem, for some recordings, the exact pregnancy day is not available. Nevertheless, we can confirm that recordings from all mated females were made no earlier than 3 days and no later than 17 days after mating, with the majority taking place between days 9 and 15 post mating.

Stimuli and stimulus delivery—For urine collection, mice were gently held over a plastic sheet until they urinated. The urine was transferred to a plastic tube with a micro-pipette and then flash frozen in liquid nitrogen and subsequently stored at −80°C. For stimulation, urine was diluted in Ringer's solution (1/10). To reduce the effects of sample-to-sample variation in individuals' urine, stimuli were pooled samples collected from at least 3 different days. Each female was exposed to 9 different stimuli as listed in Figure 1. Castrated male urine was collected from 8 weeks old males that were castrated (testis removal under

Isoflurane anesthesia) at the age of 4 weeks (before puberty). Castrated male urine and the female urine mix included a mix of at least 3 individuals from each strain (in the case of females, the estrus stage was not controlled). Predator urine included a mix of four species of predator urine (bobcat, lion, fox and wolf, purchased from PredatorPee (Maine, US).

In each trial, 2μl of the stimulus were applied directly into the nostril (stimulus application). This application is given manually by the experimenter, upon prompting by a sound sequence generated by the session management program (custom-written in MATLAB, see below). 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 trunk (SNT) cuff electrode to induce VNO pumping and stimulus entry to the VNO lumen (SNT stimulation). Following another delay of 40 s, the nasal cavity and the VNO were flushed with 1–2 ml of Ringer's solution, which flowed from the nostril into the nasal cavity and drained via the nasopalatine duct using a solenoid-controlled suction tube. The flushing procedure was 40s long and included a single sympathetic trunk stimulation to facilitate stimulus elimination from the VNO lumen. In each session, the 9 different stimuli were presented in a pseudorandom order, typically five times each, with a 10 s inter trial interval between them. In a subset of experiments, an additional block of repetitive exposure was included at the end of the session. These blocks included only 2 stimuli: stud male urine and a mix of urine from males, females and predators (denoted as MFP mix). The MFP mix was used as a control stimulus, with the intention of increasing the chance to obtain a neuronal response. We reasoned that although responses to male female and predator stimuli may not sum linearly, and in many cases even sub-linearly (Hendrickson et al., 2008; Ben-Shaul et al., 2010), the mix includes a rich array of molecules and is thus more likely to elicit a response than each of its constituents. The males in the MFP mix were the two individuals from the non-stud strain, while the females were from both strains. In naive females the "stud" was randomly selected out of the 4 males. Each stimulus was presented in 4 consecutive trials. Each trial was composed of a 10 s inter trial interval, stimulus application to the nostril, 20 s delay, stimulation of the SNT via the cuff electrode and additional 40 s delay. Importantly, in these sessions, the stimulus was not washed after each delivery. At the end of the four repetitions, the nasal cavity and VNO were flushed with 1–2 ml of Ringer's solution. This subset of experiments included 4 sessions from 3 unmated females and 4 sessions from 3 mated females (one mated with BC male and 2 mated with C57 males).

Experimental design—The experimental procedures are described in detail in (Yoles-Frenkel et al., 2017). Briefly, anesthesia was induced with an intraperitoneal injection of a ketamine-xylazine mix (10 mg/kg xylazine and 100 mg/kg ketamine) or 3% isoflurane mixed with oxygen gas in an anesthesia chamber. A tracheotomy was performed with a polyethylene tube, and a cuff electrode was placed around the sympathetic nerve trunk. Incisions were closed with veterinary glue, after which the mouse was placed in a custom-built stereotaxic apparatus. After placing the mouse on a stereotaxic stage, anesthesia was maintained with 1% isoflurane and monitored using a heart rate monitor and by testing the foot withdrawal reflex. A craniotomy was made, the dura was removed around the penetration site, and electrodes were advanced into the AOB at an angle of ~30° with a manual micromanipulator (model MM-33, Sutter Instruments). All recordings were made

with 32-channel probes (A4×8-5 mm-100-200-177-A32 or A4×8-5 mm-50-200-177-A32 configurations; NeuroNexus, Michigan, US). Before recordings, electrodes were dipped in fluorescent dye (DiI, Invitrogen) to allow subsequent confirmation of electrode placement within the AOB external cell layer that contains MTC bodies (Larriva-Sahd, 2008). In the beginning of every experimental session, we recorded ~10 minutes of neuronal activity, with no stimulus presentation. This period was followed by recording of neuronal activity during stimulus presentation.

Hardware and experimental control codes—The experiments were controlled using custom-written MATLAB code. The MATLAB code determined the pseudo-random order of stimuli, and interacted with the data acquisition card (I/O board, e.g., a National Instruments USB 6343 board) which controlled the speaker, a solenoid valve and the electrical stimulus isolator (AM-systems model 2200). Neuronal data were recorded using an INTAN board (RHD2000 V1, Intan Technologies).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data processing—Signals were sampled at 25 kHz and bandpass filtered (300–5000 Hz). Spike waveforms were extracted using custom-written MATLAB code. Spikes were sorted automatically using the KlustaKwik program (Harris et al., 2000) and then manually verified and adjusted using the Klusters software (Hazan et al., 2006). Spike clusters were evaluated by their waveforms, projection on principal component space (calculated for each session individually), and autocorrelation functions. A cluster was designated as a single unit if it showed a distinct spike shape, was fully separable from both the origin (noise) and other clusters along at least one principal component projection, and if the inter-spike interval histogram demonstrated a clear trough around time 0 of at least 10 ms. Clusters not meeting these criteria were designated as *multi-units*. Multi-units are assumed to be composed of a mix of a small number of units with small signal amplitudes that do not allow confident separation according to their unique spike form.

Selection of units for analysis—Unless indicated otherwise, we considered all neurons that were identified as AOB neurons. Specifically, we included neurons that showed significant stimulation-locked responses to at least one of the tested stimuli. A stimulation-locked response is considered significant if the distribution of single-trial firing rates (typically, five single-trial values for each stimulus), quantified for 40 s following VNO stimulation is significantly different from the distribution of the pre-stimulus firing rates of the same neuron. The pre-stimulus firing rate distribution was evaluated during the 15 s period before stimulus application, pooled across all trials of all stimuli for each neuron. The response of a neuron to a given stimulus is considered significant if the set of firing rate responses following the 5 repetitions of odor exposures differ at the $p < 0.05$ significance level from pre-stimulus firing rate, determined using ANOVA (*anova1* function in MATLAB). Once included, all individual trials for all stimuli from that neuron were used for the analysis.

Data analysis—All data analyses and visualizations were performed with built in and custom-written MATLAB programs. Response magnitude (R) is defined as the difference

between firing rate following SNT activation and the baseline firing rate during the 15 s inter trial interval (ITI). For the repetitive exposure experiment which did not include flushing of the nasal cavity between consecutive stimulations, the activity before a given stimulation may be influenced by the previous presentation. Hence the analysis of this set of experiments is not based on response magnitude as defined above, but rather on the actual firing rate in the 40 sec post-stimulation period. Table S1 lists all the statistical tests that were used in each of the panels.

Analysis of response pattern frequencies—Expected values for response patterns to male stimuli (Figure 2B) were calculated using the (empirical) marginal response probabilities to each of the stimuli (shown in Figure 2C), and the assumption of independence in responses to them. The estimated marginal probability (of a response to a given stimulus) is the probability that a unit in our dataset displays a significant response to that stimulus. For example, if the marginal probabilities to obtain a response to the four individual stimuli are P_A, P_B, P_C, and P_D, respectively, then the expected probability to observe a neuron that responds to all of them is given by the product of these probabilities. Alternatively, the probability to observe a neuron that responds to A, and B, but not to C or D, is given by: $P_A \bullet P_B \bullet (1- P_C) \bullet (1-P_D)$. The expected *number* of neurons conforming to this pattern is the product of this probability and the total number of neurons in the dataset. To determine if a deviation of the observed number from the expected number is statistically significant, we used the binomial distribution. Given N neurons and a probability P for a given response pattern (as calculated above, under the null hypothesis of independence), to determine if a value x is significantly larger than expected, we apply the cumulative binomial distribution to derive the probability to obtain a value that is larger than x under the null hypothesis. To determine is the value is significantly smaller than expected we apply the cumulative binomial distribution to derive the probability to obtain a value smaller than or equal to x under the null hypothesis. It is these probabilities that we use as a p value in our analysis. A significant difference between the observed number of neurons responding to a specific pattern (indicated by the blue bars in Figure 2B), and the expected value (indicated by the orange bars), implies that responses to each of the individual stimuli are not independent.

Preference indices—In order to avoid inter-neuron variability and to compare responses to two different stimuli, we applied preference indices of the form:

$$index = \frac{\text{response to A} - \text{response to B}}{\text{response to A} + \text{response to B}} \,. \tag{1}$$

Where A and B are two stimuli under comparison. In this calculation, the preference of each neuron is normalized to values between −1 and 1, where 1 indicates an exclusive response to A, and −1 indicates an exclusive response to B. A value of 0 indicates equal responses to both stimuli. Because the index is only meaningful for positive responses, it was only computed for the set of cells that showed a significant excitatory response to one or more of the stimuli under comparison, where all responses with rate decreases were set to zero.

Strain selective index: for the strain selective index, A = max ΔR BC (the larger of the responses to the two BC males), and B = max ΔR C57 (the larger of the two responses to the two C57 males). For this analysis we only included neurons that responded to one of the four male stimuli.

Stud strain selective index: for the stud strain selective index, A = max ΔR stud (the larger of the responses to the two stud strain males), B = max ΔR unfamiliar male (the larger of the two responses to the two unfamiliar strain males). For this analysis we only included neurons that responded to one of the 4 male stimuli.

Stud selective index: for the stud selective index, A = ΔR to the stud male individual, B = ΔR to the non-stud individual from the same strain. For this analysis we included neurons that responded to at least one of the two males from the stud strain.

Sex selectivity index (SSI): For the SSI, A = mean ΔR to male stimuli, B = mean ΔR to female stimuli. For this analysis we only included neurons that responded to one of the four males or one of the two female stimuli.

Attenuation index: For the attenuation index, A is the firing rate during the 40 s period after the first exposure, while B is the firing rate following the last (4th or 5th) exposure. For this analysis, we only included neurons that responded to one of male, female, or predator stimuli. The AI measures the decrease in response magnitude from the 1st to the last (4th or 5th) presentation for each neuron. The AI essentially normalizes the firing rate of each neuron (so that neurons with higher rates do not dominate the metric) and thus assumes values between −1 and 1.

Sparseness—Lifetime sparseness is a measure that quantifies selectivity of individual neurons based on a given stimulus set. Lifetime sparseness (S) was computed using the definition given in (Vinje and Gallant, 2000):

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

where r_i is the response of neuron to the ith stimulus (averaged across all trials) and N is the number of stimuli (N = 9). S varies between 0 and 1, such that 0 indicates uniform responses to all stimuli whereas 1 indicates a response to only one stimulus. Sparseness was calculated for the dataset of single units.

Measures of population distance—Distances between response vectors were calculated using both the *Euclidean* distance and the *correlation* distance (defined as 1 minus the correlation between responses to different stimuli). Distances were calculated to all stimulus pairs (for example, for 7 stimuli, there are 21 unique pairs). Pairwise distances between response vectors were calculated using MATLAB *pdist* function with the Euclidean and correlation distances. Correlation between the pairwise distances was calculated for the response magnitude using the *corrcoef* function in MATLAB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Neuronal correlates of the Bruce effect are tested in the female mouse AOB

- Initial representations of chemosensory stimuli are unaltered by mating

- Responses to extended presentation of stud urine are attenuated

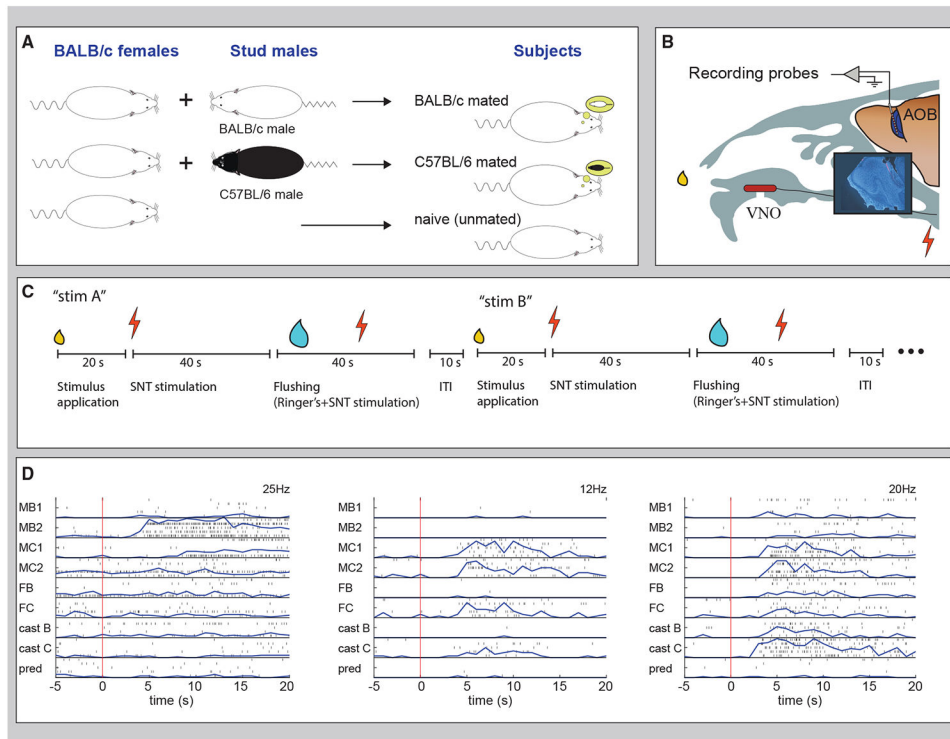- Distinct temporal scales may reconcile memory formation and representational stability

Figure 1. Experimental design and AOB responses to different chemosignals

(A) Definition and icons used for the female groups in this paper.

(B) Schematic of the recording setup. Inset shows a DAPI-stained sagittal brain slice showing electrode positioning within the cellular layer of the AOB.

(C) Timeline of the experiment, showing two trials (out of the entire session, which typically included five interleaved presentations of each of the nine stimuli in a random order).

(D) Examples of responses of three neurons recorded in naive females in response to the nine stimuli. Each plot shows single-trial spike times and the mean firing rates (PSTH [peri stimulus time histograms]). Red lines indicate sympathetic nerve trunk stimulation. Numbers on the upper right side of each set of responses indicate the range of the vertical scale (common to all stimulus PSTHs for each of the neurons). VNO, vomeronasal organ; AOB, accessory olfactory bulb; stimulus name abbreviations: MB1 and MB2 are two distinct male BALB/c individuals; MC1 and MC2 are two distinct male C57BL/6 individuals; FB and FC are urine mixes from BALB/c and C57BL/6 females; castB and castC are urine mixes from castrated BALB/c and C57BL/6 males; Pred is a mix of urine from four predator species.
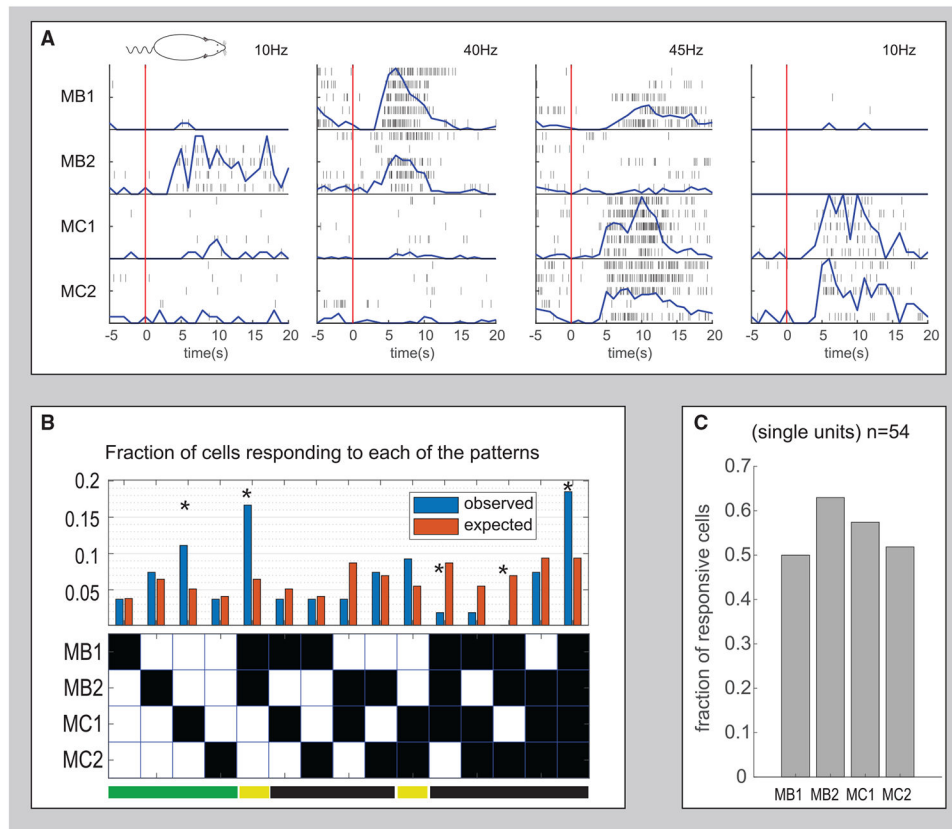
Figure 2. Responses to male urine in naive females

(A) Examples of responses of four different single units recorded in naive females to the four male stimuli.

(B) Distribution of significant response patterns to each combination. Blue and orange bars show the observed and expected distribution, respectively to patterns indicated in the table below (a filled square in the table indicates a significant response to the stimulus shown on the left). Significant differences between the observed and expected distributions are indicated with asterisks (p < 0.05, according to the binomial distribution, see STAR Methods). Stimulus name abbreviations are defined in the legend for Figure 1. (n = 54 single units that respond to at least one of the male stimuli). Green and yellow horizontal bars indicate individual and strain-specific response patterns, respectively. Black horizontal bars indicate responses to stimuli from both strains.

(C) Fraction of responsive neurons to each of the four stimuli across all naive females. See Figure S1 for additional analysis using multi-unit data and exact statistics for comparisons of observed and expected values.
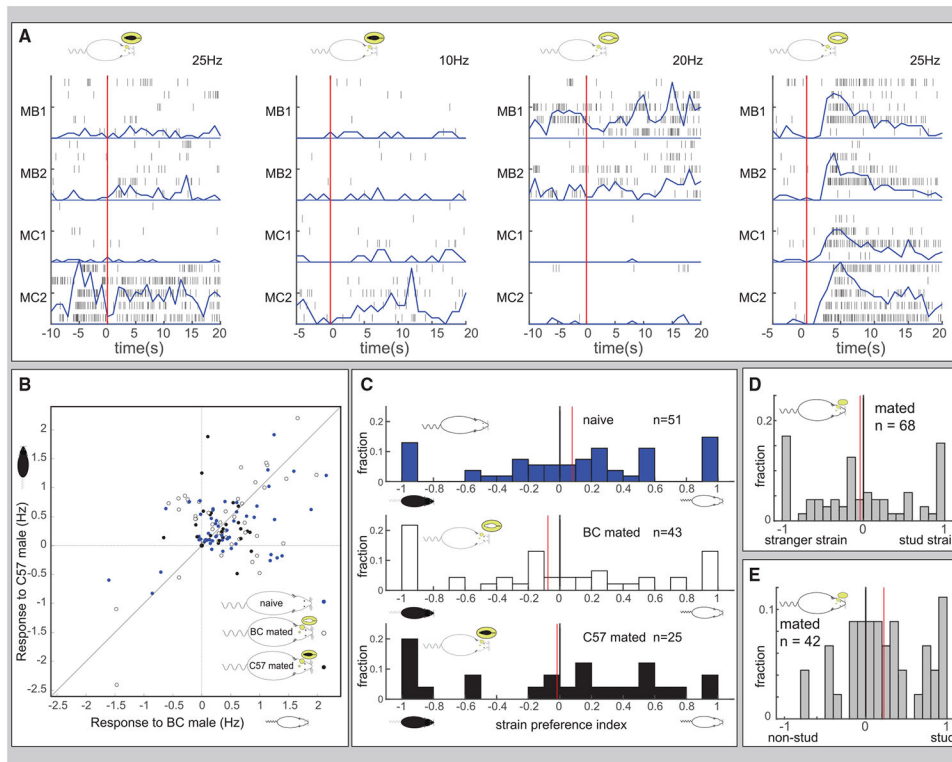
Figure 3. Response strength to male stimuli in naive and mated females

(A) Examples of single-unit responses to the four male stimuli in mated females (stud strain indicated by the icons next to each panel). Values above each panel indicate the maximal firing rate at the upper end of the y axes.

(B) Scatterplot showing response magnitude (defined as the maximal response across both male individuals) in each of the three female groups.

(C) Strain preference indices in each of the female groups. p value for rejecting the null hypothesis that medians are 0: naive, 0.27; BC mated, 0.42; C57 mated, 0.99 (two-sided Wilcoxon signed rank test). Pairwise group comparison p values: naive versus BC mated, 0.24; naive versus C57 mated, 0.75; BC mated versus C57 mated, 0.56 (two-sided Wilcoxon rank-sum test).

(D) Stud strain preference index in both mated female groups. Two-tailed Wilcoxon signed rank test, p = 0.4885.

(E) Stud-selective index in both mated female groups. Mean = 0.22. Wilcoxon signed rank test p = 0.008. Red vertical lines in (C–E) indicate mean values.
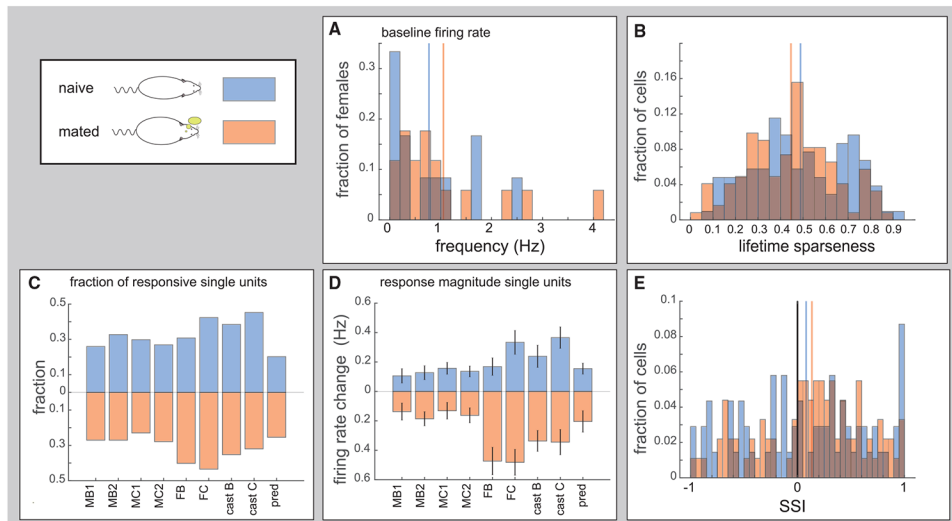
Figure 4. Responses to the entire panel of stimuli in naive and mated females

(A) Distribution of baseline firing rates in naive and mated females. Horizontal bars represent the mean baseline firing rates. p = 0.4127, two sample two-tailed Wilcoxon rank-sum test.

(B) Lifetime sparseness of single neurons in the two groups. Horizontal bars represent the mean sparseness (naive, 0.4887; mated, 0.4463). p = 0.16236 (two-sample two-tailed Wilcoxon rank-sum test).

(C and D) (C) Fraction of responding single neurons to each of the stimuli in the two groups. (CC = 0.6064, p = 0.0834). (D) Mean (and standard errors) of firing rate changes in each of the female groups (CC = 0.68, p = 0.04). In (B)–(D), n naive = 104, n mated = 122 single units. Comparison of firing rate distributions under the two conditions using rank-sum test yielded two values below 0.05: 0.0002 for the FB stimulus, and 0.0497 for the cast B stimulus. After correction for multiple comparisons, the only significant difference is for the FB stimulus.

(E) SSI in the two female groups. Horizontal bars represent the mean SSI values. n naive = 69, n mated = 91. Mean SSI in naive females: 0.0808, p = 0.2461 (two-sided Wilcoxon signed rank test). Mean SSI in mated females: 0.135, p = 0.0074. Difference between the SSI distributions in mated and naive females, p = 0.5933 (two-sided Wilcoxon rank-sum test). These analyses are based on single-unit activity from 12 naive and 17 mated females.
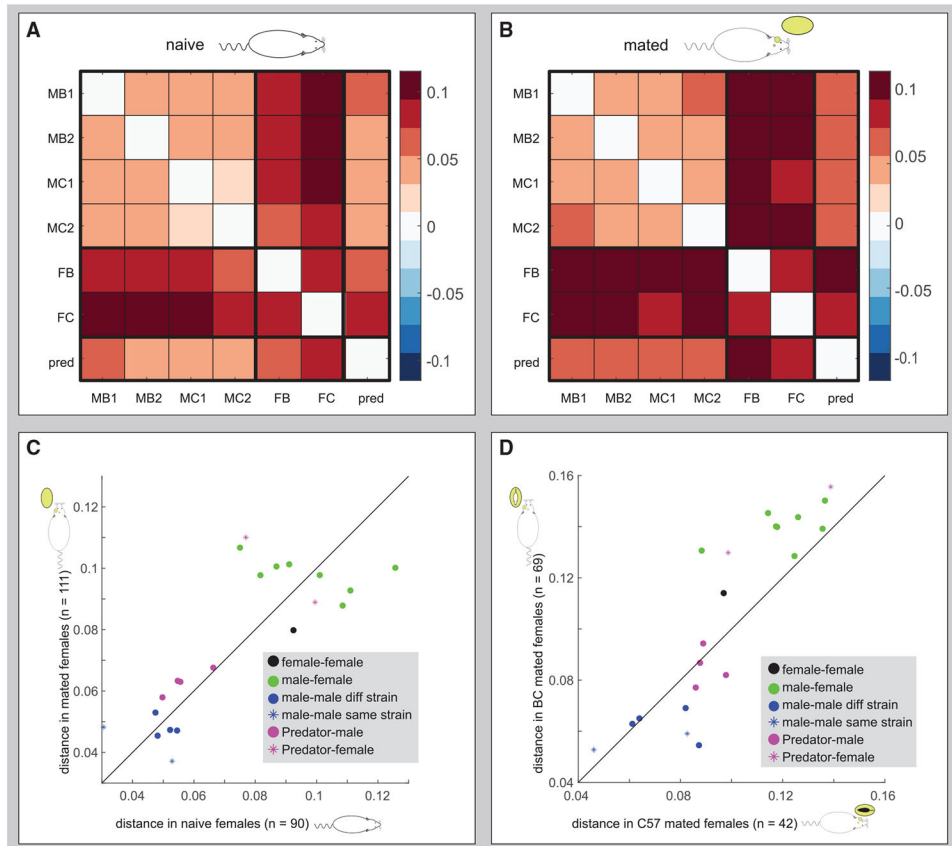
Figure 5. Similar population level response patterns in mated and naive females

(A) Population response distance matrix in naive females calculated using the Euclidean distance metric. n = 90 single units.

(B) Like (A), but in mated females. n = 111 single neurons. In this analysis, we only include units that respond to at least one of the non-castrated male, female, or predator stimuli.

(C) Correlation of pairwise distances between mated and naive values: CC = 0.795, p = 0.000016.

(D) Correlation of pairwise distances between C57 mated (n = 42 single units) and BC mated females (n = 69 single units): CC = 0.876, p = 0.00000018. In (C) and (D), each dot corresponds to one pairwise comparison, where the markers indicate the class of the stimuli. For example, green dots indicate comparisons between a male individual stimulus and a female mix (eight different combinations).
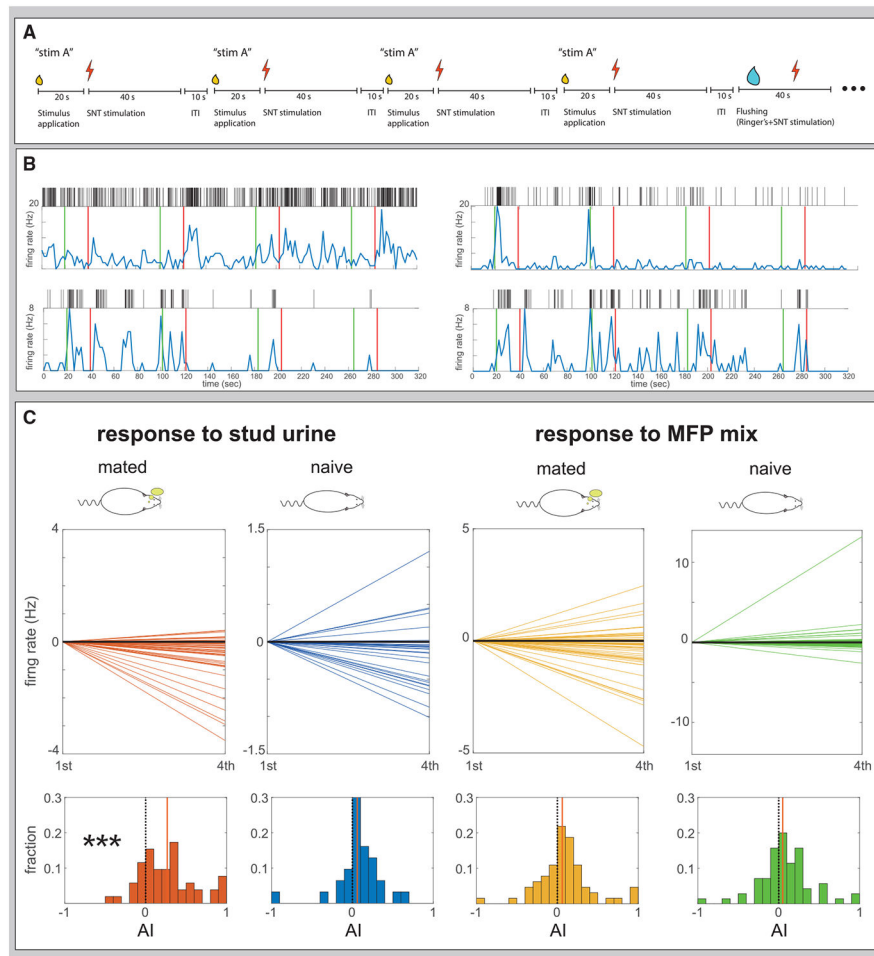
Figure 6. Analysis of responses to extended stimulation

(A) Schematic of the prolonged stimulus presentation paradigm. The schematic shows presentation of one of the two stimuli, after which the other stimulus is presented.

(B) Examples of neuronal responses during the prolonged stimulus presentation. Green and red vertical lines indicate times of stimulus application, and nerve stimulation, respectively. All examples are from mated females in response to stud stimuli (C). Firing rate change in the fourth trial relative to the first (top, each line represents one unit) per group, and distribution of the AI for units in the group (bottom). Each line represents one unit. In all histograms, broken black vertical lines indicate 0 and red vertical lines indicate the median value. Triple asterisks (***) indicate that the median is significantly larger than 0. Unit group identities are indicated above. Values for each of the four groups: mated females, stud stimulus, n = 52/51 (top panel and bottom plot respectively), median = 0.27, p = 8.5×10^{-5} (rank-sum test); naive females, stud stimulus, n = 31/31, median = 0.06, p = 0.15; mated females, MFP stimulus, n = 64/64, median = 0.06, p = 028; naive females, MFP stimulus, n = 70/68, median = 0.05, p = 0.28. Note that cases where n values for the AI distributions (histograms) are smaller than the values for the firing rate values (line plots) are due to undefined AI values following division by zero. Pairwise group comparison of AI values (p values, one-tailed rank-sum test): mated females, stud stimulus versus naive females, stud

stimulus, p = 0.015; mated females, stud stimulus versus mated females, MFP stimulus, p = 0.0024; mated females, stud stimulus versus naive females, MFP stimulus, p = 0.0015.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Mouse urine (stimulus)	Urine collected in-house. Mice purchased from Envigo Laboratories (Israel)	N/A
Predator urine (stimulus)	PredatorPee	N/A
Chemicals, peptides, and recombinant proteins		
Xylazine (anesthesia)	Eurovet	Sedaxylan
Ketamine (anesthesia)	Vetoquinol	clorketan
Isoflurane (anesthesia)	Piramal Critical Care	NDC 60,307-110-25
Veterinary glue (for various gluing stages during the surgery)	3 M Animal Care Products	1469SB
Experimental models: Organisms/strains		
Mice, BALB/c strain	Envigo Laboratories (Israel)	BALB/cOlaHsd
Mice, C57BL/6 strain	Envigo Laboratories (Israel)	6JRCCHSD
Software and algorithms		
Spike sorting software	Harris et al., 2000, Hazan et al., 2006	N/A
Experiment control software	Yoles-Frenkel et al., 2017	https://github.com/yorambenshaul/VNS_EXP
Other		
DAQ board (for all analog and digital channels)	INTAN	RHD2000 V1 INTAN
DAQ board (for output signals used in experimental control)	National Instruments	USB-6343
Stimulus isolator (for sympathetic nerve trunk stimulation)	AM-Systems	2200
Insulated platinum wire (connecting the cuff electrode to the stimulator connector. Diameter: 0.002 inch bare, 0.004 inch coated.)	AM-systems	771,000
Platinum Sheet (for cuff electrode. 0.025 mm thickness.)	Sigma Aldrich	267,244-1.4G
Silicone (mixture for insulating cuff electrodes)	NuSil Technology	Silicone (Silicone mixture for insulating cuff electrodes)
Polyethylene Tube (used in the mouth bar for sucking liquids from the nasopalatine duct)	AM-systems	804,300
Stainless Steel Tube (used for stabilization in mouth bar)	AM-systems	845,600
Solenoid pinch valve (for controlling suction tube)	Takasago electric	PM-0815W
Tubing for pinch valve (for pinch valve)	Takasago electric	HUJ71/2405/3036
Silk Sutures (for preventing the cheeks from being sucked during the wash)	Assut Sutures	4-0 Silk black braided. 75 cm HS 17
Tube for tracheotomy	AM-systems	801,600